CADPE Inhibits PMA-Stimulated Gastric Carcinoma Cell Invasion and Matrix Metalloproteinase-9 Expression by FAK/MEK/ERK-Mediated AP-1 Activation

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Abstract
Metastasis is one of the main causes of death for patients with malignant tumors. Aberrant expression of matrix metalloproteinase-9 (MMP-9) has been implicated in the invasion and metastasis of various cancer cells. Here, we found that caffeic acid 3,4-dihydroxy-phenethyl ester (CADPE) could inhibit the migration and invasion of human gastric carcinoma cells in Transwell migration assays. To understand the underlying mechanism, we showed that CADPE significantly inhibited phorbol 12-myristate 13-acetate (PMA)–induced increases in MMP-9 expression and activity in a dose-dependent manner. The inhibitory effect of CADPE on MMP-9 expression correlated well with the suppression of MMP-9 promoter activity and the reduction of MMP-9 mRNA. Reporter gene assay and electrophoretic mobility shift assay showed that CADPE inhibited MMP-9 expression by suppressing the activation of the nuclear transcription factor activator protein-1 (AP-1) and c-Fos, but not NF-κB. Moreover, CADPE inhibited PMA-induced phosphorylation of protein kinases involved in AP-1 activation, such as focal adhesion kinase (FAK), mitogen-activated protein kinase/extracellular signal–regulated kinase (ERK) kinase (MEK), and ERK1/2, whereas CADPE had little effect on the phosphorylation of p38 and c-jun NH2-terminal kinase. Taken together, our findings indicate that CADPE could be a unique antitumor agent that specifically inhibits MMP-9 activity by targeting the activation of FAK/MEK/ERK protein kinases and AP-1 transcription factor.

Introduction
Gastric carcinoma is the second most common cause of death and the third most common cancer worldwide (1). The current overall 5-year survival figures for gastric cancers in western patients are in the range of 5% to 17%. The incidence is also high in Europe, South America, and Eastern Asia (2–4). Because of its limited treatment efficiency and poor prognosis, the therapeutics of gastric cancer remains a major clinical challenge (5). Loss of control of tumor cell invasion and metastasis is the main cause of death in gastric cancer patients. The formation of metastatic nodules to gastric carcinoma is a multistep and complex process that includes cell proliferation, digestion of the extracellular matrix (ECM), cell migration to circulation system or lymph nodes, and remigration and growth of tumors at metastatic sites. It is widely believed that the aberrant expression of matrix metalloproteinases (MMP) is involved in these processes (6). MMPs are well-known ECM-degrading enzymes, which comprise a family of 24 members. Based on their substrates, MMPs are divided into four subclasses: collagenase, gelatinase, stromelysin, and membrane-associated MMPs (7). As a main ECM-degrading enzyme family, MMPs have essential roles in physiologic processes such as tissue development, remodeling, and wound healing (8). However, they are also involved in some tissue destructive diseases, such as atherosclerosis; inflammation; rheumatoid arthritis; and tumor invasion, metastasis, and neoangiogenesis (9, 10). Recent studies showed that MMPs were important regulators of the tumor microenvironment, including tumor progression, metastatic niche formation, and inflammation in cancer (11). Among human MMPs, MMP-2 (gelatinase-A) and MMP-9 (gelatinase-B) are key enzymes in the degradation of type IV collagen, which is an important component of ECM. These two members are mostly associated with tumor migration, invasion, and metastasis for various cancers (12). An enhanced expression of MMP-9 has been shown to be associated with the progression and invasion of tumors, whereas MMP-2 is usually expressed constitutively (13, 14).
The expression of MMP-9 can be stimulated by various agents, such as inflammatory cytokine, growth factor, and phorbol myristate acetate (PMA). PMA is a well-known inflammatory stimulator and tumor promoter that activates almost all protein kinase C (PKC) isozymes and increases the invasiveness of various types of cancer cells by activating MMP-9 (15). Those stimulators can upregulate the expression of MMP-9 by modulating the activation of transcription factors such as activator protein-1 (AP-1) and NF-kB through the Ras/Raf/extracellular signal-regulated kinase (ERK), c-jun NH2-terminal kinase (JNK), and phosphoinositide 3-kinase/Akt signaling pathways (16-19). AP-1 has been shown to regulate the expression of a number of genes, some of which are involved in tumorigenesis (20, 21). Thus, it will be an effective way to find tumorigenesis and metastasis inhibitors from the agents that can suppress the activities of AP-1 and MMP-9.

Caffeic acid 3,4-dihydroxyphenethyl ester (CADPE) was originally isolated from Tauricium pilosum as a substance named teucrol in 2000 (22) and can be synthesized by a chemical process (23). Caffeic acid (CA) is a phenolic compound and is largely found in food plants. CA has been reported to possess a wide spectrum of biological effects (e.g., antioxidant, anti-inflammatory, antitumor angiogenesis and antitumor invasion, and metastasis properties; ref. 23). A recent study showed that both CA and CADPE could inhibit tumor angiogenesis in human renal carcinoma cells by suppressing hypoxia-induced signal transducer and activator of transcription-3 phosphorylation, signal transducer and activator of transcription-3 nuclear translocation, hypoxia-inducible factor-1α induction, and vascular endothelial growth factor expression (24). However, the exact mechanism of CADPE in the regulation of MMPs in cancer cells has not been reported.

In this study, human gastric carcinoma cell line (MGC-803) was used to investigate the effect of CADPE on MMP-induced MMPs expression and the underlying molecular mechanism. We show that CADPE inhibits the migration and invasion of gastric cancer cells by suppressing MMP-9 expression and blocking the activation of focal adhesion kinase (FAK)/mitogen-activated protein kinase (MAPK)/ERK kinase (MEK)/ERK1/2 protein kinases and AP-1 transcription factor.

Materials and Methods

Materials and cells

CADPE was synthesized by Dr. Xiaoyuan Lian (The Institute of Biomedical Sciences, East China Normal University, Shanghai, China; ref. 23). A 100 mmol/L stock solution of CADPE was prepared in DMSO. CA and PMA were purchased from Sigma-Aldrich. Matrigel was purchased from BD Biosciences. Kinase inhibitors PD188059, SB203580, SP600125, and U0126 were purchased from Calbiochem. Antibodies against MMP-9, MMP-2, c-Fos, c-Jun, p65, total and phosphorylated FAK, MEK, ERK1/2, stress-activated protein kinase/JNK, and p38 MAPK were from Cell Signaling Technology; phospho-PKC antibody sample kit (49921) was obtained from Cell Signaling Technology; and antibody against β-actin was purchased from Sigma-Aldrich.

Human gastric carcinoma cell lines MGC-803, HGC-27, and AGS and human breast carcinoma cell line MDA-MD-231 were obtained from the China Type Culture Collection (Shanghai, China). MGC-803 and HGC-27 were cultured in RPMI 1640 containing 10% fetal bovine serum. AGS and MDA-MD-231 were cultured in DMEM and L-15 medium containing 10% fetal bovine serum, respectively.

Cell viability assay

For the cell viability assay, 2 \times 10^4 MGC-803 cells per well were treated with different concentrations of CADPE for 24 hours. Cell viability was determined by the (4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) method, following the manual of CellTiter 96 Aqueous One Solution Cell Proliferation assay (Promega). Absorbance was measured with a VERSAmax microplate reader.

Transwell migration assay

The Transwell migration assay was done according to a previously reported method with some modifications (27). Briefly, Transwell membrane (8-μm pore size, 6.5-mm diameter; Corning Costar Corporation) was used. The bottom chambers of the Transwell were filled with migration-inducing medium (with 10% fetal bovine serum). The top chambers were seeded with 2 \times 10^4 MGC-803 cells per well with different concentrations of CADPE (0, 5, 10, and 20 μmol/L). After 8 to 10 hours, the filters were fixed with 4% paraformaldehyde for 30 minutes at room temperature; subsequently, the cells on the upper side of the membrane were scraped with a cotton swab. Filters were stained with hematoxylin for light microscopy. Images were taken using an Olympus inverted microscope and migratory cells were evaluated by manual counting. Percentage inhibition of migratory cells was quantified and expressed based on untreated control wells.

Matrigel-based Transwell invasion assay

Cell invasion assays were carried out as previously reported (27) with slight modifications. Transwell membrane coated with Matrigel (100 μg/mL, 100 μL per well) was used for invasion assay. Cells (5 \times 10^4) were seeded onto the upper wells in the presence of different concentrations of CADPE or MMP-9 inhibitor (28) with or without PMA. The bottom chambers of the Transwell were filled with condition medium. The inserts were incubated at 37°C for 24 hours. Cells that had invaded the lower surface of the membrane were fixed, stained, and counted under a light microscope. Percentage inhibition of invasive cells was quantified and expressed based on untreated control wells.

Gelatin substrate gel zymography

Gelatin zymography was carried out as previously reported (18). The MGC-803 cells were plated onto six-well plates at...
a density of $2 \times 10^5$ cells per well and incubated until they reached 80% confluence; the medium was then changed to fresh serum-free medium with or without CADPE or specific inhibitors of MAPKs (PD98059, U0126, SP600125, and SB203580). After 2 hours of pretreatment, 100 nmol/L PMA was added to the medium for 24 hours, and the supernatant was collected and concentrated. The resultant supernatant was subjected to SDS-PAGE in 8% polyacrylamide gels that were copolymerized with 1 mg/mL gelatin. After the electrophoresis runs, the gels were washed several times with 2.5% Triton X-100 for 1 hour at room temperature to remove the SDS and incubated for 12 hours at 37°C in a buffer containing 5 mmol/L CaCl₂ and 1 μmol/L ZnCl₂. The gels were stained with Coomassie brilliant blue R250 (0.25%; Bio-Rad) for 1 hour and then destained for 1 hour in a solution of acetic acid and methanol. Proteolytic activity was evidenced as clear bands against the blue background of the stained gelatin.

**Western blot assay**

For the Western blot assay, MGC-803 cells were treated with different concentrations of CADPE in the presence of 100 nmol/L PMA for 24 hours. Cell lysates were prepared in radioimmunoprecipitation assay buffer (20 mmol/L Tris, 2.5 mmol/L EDTA, 1% Triton X-100, 0.1% SDS, 40 mmol/L NaF, 10 mmol/L Na₄P₂O₇, and 1 mmol/L phenylmethylsulfonyl fluoride). Aliquots of cellular protein (40 μg/lane) were electrophoresed on 10% to 12% SDS-PAGE and transferred onto a polyvinylidene difluoride membrane (Millipore Corp.). The membrane was blocked with 5% skim milk in PBS containing 0.1% Tween 20 and then reacted with specific antibodies. Detection of specific proteins was carried out with an enhanced chemiluminescence Western blotting kit following the manufacturer’s instructions (Amersham-Pharmacia). To examine the activation of transcription factors, nuclear fractions were obtained from cells treated with PMA for 1 hour after 4 hours of pretreatment with CADPE. To assess the changes in signaling pathway, starved MGC-803 cells were treated with CADPE for 2 hours and then stimulated with 100 nmol/L PMA.

**Reverse transcription-PCR**

In the reverse transcription-PCR (RT-PCR) analysis, total RNA was extracted from the treated cells. For reverse transcription reaction, cDNA was synthesized from 1 μg of total RNA using Moloney murine leukemia virus reverse transcriptase (Promega). The PCR primers used are as follows: MMP-9 sense, 5′-TCCCTGGAGACCTGAGAACC-3′; MMP-9 antisense, 5′-GGCAAGTCTTCCGAGTAGTTT-3′; MMP-2 sense, 5′-GAGATGATGCCTTTGCTCG-3′; MMP-2 antisense, 5′-ATCGGCGTTCCCATACTT-3′; MMP-7 sense, 5′-CTTCTCTGAGTCAACCTC-3′; MMP-7 antisense, 5′-GGATGATGCCTTTGCTCG-3′; TIMP-1 sense, 5′-GGGAAGGAGCAATGCTGTATG-3′; TIMP-1 antisense, 5′-CGAGATCTGGAGAGGAGGTGG-3′; TIMP-2 sense, 5′-GGAGGAGCAATGCTGTATG-3′; TIMP-2 antisense, 5′-TTAGGCTGCTCCTCCGAGGTGCCAC-3′; β-actin sense, 5′-GCCATCGTCACCAACTGGGAC-3′; and β-actin antisense, 5′-CGATTTCGCCGTCGGCGGTG-3′. PCR products were analyzed by agarose gel electrophoresis and visualized by treatment with ethidium bromide.

**Construction of human MMP-9 promoter**

A 700-bp fragment at the 5′-flanking region of the human MMP-9 gene was amplified by PCR from human genomic DNA. Specific primers were designed to contain the appropriate restriction enzyme site: sense 5′-CGG GGTACCTGCTACTGTCCCCTTACTG-3′ (KpnI) and antisense 5′-GAGGAAGCTGAGTCAAAGAAGGC-3′. The amplified promoter DNA was digested with KpnI and BglII and then cloned upstream of the luciferase gene in pGL3 plasmid. The DNA sequence of the MMP-9 promoter was confirmed, and the resultant reporter plasmid was named pGL3-MMP-9-WT. The AP-1, NF-κB, and SP-1 mutants from pGL3-MMP-9-WT were generated using the QuickChange Site-Directed Mutagenesis Kit (Stratagene); all the mutants were confirmed by DNA sequencing.

**Transient transfection and luciferase reporter gene assays**

MMP-9 wild-type (pGL3-MMP-9-WT), AP-1 site-mutated (pGL3-MMP-9-Mut-AP-1-1), NF-κB site-mutated (pGL3-MMP-9-Mut-NF-κB), and SP-1 site-mutated (pGL3-MMP-9-Mut-SP-1) were used in transient transfection assays. MGC-803 cells were plated onto six-well plates at a density of $2 \times 10^5$ cells per well and grown overnight. Cells were cotransfected with 1 μg of MMP-9 promoter-luciferase reporter constructs and 0.2 μg of the Renilla reporter plasmid for 6 hours using Lipofectamine reagent (Invitrogen) according to the manufacturer’s protocol. After transfection, the cells were cultured in condition medium with PMA and incubated with different concentrations of CADPE for 24 hours. Luciferase and Renilla activities were determined by following the manufacturer’s protocol (Dual-Luciferase Reporter Assay System, Promega). Luciferase activity was normalized with the Renilla activity in the cell lysate and expressed as an average of three independent experiments.

**Electrophoretic mobility shift assay**

Cultured cells were collected by centrifugation, washed, and resuspended in buffer A [10 mmol/L HEPES (pH 7.9), 10 mmol/L KCl, 0.1 mmol/L EDTA, 0.1 mmol/L EGTA, 1 mmol/L DTT, and 0.5 mmol/L phenylmethylsulfonyl fluoride]. After 15 minutes on ice, the cells were vortexed in the presence of 0.5% NP40. The nuclear pellet was then collected by centrifugation and extracted with buffer B [20 mmol/L HEPES (pH 7.9), 0.4 mol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L DTT, and 1 mmol/L PMSF] for 15 minutes at 4°C. Double-stranded oligonucleotides containing the consensus sequences for AP-1 upstream (5′-GAGGAAGCTGAGTCAAAGAAGGC-3′), CGATGTCGAG-3′; β-actin sense, 5′-GCCATCGTCACCAACTGGGAC-3′; and β-actin antisense, 5′-CGATTTCGCCGTCGGCGGTG-3′. PCR products were analyzed by agarose gel electrophoresis and visualized by treatment with ethidium bromide.
AP-1 proximal (5′-CTGACCCCTGAGTCAGCACTT-GC-3′), and NF-κB (5′-CCCCAGTGGAATTCCCA-GCCTTG-3′) were end-labeled with [γ-32P]ATP using T4 polynucleotide kinase and used as probes for electrophoretic mobility shift assay (18). The nuclear extracts (5 μg) were incubated at 4°C for 30 minutes in 25 mmol/L HEPES buffer (pH 7.9), 0.5 mmol/L EDTA, 0.5 mmol/L DTT, 0.05 mol/L NaCl, and 2.5% glycerol with 1 μg of poly(ddeoxyinosinic-deoxyctydilic acid) and 0.5 pmol of labeled probe. The DNA-protein complex...
was separated by electrophoresis at 4°C in 6% polyacrylamide gels in 0.5× Tris-borate EDTA buffer. For competition assay to confirm the binding specificity, nuclear extracts were preincubated at 4°C for 30 minutes with a 100-fold excess of an unlabeled oligonucleotide. Gels were dried and imaged using the Personal Molecular Imager system (Bio-Rad).

**Statistical analysis**

The results are presented as mean ± SE, and statistical comparisons between groups were done using one-way ANOVA followed by Student’s t test. *P* ≤ 0.05 was considered statistically significant.

**Results**

**Effects of CADPE on the viability and invasion of gastric carcinoma cells**

As a synthetic derivative of CA, CADPE preserved the key structure of CA (Fig. 1A). To evaluate the effect of CADPE on cell proliferation, we treated MGC-803 cells with different concentrations of CADPE or CA in serum-free and serum-containing medium, respectively, for MTS assays. Only 9% and 15% decreases in cell viability were found in cells treated with 25 μmol/L CADPE in serum-containing and serum-free medium (Fig. 1B). No significant cytotoxicity was found for CA at the concentration of 100 μmol/L (data not shown). To investigate the effects of CADPE on tumor cell migration and invasion, the Transwell migration assay and the Matrigel-based Transwell invasion assay were done using MGC-803 cells at different concentrations of CADPE, ranging from 5 to 20 μmol/L. As shown in Fig. 1C, CADPE significantly inhibited MGC-803 cancer cell migration (Fig. 1C). Moreover, CADPE dramatically inhibited PMA-induced cell invasion in a dose-dependent manner (Fig. 1D), suggesting that CADPE is an effective inhibitor of cancer cell migration, invasion, and metastasis.

![FIGURE 2. Inhibition of MMP-9 activity by CADPE in tumor cells.](image)

**FIGURE 2. Inhibition of MMP-9 activity by CADPE in tumor cells.** A, CADPE inhibits PMA-induced MMP-9 activity. MGC-803, HGC-27, AGS, and MDA-MB-231 cells were preincubated with varying concentrations of CADPE for 24 h, followed by PMA stimulation for 24 h. Conditional media were collected and MMP activity was analyzed by gelatin zymography. B, CADPE inhibits EGF-induced MMP-9 activity. MGC-803 cells were incubated with varying concentrations of CADPE in the presence of EGF for 24 h and gelatin zymography was done. C, CA inhibits PMA-induced MMP-9 activity. MGC-803 cells were incubated with 25 to 100 μmol/L CA in the presence of PMA, and MMP-9 enzyme activity in the conditioned medium was analyzed by zymography. D, MMP-9 and MMP-2 derived from PMA-treated conditioned medium were incubated with CADPE (5-20 μmol/L) for 30 min and then subjected to gelatin zymography.
CADPE inhibits MMP-9 expression and activity

MMP-9 and MMP-2 are important ECM-degrading enzymes. It has been reported that both enzymes were involved in cancer cell invasion and metastasis (14, 29). The fact that CADPE inhibited cancer cell invasion prompted us to examine the effect of CADPE on MMPs activity using gelatin zymography. The secretion of MMP-9 in the conditioned medium of MGC-803 was dramatically induced by PMA (10-100 nmol/L) in a dose-dependent manner, whereas no detectable change of MMP-2 (data not shown) was found in the same condition. To examine the effects of CADPE on MMP-9 expression in cancer cells, we chose 100 nmol/L PMA to induce the activation of MMP-9 in different cancer cells. As shown in Fig. 2A, treatment of MGC-803 cells with CADPE (5-20 μmol/L) suppressed PMA-induced MMP-9 activity in a dose-dependent manner, whereas the activity of MMP-2 did not significantly decrease. Similar results were obtained in three other cancer cell lines, including two gastric cancer cell lines (AGS and HGC-27) and one breast cancer cell line (MDA-MB-231; Fig. 2A), suggesting that CADPE significantly inhibits the secretion of MMP-9 in cancer cells.

**FIGURE 2.** Inhibition of PMA-induced MMP-9 expression by CADPE. A, CADPE inhibits MMP-9, but not MMP-2, expression. The expression levels of MMP-9 and MMP-2 in MGC-803 cells treated with CADPE in the presence of PMA for 24 h were evaluated by Western blot analysis. Expression of β-actin in cell lysates was used as a control. B, CADPE inhibits MMP-9, but not MMP-2, mRNA level. MGC-803 cells were treated with or without CADPE (20 μmol/L) in the presence of PMA for 0, 3, 6, 12, 18, and 24 h, and mRNA levels of MMP-9 and MMP-2 were examined. C, effects of CADPE on the mRNA levels of MMPs and their regulators. MGC-803 cells were incubated with CADPE for 24 h. The mRNA expression levels of MMP-9, MMP-2, MMP-3, MMP-7, TIMP-1, and TIMP-2 were analyzed by RT-PCR; β-actin expression was included as an internal control. The expression levels of MMP-9 in CADPE-treated or untreated MGC-803 cells were determined by real-time PCR analysis. D, MMP-9 inhibitor can block PMA-induced MGC-803 cell invasion in the Matrigel invasion assay.
invasive cancer cell lines. Compared with CADPE, CA also decreased MMP-9 activity in MGC-803 cells, but at a much higher concentration (25-100 μmol/L). CA had little effect on MMP-2 activity (Fig. 2C). Furthermore, we show that CADPE inhibited epidermal growth factor (EGF)-induced MMP-9 expression and activity in MGC-803 cells (Fig. 2B). To investigate whether CADPE directly affects MMP-9 enzyme activity, conditioned medium derived from PMA-treated MGC-803 was incubated with different concentrations of CADPE in the gelatin zymography assays. As shown in Fig. 2D, there was no visible difference between the CADPE- treated and untreated groups (Fig. 2D), suggesting that CADPE has no direct influence on MMP-9 activity. Taken together, our data suggest that CADPE inhibits PMA- and EGF-induced MMP-9 activation in different cancer cell lines.

**CADPE suppresses MMP-9 transcription in a dose-dependent manner**

As shown in Fig. 3A, the expression levels of MMP-9 gradually decreased in a dose-dependent manner in Western blot assay, indicating that CADPE inhibits MMP-9 enzyme activity by reducing the protein level of MMP-9 (Fig. 3A). Similar to prior observations,

![FIGURE 4. Analysis of CADPE on MMP-9 promoter activity through AP-1 and NF-κB binding sites.](image)

A. CADPE inhibits PMA-induced MMP-9 luciferase activity. B and C, mutations at NF-κB and SP-1 binding sites have little influence on the inhibitory effects of CADPE in MGC-803 cells. D, mutations at the two AP-1 binding sites of the MMP-9 promoter diminish the inhibitory effects of CADPE, suggesting that CADPE suppresses the expression of MMP-9 through AP-1 binding sites. E and F, CADPE inhibits the AP-1-luciferase, but not the NF-κB-luciferase, activity. MGC-803 cells were transfected with reporter vectors containing binding sites for AP-1 and NF-κB. The cells were cultured in the presence of CADPE (5-20 μmol/L) for 24 h, and luciferase activity was measured. Columns, mean of at least three independent experiments; bars, SE. *, P < 0.05.
CADPE had little effect on the protein expression of MMP-2 (Fig. 3A). Furthermore, we performed RT-PCR to determine the CADPE regulation of MMPs at the mRNA level. In MGC-803 cells, the mRNA level of MMP-9 was induced by PMA after 3 hours, peaked in 12 hours, and persisted for less than 24 hours (Fig. 3B). CADPE significantly inhibited PMA-induced MMP-9 mRNA expression in a time- and dose-dependent manner (Fig. 3B and C). CADPE had no effect on the mRNA level of MMP-2 (Fig. 3B and C). The inhibitory effect of CADPE on MMP-9 mRNA expression was further confirmed by real-time quantitative PCR (Fig. 3C).

Because the activity of MMP-9 is tightly regulated by endogenous inhibitors, tissue inhibitors of metalloproteinases (TIMP), we examined the expression levels of TIMP-1 and TIMP-2 by RT-PCR. As shown in Fig. 3C, TIMP-1, but not TIMP-2, can be slightly stimulated by PMA. However, CADPE had no effect on the mRNA levels of both TIMP-1 and TIMP-2 (Fig. 3C). Furthermore, we examined the expression level of MMP-7 in CADPE-treated MGC-803 cells and showed that MMP-7 remained essentially unchanged (Fig. 3C). These results indicate that CADPE selectively suppressed MMP-9 expression both at the protein and mRNA levels in a time- and dose-dependent manner.

To understand the relationship of cell invasion and MMP-9 in gastric cancer, we performed a Matrigel invasion assay with MMP-9 inhibitor. Results showed that MMP-9 inhibitor blocked MGC-803 cell invasion in a dose-dependent manner, suggesting that MMP-9 was largely responsible for the invasion of MGC-803 cells (Fig. 3D). The inhibitory effect of MMP-9 inhibitor on MGC-803 cell invasion was almost the same as that of CADPE (Supplementary Fig. S2B). The specificity of MMP-9 inhibitor was tested by gelatin zymography assay in different gastric carcinoma cell lines (Supplementary Fig. S1). We also detected the effects of MMP-9 inhibitor on the migration of gastric cancer cells. Results showed that MMP-9 inhibitor had less inhibitory effects when compared with CADPE (Supplementary Fig. S2A).

CADPE inhibits MMP-9 expression by suppressing AP-1 binding and AP-1–dependent transcription activity

To understand the molecular mechanism underlying the inhibitory effects of CADPE on MMP-9 expression, we find that there are two AP-1 binding sites (located at −79 bp and −533 bp) and an NF-κB binding site (located at −600 bp) in the MMP-9 promoter. It has been shown that NF-κB and AP-1 play an important role in controlling basal and cytokine-induced MMP-9 expression in various cancer cell lines (18). To determine the effect of CADPE on the promoter activity of MMP-9, luciferase-report gene that contains the MMP-9 promoter region was transiently transfected into MGC-803 cells. As shown in Fig. 4A, MMP-9-luciferase activity was activated up to 20-fold in cells treated with PMA. CADPE inhibited the PMA-induced MMP-9-luciferase activity in a dose-dependent manner (Fig. 4A), suggesting that CADPE could inhibit MMP-9 expression at the transcriptional level.

To determine which of these transcription factors may participate in the regulation of MMP-9 transcription in MGC-803 cells, we mutated the potential binding sites for different transcription factors found in the MMP-9 promoter, including NF-κB, SP-1, and two AP-1 sites (Fig. 4B-D). MGC-803 cells were transiently transfected with MMP-9 reporter genes with mutations in different transcription binding sites. Mutations at the NF-κB and SP-1 binding sites have little effect on the inhibitory effects of CADPE on PMA-induced MMP-9 activity (Fig. 4B and C). However, mutations at the two AP-1 binding sites completely abolished the inhibitory effects of CADPE on PMA-induced MMP-9 promoter activity (Fig. 4D), suggesting that the regulation of CADPE on the MMP-9 promoter region was facilitated by AP-1 transcription factor. To further confirm this observation, the luciferase report vectors that contain tandem repeats of the AP-1 or NF-κB binding sites were used to examine the effects of CADPE in the luciferase assays. As shown in Fig. 4E, luciferase activity in the cells transfected with the AP-1 reporter was significantly reduced by treatment with CADPE in the range of 5 to 20 μmol/L, whereas no statistically significant changes were found in the cells transfected with the NF-κB reporters in the presence of CADPE (Fig. 4F). These results suggest that AP-1 transcription factor and AP-1 binding sites in the MMP-9 promoter region contribute to the inhibition of PMA-dependent MMP-9 activation by CADPE.

CADPE decreases transcription factor binding to AP-1 motifs in the MMP-9 promoter region

To determine whether CADPE inhibited the transcriptional binding activity of AP-1 to its DNA motifs, we performed electrophoretic gel mobility shift assay using the consensus sequences of AP-1 or NF-κB as probes in CADPE-treated cells. MGC-803 cells were pretreated with different concentrations of CADPE for 4 hours, followed by treatment with 100 nmol/L PMA for 1 hour. Then, nuclear extracts were prepared and analyzed for AP-1 and NF-κB DNA binding activities, respectively. As shown in Fig. 5A, CADPE significantly decreased PMA-induced AP-1 DNA binding ability, whereas CADPE has no effect on PMA-induced NF-κB binding activity (Fig. 5B). These data were consistent with the reporter gene analysis, suggesting that CADPE blocks MMP-9 expression, at least in part, by inhibiting the expression or DNA binding activity of AP-1 transcription factor. To determine which subunit of AP-1 transcription factor is regulated by CADPE, we examined the expression levels of c-Fos and c-Jun with CADPE treatment. Our data showed that CADPE significantly reduced PMA-induced c-Fos expression but had a little effect on the expression of c-Jun or p65 in Western blot assays (Fig. 5C), suggesting that CADPE inhibits AP-1 transcription activity by suppressing the expression of c-Fos in the gastric cancer cells.
CADPE blocks PMA-induced activation of FAK, MEK, and ERK1/2 in gastric cancer cells

Activation of one or more mitogen-activated protein kinase (MAPK) pathways is important for the MMP-9 induction by PMA in various cell types (30). As shown in Fig. 6A, the phosphorylation of ERK, JNK, and p38 was increased by the stimulation with PMA. Addition of CADPE decreased only ERK phosphorylation in MGC-803 cells (Fig. 6B). On the other hand, the levels of phosphorylated JNK and p38 had little change after CADPE treatment (Fig. 6B). We further examined the phosphorylation of upstream regulators in the ERK signaling pathway by Western blot with specific phosphorylation antibodies. CADPE significantly inhibited MEK and FAK phosphorylation induced by PMA treatment (Fig. 6B). As PKC is also involved in MMP-9 expression, we examined the effects of CADPE on PKC activation using specific phospho-antibodies for different PKC isoforms as described in Materials and Methods. Our results showed that CADPE has little effect on PKC activation (Fig. 6B), suggesting that the effects of CADPE are mediated by pathways other than PKC. To further confirm our conclusion, we measured MMP-9 activity using specific protein kinase inhibitor assays. Overnight starved MGC-803 cells were pretreated with PD98059, U0126, SP600125, and SB203580 (inhibitors of MEK, ERK, JNK, and p38, respectively) for 2 hours and then stimulated with PMA for 24 hours; conditioned media were collected for gelatin zymography assay of MMP-9 activity. As shown in Fig. 6C, MEK and ERK inhibitors can significantly suppress MMP-9 expression and activity, whereas JNK and p38 inhibitors have little inhibitory activity on MMP-9. These results suggest that CADPE inhibits the PMA-induced activation of AP-1 by blocking FAK and MEK/ERK1/2 activation in MGC-803 cells.

Discussion

Metastasis and invasion are major properties of various malignant tumors that are associated with a poor prognosis. It was thought that MMP-9 participated in promoting these processes. Recent studies show that MMP-9 has statistically significantly different expression patterns between well-differentiated and poorly differentiated tissue samples and may play key roles during the development of gastric cancer (31). In addition, MMP-9 also correlated with the invasion, metastasis, and angiogenesis of gastric cancer cells (32, 33). Therefore, development of various compounds that can inhibit MMP-9 would be useful in the treatment of gastric carcinoma.
As a structure analogue of CADPE, CA has a wide spectrum of biological effects, including antitumor invasion by targeting MMP-9 through the AP-1 and NF-κB signaling pathways (18). The ability to inhibit tumor cell invasion was also extended to other caffeic acid derivatives, such as caffeic acid phenethyl ester, which was originally isolated from honeybee propolis. However, the inhibitory role of CADPE against MMP expression and the invasiveness of gastric carcinoma has not been reported. In this study, we show that CADPE is a potential anticancer agent due to its ability to inhibit PMA-induced phosphorylation of protein kinases (FAK, MEK, and ERK1/2), AP-1 nuclear translocation, and MMP-9 expression in invasive tumor cells.

Gelatin zymography is a classic method to detect the activity and expression of gelatinases A and B, and thus we used this method to detect the secretion of MMP-9 and MMP-2 in the condition medium. In this study, treatment of MGC-803 cells with CADPE selectively suppressed the PMA-induced activity of MMP-9, whereas the activity of MMP-2 was not affected. A similar result was also observed in two more gastric carcinoma cell lines, AGS and HGC-27, and a breast cancer cell line, MDA-MB-231. When compared with its precursor CA, CADPE showed a better inhibitory effect on MMP-9 at a relatively lower concentration, suggesting that CADPE inhibits tumor invasion through MMP-9 in these invasive tumor cell lines.

The activity of MMPs is precisely regulated at three levels: gene transcription, posttranscriptional activation of zymogens, and endogenous expression of tissue inhibitors of metalloproteinases (34). It was thought that the key step in the regulation of MMPs was at the transcription level. To determine which step was affected by CADPE, gelatin zymography, RT-PCR, and Western blot analysis were performed to show that CADPE inhibits the expression of MMP-9 at both the mRNA and protein levels but has little effect on the enzymatic activity.

**FIGURE 6.** CADPE inhibits the activation of FAK, MEK, and ERKs. A, time-dependent phosphorylation of ERK, JNK, and p38 induced by PMA in MGC-803 cells. B, effects of CADPE on the phosphorylation of ERK, JNK, p38, MEK, FAK, and PKC. MGC-803 cells were pretreated with CADPE for 4 h and stimulated with PMA for 15 min; the levels of phosphorylated protein kinases, including pERK, p-JNK, p-p38, p-MEK, p-FAK, and p-PKC, were determined by Western blotting with phospho-specific antibodies. C, inhibition of PMA-induced MMP-9 activity by protein kinase inhibitors for MEK and ERK. MGC-803 cells were pretreated with U0126 (MEK inhibitor; 10 μmol/L), SP600125 (JNK inhibitor; 10 μmol/L), PD98059 (ERK inhibitor; 20 μmol/L), and SB203580 (p38 inhibitor; 10 μmol/L) for 2 h and then treated with PMA for 24 h; MMP-9 levels were tested by gelatin zymography. D, possible molecular mechanisms underlying the antitumor activities of CADPE in PMA-treated MGC-803 cells.
MMPs activity is also regulated by tissue-specific inhibitors (TIMPs), of which there are four identified members (TIMP-1 to TIMP-4; ref. 8). These proteins bind MMPs in a 1:1 stoichiometry and directly affect the level of MMP activity. Because TIMP-1 is a major inhibitor of MMP-9, TIMP-1 and TIMP-2 are differentially regulated in vivo as well as in cultured cells (35, 36). We tested the mRNA levels of these two proteins and ruled out the effects of CADPE on TIMP-1 and TIMP-2. Therefore, the inhibitory effect of CADPE on MMP-9 activity was mainly due to the transcriptional regulation and protein expression of MMP-9, which was further confirmed by MMP-9-luciferase report gene assay.

AP-1 and NF-kB are two key transcription factors involved in the regulation of MMP-9 gene expression (19). Activation of these transcription factors is centrally involved in the process of tumor invasion and metastasis by various agents including PMA, growth factors (e.g., EGF, vascular endothelial growth factor, platelet-derived growth factor, and transforming growth factor-β; refs. 37–39), and inflammatory cytokines (e.g., CXCL12 and tumor necrosis factor-α; refs. 40, 41). Thus, the regulation of AP-1 and NF-kB downstream of the FAK, phosphoinositide 3-kinase/Akt, and MAPK pathways might be involved in PMA-induced MMP-9 expression and invasion in MGC-803 cells. Luciferase reporter gene assay and mutation analysis of the promoter revealed that the major target of CADPE in the MMP-9 promoter was AP-1, whereas NF-kB had little effect on the transcriptional regulation of MMP-9. AP-1 is composed of members of the c-Jun and c-Fos families (21, 42, 43). They associate to form a variety of homodimers and heterodimers to regulate gene expression and localize to the nucleus when AP-1 is activated. Our results showed that CADPE suppressed nuclear c-Fos in a dose-dependent manner, whereas c-Jun or p65 was not affected, suggesting that the suppression of AP-1 is responsible for CADPE-induced inhibition of MMP-9 induction and cell invasion. AP-1 activity, including transcriptional activation, protein stability, and intracellular localization, was modulated by protein kinases such as MAPKs. It was reported that the intracellular localization, protein stability, and chromatin association of c-Fos family proteins were mainly regulated by ERK phosphorylation (44, 45). In our experiments, CADPE specifically suppressed PMA-mediated MEK and ERK phosphorylation without affecting pathways involving p38 and JNK. Activation of PKC was also little affected by CADPE, suggesting that the effects of CADPE are mediated by pathways other than PKC. Furthermore, CADPE also inhibits PMA-induced phosphorylation of FAK. FAK plays a critical role in contact formation between ECM and cytoskeleton and has been linked with cancer cell migration, invasion, proliferation, and survival (46). Therefore, suppression of FAK may partially explain the inhibition of cell migration in our assays.

In summary, our studies show that CADPE inhibits tumor cell migration and invasion by blocking the phosphorylation of protein kinases (FAK, MEK, and ERK) and the activation of AP-1 transcriptional factor. Therefore, CADPE is a potential agent for clinical use in preventing the invasion and metastasis of human malignant tumors, such as gastric cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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