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Brazilin Inhibits of TPA-induced MMP-9 Expression Via the Suppression of NF-κB Activation in MCF-7 Human Breast Carcinoma Cells

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ABSTRACT - Metastasis is the primary cause of from breast cancer mortality. Cell migration and invasion play important roles in neoplastic metastasis. Matrix metalloproteinase-9 (MMP-9), which degrades the extracellular matrix (ECM), plays an important role in cancer cell invasion. NF-κB is transcription factor important in the regulation of MMP-9, as the promoter of MMP-9 gene contains binding sites for NF-κB. Brazilin, an active component of sappan wood (*Caesalpinia sappan*), decreases TPA-induced MMP-9 expression and invasion in MCF-7 cells. Also, brazilin suppressed NF-κB activation in TPA-treated MCF-7 cells. Taken together, we demonstrated that the inhibition of TPA-induced MMP-9 expression and cell invasion by brazilin is mediated by the suppression of the NF-κB pathway in MCF-7 cells. This result suggest brazilin provide a potential therapeutic approach for the treatment of breast cancer.

Key words: Brazilin , MMP-9, metastasis, invasion, NF-κB, MCF-7

Introduction

Brazilin (7, 11b-dihydrobenz[b]indeno[1,2-d]pyran-3,6a,9, 10 (6H)-tetrol), the major component of Caesalpinia sappan L., is a natural red pigment. It is usually used for histological staining[1,2]. Previous studies, brazilin was demonstrated various biological effects including anti-hepatotoxicity[3], anti-platelet activity[4], inhibition of protein kinase C and insulin receptor kinase[5], induction of immunological tolerance[6,7], and anti-inflammatory activities[8-10]. Furthermore, recent studies revealed that brazilin involve regulation of transcription factors NF-κB and AP-1[9].

Breast cancer is one of the leading causes of malignancy related death in woman[11]. Most of breast cancer death cases are caused by distant metastasis from the primary tumor site. Despite successful treatment of the primary malignancy, relapse and subsequent metastatic spread can still occur at other areas of the body through the bloodstream or lymphatic channels. This leads to local, regional or distant metastasis, including bone, lung, liver, kidney, thyroid and brain[12]. Invasion and metastasis are the fundamental properties and major causes of morbidity and mortality in breast cancer patients. These processes require degradation of the extracellular matrix (ECM), which provides biochemical and mechanical barriers to cell movement in cancer cells[13]. ECM consists of type IV collagen, laminin, heparan sulfate proteoglycan, nidogen and fibronectin[14]. ECM degradation requires extracellular proteinases, of which the matrix metalloproteinases (MMPs) have been shown to play a critical role in breast cancer.

MMPs are a family of zinc- and calcium-dependent endopeptidases, consisting of four subclasses based on substrate, including collagenases, gelatinases, stromelysins and membrane-associated MMPs. MMP-9 is reported to be a key enzyme for degrading type IV collagen, which is a major component of the basement membrane. Elevated MMP-9 levels are functionally linked to elevated metastasis in many tumors, including brain[15], prostate[16], bladder[17] and breast[18,19]. Several mechanisms regulate MMP-9 activity, including gene transcription, proenzyme activation, and endogenous inhibitors such as tissue inhibitors of metalloproteinases (TIMPs). A variety of stimuli, including cytokines and phorbol ester, can stimulate MMP-9 synthesis and secretion during various pathological processes such as tumor invasion, atherosclerosis, inflammation, and rheumatoid arthritis. MMP-2, on the other hand, is usually expressed constitutively[19,20]. Cytokine and TPA treatments can induce MMP-9 expression via activation of transcription factors such as nuclear factor-κB (NF-κB). NF-κB is transcription factor important in the regulation of MMP-9, as the promoter of MMP-9 gene contains binding sites for NF-κB[21]. Therefore, it was hypothesized that brazilin may have anticancer properties inhibiting cell invasion.

In this study, brazilin was examined for its potential on

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TPA induced cell invasion and MMP-9 expression in MCF-7 cells with related molecular mechanisms. Our results demonstrated that brazilin suppresses TPA-induced MMP-9 expression by blocking the NF-κB signaling pathways and the suppression of MMP-9 expression is correlated well with its inhibition of cell invasion.

Materials and Methods

Cells and Material

MCF-7 cells were obtained from the American Type Culture Collection (Manassas, VA). Cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics at 37°C in a 5% CO_2 incubator. Brazilin was purchased from Sigma (St. Louis, MO. USA) and dissolved in dimethyl sulfoxide (DMSO). 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA) and 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), DMSO and anti-β-actin antibodies were obtained from Sigma (St. Louis, MO. USA). Primary antibodies for MMP-9, p50, p65, PCNA, and Horseradish peroxidase (HRP)-conjugated IgG were purchased from Santa Cruz Biotechnology (SantaCruz, CA. USA). [c32-P]ATP was obtained from Amersham (Buckinghamshire, UK). High glucose-containing Dulbecco's modified Eagle's medium (DMEM), FBS and phosphate-buffered saline (PBS) were obtained from Gibco-BRL (Gaithersburg, ME. USA).

Determination of cell viability

The effect of brazilin on MCF-7 cell viability was determined using an MTT assay. Briefly, cells were seeded to 3×10^4 cells/well, allowed to attach. After 24 h, cells were treated with various brazilin concentrations (10, 20, 50, 100 and 200 μM). After incubation for 24 h, cells were washed with PBS, MTT (0.5 mg/ml PBS) was added to each well and the plates were incubated at 37°C for 30 min. Formazan crystals were dissolved with DMSO (100 μl/well) and detected at 570 nm using a microplate reader (Model 3550, BIO-RAD, Richmond, CA, USA).

Western blot analysis

MCF-7 cells (5×10^5) were pre-treated with brazilin (20 μM and 50 μM) for 1 h and then incubated with TPA for 24 h. Cells were lysed with ice-cold M-PER® Mammalian Protein Extraction Reagent (Pierce Biotechnology, Rockford, IL). The protein concentration in the lysate was determined using the Bradford method[22]. Samples (20 μg) were separated by SDS-PAGE with 10% acrylamide, and transferred to hybond™-PVDF membranes using a Western blot apparatus. The PVDF membranes were blocked with 2% bovine serum albumin or 5% skim milk, and then incubated overnight with 1 μg/ml primary antibodies for MMP-9, β-actin, p50, p65, or PCNA. HRP-conjugated IgG was used as a secondary antibody. Protein expression levels were determined by signal analysis using an image analyzer (Fuji-Film, Japan).

Gelatin Zymography assay

Conditioned media were collected after 24 h stimulation, mixed with non-reducing sample buffer, and electrophoresed in a polyacrylamide gel containing 0.1% (w/v) gelatin. The gel was washed at room temperature for 30 min with 2.5% Triton X-100 solution, and subsequently incubated at 37°C for 16 h in 5 mM $CaCl_2$, 0.02% Brij, and 50 mM Tris–HCl (pH 7.5). The gel was stained for 30 min with 0.25% (w/v) Coomassie brilliant blue in 40% (v/v) methanol/7% (v/v) acetic acid and photographed on an image analyzer (Fuji-Film, Japan). Proteolysis was imaged as a white zone in a dark blue field. Densitometric analysis was performed using Multi Gauge Image Analysis software (Fuji-Film, Japan).

Quantitative real-time PCR assay

Total RNA was extracted from cells using a FastPure™ RNA Kit (TaKaRa, Shiga, Japan). The RNA concentration and purity were determined by absorbance at 260/280 nm. cDNA was synthesized from 1 μg total RNA using a PrimeScript™ RT reagent Kit (TaKaRa, Shiga, Japan). MMP-9 and GAPDH mRNA expression were determined by real-time PCR using the ABI PRISM 7900 sequence detection system and SYBR® Green (Applied Biosystems, Foster City, CA, USA). The primers were: MMP-9 (NM 004994) sense, CCTGGAGACCTGAGAACCAATCT; antisense, CCACC-CGAGTGTAACCATAGC and GAPDH (NM 002046) sense, ATGGAAATCCCATCACCATCTT; antisense, CGCCCCA-CTTGATTTTGG. To control for variation in mRNA concentration, all results were normalized to the housekeeping gene, GAPDH. Relative quantitation was performed using the comparative $\Delta\Delta C_t$ method according to the manufacturer's instructions.

Preparation of nuclear extract

MCF-7 cells (2×10^6) were treated with brazilin in the presence or absence of TPA for 4 h. Cells were immediately washed twice, scraped into 1.5 ml of ice-cold PBS (pH 7.5), and pelleted at 1,500 g for 3 min. Cytoplasmic and nuclear extracts were prepared from cells using the NE-PER® Nuclear and Cytoplasmic Extraction Reagents (Pierce Biotechnology, Rockford, IL).

Electrophoretic mobility shift assay (EMSA)

NF-κB activation was assayed with a gel mobility shift assay using nuclear extracts. An oligonucleotide containing the κ-chain (κB, 5'-CCGGTTAACAGAGGGGGCTTTC-

CGAG-3') binding site was synthesized and used as a probe for the gel retardation assay. The two complementary strands were annealed and labeled with [α-^{32}P]dCTP. Labeled oligonucleotides (10,000 cpm), 10 μg of nuclear extracts, and binding buffer (10 mM Tris-HCl, pH 7.6, 500 mM KCl, 10 mM EDTA, 50% glycerol, 100 ng poly (dI·dC), 1 mM DTT) were then incubated for 30 min at room temperature in a final volume of 20 μl. The reaction mixtures were analyzed by electrophoresis on 4% polyacrylamide gels in 0.5X Tris-borate buffer. The gels were dried and examined by autoradiography. Specific binding was controlled by competition with a 50-fold excess of cold κB oligonucleotide.

Invasion assay

The invasion assay was carried out in 24-well chambers (8 μm pore size) coated with 20 μl matrigel diluted DMEM. The matrigel coating was re-hydrated in 0.5 ml DMEM for 30 min immediately before the experiments. 2×10^5 cells were added to the upper chamber with chemoattractant in the bottom well. Conditioned medium (0.5 ml) was added to the lower compartment of the invasion chamber. The chambers were incubated for 24 h. After incubation, cells on the upper side of the chamber were removed using cotton swabs, and cells that had migrated were fixed and stained with Toluidine blue solution. Invading cells were counted in five random areas of the membrane using a light microscope. Analyzed data are the means ± SE from three individual experiments performed in triplicate.

Statistical analysis. Statistical data analysis was performed using ANOVA and Duncan's test. Differences with a $p < 0.05$ were considered statistically significant.

Results

Effect of brazilin on of MCF-7 cell viability

We first investigated the cytotoxicity of brazilin (Fig. 1A) on MCF-7 cells, the cells were seeded into 96-well culture plates at a density of 1×10^5 cells/well. Effect of brazilin on MCF-7 cellular toxicity was analyzed using the MTT assay. Treatment of MCF-7 cells with indicated concentrations of brazilin for 24 h did not show significant cytotoxicity (Fig. 1B).

Brazilin inhibits TPA-induced MMP-9 expression in MCF-7 cells

To investigate the effect of brazilin on TPA-induced MMP-9 expression, we performed western blot analysis, real-time PCR, and zymography in MCF-7 cells. Western blot analysis revealed that brazilin treatment in MCF-7 cells blocked the up-regulation of TPA-induced MMP-9 protein expression (Fig. 2A). Real-time PCR revealed that TPA increased the MMP-9 level in MCF-7 cells, and that brazilin blocked TPA-induced MMP-9 up-regulation in a dose-dependent manner (Fig. 2B). To determine the effect on TPA-induced MMP-9 secretion by brazilin, we performed zymography; MCF-7 cell treatment with TPA resulted in increased MMP-9 secretion. Brazilin significantly diminished TPA-induced MMP-9 secretion (Fig. 2C). These results indicate that brazilin is a potent inhibitor of TPA-induced MMP-9 expression in MCF-7 cells.

Brazilin suppresses TPA-induced NF-κB DNA binding activity in MCF-7 cells

To determine the mechanism of brazilin -mediated inhibition of MMP-9 expression, the effect of brazilin on TPA-induced activation of NF-κB was evaluated using EMSA. As shown in Fig. 3, TPA increased substantially NF-κB binding activity. Pre-treatment with brazilin inhibited TPA-stimulated NF-κB binding activity. Brazilin itself had no effect on NF-κB binding activity. These results suggest that brazilin specifically blocks NF-κB activation in MCF-7 cells.

Brazilin inhibits TPA-induced invasion of MCF-7cells

It has been reported that the up-regulation of MMP-9 expression contributes to invasion of cancer cells[23,24]. An *in vitro* invasion assay was used to investigate the inhibitory effects of brazilin on the invasive potency of breast carcinoma

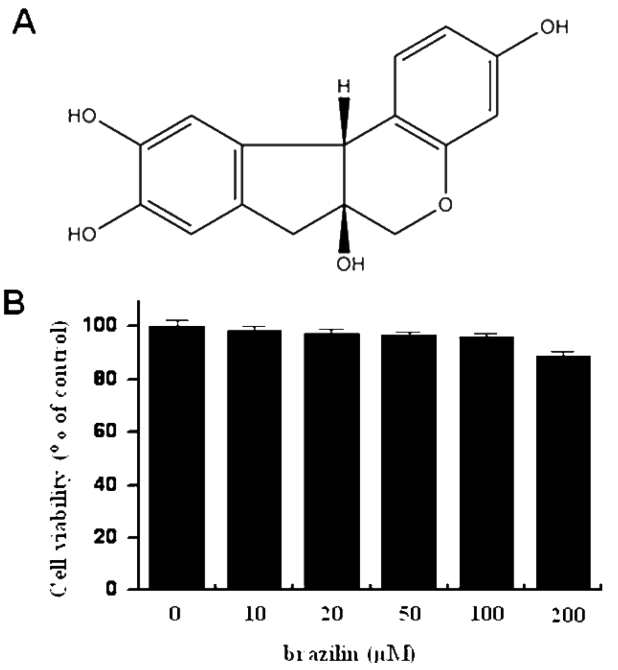

Fig. 1. Structure of brazilin and effects of brazilin on cell viability of MCF-7 cell. Chemical structure of brazilin (A). To cytotoxicity test of brazilin, Cells were cultured in 96-well plates until 70% confluence and various concentrations of brazilin were added to cells for 24h. MTT assay was used to detect the viability of the cells (B). The optical density value of control was regarded as 100%. Data points are the mean ± SE of three independent experiments.

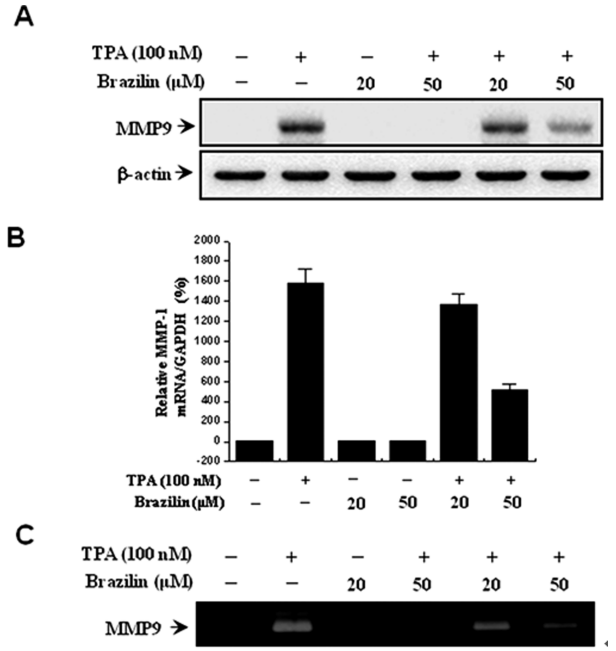

Fig. 2. Brazilin inhibits TPA-induced MMP-9 expression in MCF-7 cells. MCF-7 cells in monolayer were treated with the indicated brazilin concentrations in the presence of TPA for 24 h. Cell lysates were analyzed by Western blot with anti-MMP-9. The blot was reprobed with anti-β-actin to confirm equal loading (A). Conditioned medium was prepared and used for gelatin zymography (B). MMP-9 mRNA levels were analyzed by real-time PCR and GAPDH was used as an internal control (C). Each value represents the mean ± SEM of three independent experiments. $^{*}p < 0.01$ vs. TPA.

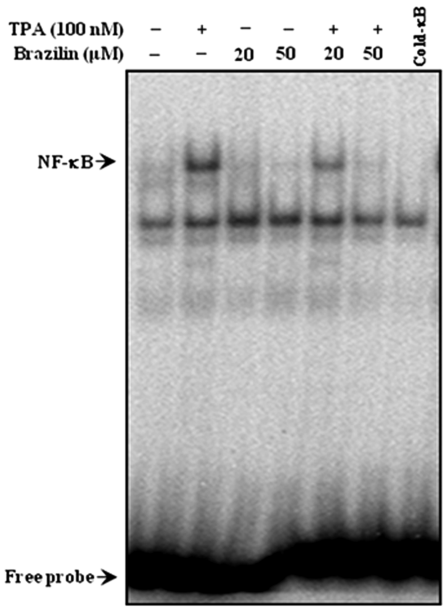

Fig. 3. Brazilin blocks TPA-induced NF-κB activation in MCF-7 cells. Cells were treated with brazilin in the presence of TPA. Following 3 h of incubation, nuclear extracts were prepared as described in Materials and Methods. NF-κB DNA binding was analyzed by electrophoretic mobility shift analysis as described in Materials and Methods.

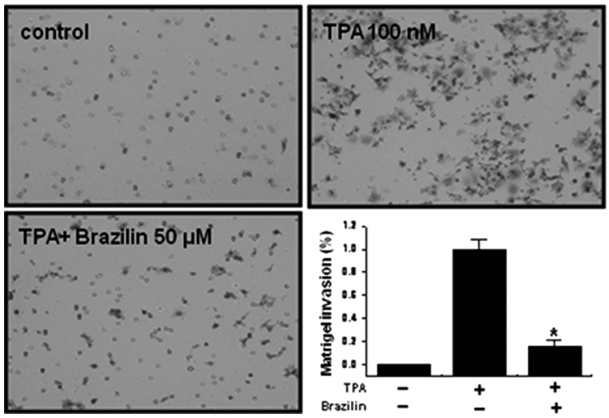

Fig. 4. Effect of brazilin on TPA-induced Matrigel invasion in MCF-7 cells. Cells were seeded onto the upper chamber and drugs placed in the well. After a 24 h incubation, cells on the bottom of filter were fixed, stained, and counted. 1, Control; 2, TPA alone (100 nM); 3, TPA with brazilin (50 μM). Each value represents the mean ± SEM of three independent experiments. $^{*}p < 0.01$ vs. TPA.

MCF-7 cells. TPA treatment increased MCF-7 cell invasion when compared with untreated control cells, as determined by a Matrigel invasion assay. Brazilin inhibited the TPA-induced MCF-7 cell invasion (Fig. 4).

Discussion

Breast cancer is the main cause of death from cancer in women globally. It is the second leading cause of woman death in the United States. Metastasis is the primary cause of breast cancer mortality. Tumor metastasis is a multistep process by which a subset of individual cancers disseminate from a primary tumor to distant secondary organs or tissues in a complex process that includes cell proliferation, ECM degradation, cell migration, and tumor growth at metastatic sites[19,25]. MMP-9 has been regarded as major critical molecules in processing tumor invasion and metastasis. MMP-9 activation has been shown to be especially associated with tumor progression and invasion, including mammary tumors[27]. In previous reports, inflammatory cytokines, growth factors, or phorbol esters stimulated MMP-9 by activating different intracellular-signaling pathways in breast cancer cells[28-30]. The inhibitory effects on expression are important for the development of a therapeutic experimental model of tumor metastasis.

NF-κB is transcription factor important in regulating MMP-9, as the MMP-9 gene promoter contains binding sites for transcription factors[21]. NF-κB comprises a family of inducible transcription factors which regulate host inflammatory and immune responses[31]. Diverse signal transduction cascades

mediate NF-κB pathway stimulation[31]. NF-κB elements are centrally involved in MMP-9 gene induction by TPA[25,32]. Our results show that brazilin inhibited MMP-9 expression by suppression of NF-κB in breast carcinoma cells.

In this study, we have for the first time provided evidence that brazilin inhibits TPA-induced expression of MMP-9 in breast carcinoma cells. Furthermore, we also demonstrated the molecular mechanism of brazilin. Our results also showed that brazilin blocked TPA-induced NF-κB activation. Recent studies have clearly implicated multiple targets of brazilin action. Recent several studies focused on heme oxygenase-1 activation by brazilin, but many studies have been growing interest in effect of anti-inflammatory by brazilin[9,10,33]. Bae *et al.* demonstrated that NF-κB is molecular target in brazilin treated cells[9]. These results indicate that brazilin can affect proliferation signals and apoptotic signals via modulation of NF-κB activity.

In conclusion, our results have demonstrated that brazilin is a potent inhibitor of TPA-induced MMP-9 expression and strongly blocks the ability of NF-κB signalling pathway in breast carcinoma cells. This is the first study showing brazilin suppress TPA-stimulated cancer cell invasion by inhibiting MMP-9 expression. Thus, brazilin may be a potential candidate to prevent breast tumor invasion and metastasis *in vivo*.

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