Sulforaphane controls TPA-induced MMP-9 expression through the NF-κB signaling pathway, but not AP-1, in MCF-7 breast cancer cells

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Sulforaphane [1-isothiocyanato-4-(methylsulfinyl)-butane] is an isothiocyanate found in some cruciferous vegetables, especially broccoli. Sulforaphane has been shown to display anti-cancer properties against various cancer cell lines. Matrix metalloproteinase-9 (MMP-9), which degrades the extracellular matrix (ECM), plays an important role in cancer cell invasion. In this study, we investigated the effect of sulforaphane on 12-O-tetradecanoyl phorbol-13-acetate (TPA)-induced MMP-9 expression and cell invasion in MCF-7 cells. TPA-induced MMP-9 expression and cell invasion were decreased by sulforaphane treatment. TPA substantially increased NF-κB and AP-1 DNA binding activity. Pre-treatment with sulforaphane inhibited TPA-stimulated NF-κB binding activity, but not AP-1 binding activity. In addition, we found that sulforaphane suppressed NF-κB activation, by inhibiting phosphorylation of IκB in TPA-treated MCF-7 cells. In this study, we demonstrated that the inhibition of TPA-induced MMP-9 expression and cell invasion by sulforaphane was mediated by the suppression of the NF-κB pathway in MCF-7 cells. [BMB Reports 2013; 46(4): 201-206]

INTRODUCTION

Sulforaphane [1-isothiocyanato-4-(methylsulfinyl)-butane] is one of the most abundant isothiocyanates in some cruciferous vegeta-

bles, especially broccoli (1). Sulforaphane has been shown to display anti-cancer properties against various cancer cell lines (2-4). In recent studies, NF-κB was reported to be a molecular target for sulforaphane mediated cancer chemoprevention through anti-inflammatory mechanisms (5-9). Sulforaphane has also been reported to inhibit the cell-mediated immune response in B16F-10 melanoma-induced metastasis-bearing C57BL/6 mice (10, 11).

Breast cancer is one of the leading causes of malignancy-related death in women (12). Most cases of breast cancer death 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 (13). This process requires degradation of the extracellular matrix (ECM) and basement membranes, which provides biochemical and mechanical barriers to cell movement in cancer cells (14). Many studies have assessed matrix metalloproteinase (MMP) expression correlated with invasive ability or metastatic potential of tumor (15), and the many factors that influence the transcription of MMP genes include cytokines, growth factors and phorbol ester (16). Among the MMPs, MMP-9 is a key enzyme for degrading type IV collagen, which is localized in the basement membrane (17). Elevated MMP-9 levels are functionally linked with elevated metastasis in many tumors (18, 19), and MMP-9-deficient mice exhibit suppression of metastasis (20).

Cytokine and phorbol-ester treatments have been shown to MMP-9 expression, via activation of transcription factors such as NF-κB and activator protein-1 (AP-1) (15-17). Also, the mitogen-activated protein kinase (MAPK) signaling pathway is important for NF-κB activation and AP-1 activation (15, 18). Consequently, MMP-9 expression and/or its upstream regulatory pathways would be critical targets in treating malignant tumors, including breast cancer. Therefore, we hypothesized that sulforaphane may have anticancer properties, by inhibiting

MMP-9 expression and cell invasion in breast cancer cells.

Our results demonstrated that sulforaphane suppresses TPA-induced MMP-9 expression through inhibition of the NF-κB, which occurs by blocking IκB phosphorylation, and the suppression of MMP-9 expression correlated well with its ability to inhibit cell invasion.

RESULTS

Effect of sulforaphane on MCF-7 cell viability

In order to investigate the cytotoxicity of sulforaphane on MCF-7 cells, the cells were seeded into plate at a density of 1×10^5 cells/plate. MCF-7 cellular toxicity was analyzed using the MTT assay. Treatment of MCF-7 cells with (0.5-5 μM) sulforaphane for 24 h did not cause any significant change in cell viability (Fig. 1B). But, the pro-survival effect of TPA did not show significant at treatment with sulforaphane (1 μM and 5 μM) (Fig. 1C), indicating that sulforaphane does not affect cell proliferation. Therefore, we performed experiments at the optimal non-toxic sulforaphane concentration (1 μM and 5 μM).

Effect of sulforaphane on TPA-induced MMP-9 expression in MCF-7 cells

To investigate the effect of sulforaphane on TPA-induced MMP-9 expression, we performed western blot analysis, real-time PCR, and zymography in MCF-7 cells. Western blot anal-

ysis/real-time PCR/zymography revealed that sulforaphane blocked the up-regulation of TPA-induced MMP-9 protein/mRNA expression and secretion (Fig. 2A-C). These results indicate that sulforaphane is a potent inhibitor of TPA-induced MMP-9 expression and secretion in MCF-7 cells.

Effect of sulforaphane on TPA-induced NF-κB and AP-1 activation

To determine the mechanism of sulforaphane-mediated inhibition of MMP-9 expression, the effects of sulforaphane on TPA-induced activation of NF-κB and AP-1 were evaluated using EMSA and western blot analysis. As shown in Fig. 3A, TPA substantially increased NF-κB and AP-1 binding activity. Pre-treatment with sulforaphane inhibited TPA-stimulated NF-κB binding activity, but not AP-1 binding activity. Sulforaphane itself had no effect on NF-κB and AP-1 binding activity. In addition, we determined the levels of p65, p50 and p-c-Jun in the nuclear fraction. TPA treatment resulted in increase of p65, p50 and p-c-Jun. However, sulforaphane blocked the TPA-induced translocation of NF-κB to the nucleus (Fig. 3B). These results suggest that sulforaphane specifically blocked NF-κB activation in MCF-7 cells. The IκB Kinase (IKK) enzyme complexs are part of the upstream NF-κB signal transduction cascade (19). IKK phosphorylation results in the dissociation of IκB from NF-κB, and thereby activates NF-κB. Therefore, we examined the effect of TPA and sulforaphane treatment on the p-IKKαβ, p-IκBα levels
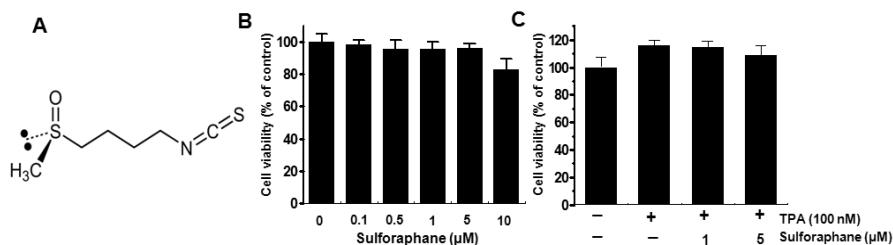

Fig. 1. Structure of sulforaphne and effect of sulforaphane on cell viability of MCF-7 viability. Chemical structure of sulforaphane (A). To examine the cytotoxicity of sulforaphane, cells were cultured in 96-well plates up to a confluency of 70%, and various concentrations of sulforaphane were added to cells for 24 h (B). MTT assay was used to detect the viability of the cells. Cells were pretreated with 1 μM and 5 μM sulforaphanes for 1 h, before incubation with 100 nM TPA for 24 h (C). The optical density of the control was regarded as 100%. Data points are the mean ± SEM of three independent experiments.
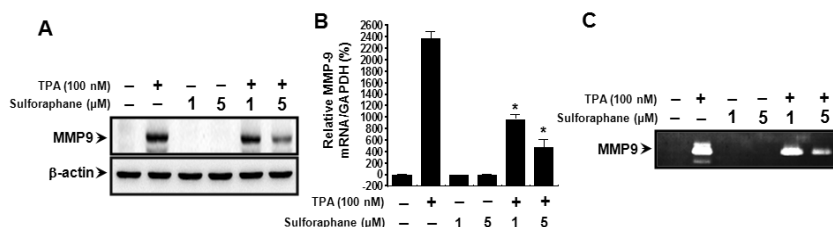

Fig. 2. Sulforaphane inhibits TPA-induced MMP-9 expression in MCF-7 cells. MCF-7 cells were treated with the indicated sulforaphane concentrations in the presence of TPA for 24 h. Cell lysates were analyzed by western blot analysis with anti-MMP-9. The blot was reprobed with anti-β-actin to confirm equal loading (A). MMP-9 mRNA levels were analyzed by real-time PCR, and GAPDH was used as an internal control (B). Conditioned medium was prepared, and used for gelatin zymography (C). Each value represents the mean ± SEM of three independent experiments. *P < 0.01 vs. TPA.
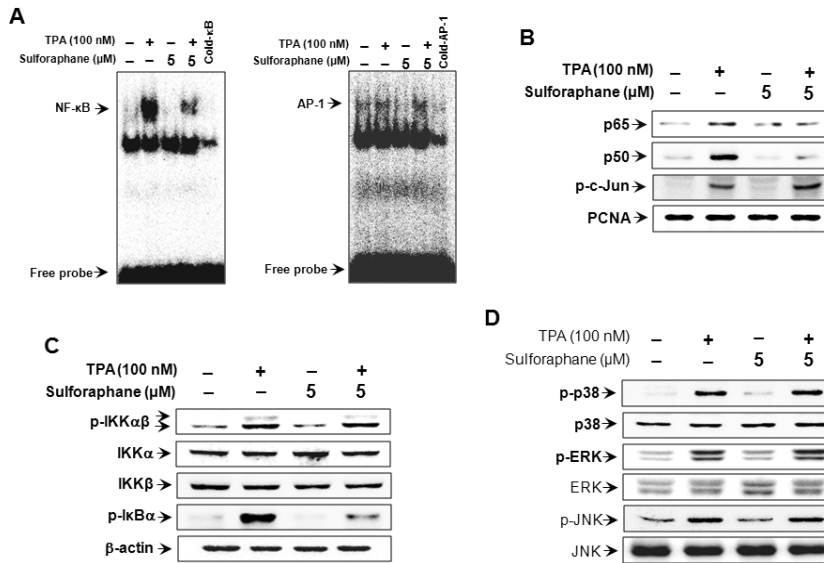
Fig. 3. Sulforaphane blocks TPA-induced NF-κB activation in MCF-7 cells. Cells were treated with sulforaphane in the presence of TPA. Following 3 h incubation, nuclear extracts were prepared as described in the Materials and Methods. NF-κB/AP-1 DNA binding was analyzed by EMSA, as described in Materials and Methods (A). Nuclear/cytoplasm extracts were subjected to western blot analysis (B, C). Cell extracts were prepared from MCF-7 cells with TPA for 15 min in the absence or presence of sulforaphane, and subjected to western blotting (D).

in the cytoplamic fraction. TPA-stimulated MCF-7 cells showed increased levels of p-IKKαβ and p-IκBα level. The p-IκBα level was significantly suppressed by treatment with sulforaphane, but not p-IKKαβ (Fig. 3C).

Effect of sulforaphane on TPA-induced MAPKs signaling pathway

MAPKs (ERK, p38 and JNK) have been shown to be upstream modulators of NF-κB and AP-1, which results in the activation of MMP-9 expression (20, 21). Thus, we investigated the effect of sulforaphane on TPA-induced activation of MAPKs. Sulforaphane showed no effect on the MAPKs (Fig. 3D). These results suggest that the MAPK pathway is not involved in the regulation of TPA-induced MMP-9 expression by sulforaphane.

Effect of sulforaphane on TPA-induced MCF-7 cell invasion *in vitro*

It has been reported that the up-regulation of MMP-9 expression contributes to the invasion of cancer cells (22, 23). An *in vitro* invasion assay was used to investigate the effects of sulforaphane on the invasive potency of breast carcinoma MCF-7 cells. TPA treatment increased MCF-7 cell invasion when compared with untreated control cells, as determined by a Matrigel invasion assay. However, sulforaphane inhibited TPA-induced MCF-7 cell invasion by 80% (Fig. 4).

DISCUSSION

In this study, we demonstrated that sulforaphane inhibited TPA-induced MMP-9 expression and cell invasion in MCF-7 cells. Furthermore, sulforaphane strongly blocked TPA-mediated activation of NF-κB, but not AP-1, in MCF-7 cells. These findings suggest that the inhibition of TPA-induced MMP-9 ex-
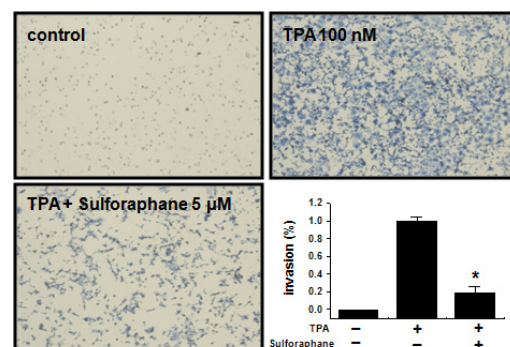

Fig. 4. Effect of sulforaphane on TPA-induced Matrigel invasion in MCF-7 cells. Cells were seeded onto the upper chamber, and TPA and sulforaphane placed in the well. Each value represents the mean ± SEM of three independent experiments. *P < 0.01 vs. TPA.

pression and cell invasion by sulforaphane is mediated by the suppression of the NF-κB pathway in MCF-7 cells.

Recent studies have clearly demonstrated that the action of sulforaphane involves multiple targets. Early research focused on Phase 2 enzyme induction by sulforaphane, as well as inhibition of enzymes involved in carcinogen activation, but recent studies have identified other activities of sulforaphane, including chemoprotection and anti-inflammation (6, 7, 11, 24, 25). Previous studies have demonstrated that NF-κB is a molecular target in sulforaphane treated cells (5-7, 18, 19). These results indicate that sulforaphane can affect proliferation signals and apoptotic signals, via modulation of NF-κB activity.

Globally, breast cancer is the main cause of death from cancer in women. Metastasis is the primary cause of breast cancer mortality. Tumor metastasis is a multistep process in a complex

process that includes cell proliferation, ECM degradation, cell migration, and tumor growth at metastatic sites (15, 26). Morphologically, tumor invasion is associated with a distorted edge of the primary tumor, where individual or cohorts of tumor cells actively invade the tissue surrounding ECM tissue (27).

MMP-9 has been regarded as major critical molecule in processing tumor invasion and metastasis. MMP-9 activation has been shown to be associated with tumor progression and invasion, including mammary tumors (28). In previous reports, inflammatory cytokines, growth factors, or phorbol esters were shown to stimulate MMP-9 by activating different intracellular-signaling pathways in breast cancer cells (29-31). The PKCs can be activated by phorbol esters in vitro, and TPA acts as a potential inducer of tumor invasion and migration in various tumor cells. Up-regulation and activation of PKCs are highly correlated with an increased invasiveness in breast carcinomas (32-34). The inhibitory effects on expression are important for the development of a therapeutic experimental model of tumor metastasis.

The three major MAPKs families, JNK, ERK, and p38 kinase, are expressed, and the active phosphorylated forms of these proteins have been detected in MCF-7 cells (12). The results of the present study suggest that sulforaphane does not inhibit the phosphorylation of MAPKs in TPA-mediated signaling pathways. These findings suggest that sulforaphane is not involved in the TPA-stimulated MAPKs pathway.

NF-κB is a transcription factor that regulates MMP-9 expression binding sites on its promoter (35, 36). NF-κB comprises a family of inducible transcription factors that regulate host inflammatory and immune responses. Diverse signal transduction cascades mediate NF-κB pathway stimulation (37). NF-κB is an inducible dimeric transcription factor that belongs to the Rel/NF-κB family of transcription factors, and consists of two major polypeptides, p65 and p50 (38). NF-κB is initially located in the cytoplasm in an inactive form complexed with IκB, an inhibitory factor of NF-κB. NF-κB elements are centrally involved in MMP-9 gene induction by TPA (15, 16, 39). Our results show that sulforaphane inhibited MMP-9 expression by suppression of NF-κB in breast carcinoma cells.

In this study, we identified the molecular mechanisms of the MAPKs, NF-κB and AP-1 signal pathways in breast cancer cells responsible for the sulforaphane inhibitory effect. Our results have demonstrated that sulforaphane is a potent inhibitor of TPA-induced MMP-9 expression, and strongly blocks the NF-κB signaling pathway in breast carcinoma cells. This is the first study demonstrating that sulforaphane suppresses TPA-stimulated cancer cell invasion by inhibiting MMP-9 expression. Thus, sulforaphane may be a potential candidate in the development of novel therapeutics to preventing breast tumor invasion and metastasis *in vivo*.

MATERIALS AND METHODS

Cells and chemicals
MCF-7 cells were purchased from the American Type Culture Collection (Manassas, VA). Cells were cultured in high glucose-containing Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics, at 37°C in a 5% CO_2 incubator. Sulforaphane was obtained from Sigma (St. Louis, MO. USA). 12-O-tetradecanoyl-phorbol-13-acetate (TPA) and 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO) and anti-β-actin and toluidine were obtained from Sigma (St. Louis, MO, USA). Primary p-c-Jun, p-IκBα, p-IKKαβ, p38, p-p38, JNK, p-JNK, ERK, and p-ERK antibodies were from Cell Signaling Technology (Beverly, MA, USA). MMP-9, p50, p65, IKKα, IKKβ, IκBα, PCNA, and Horseradish peroxidase (HRP)-conjugated IgG were from Santa Cruz Biotechnology (Santa Cruz, CA. USA). [α-^{32}P]dCTP was purchased from Amersham (Buckinghamshire, UK). DMEM, FBS and phosphate-buffered saline (PBS) were obtained from Gibco-BRL (Gaithersburg, ME, USA).

Determination of cell viability
The effect of sulforaphane on MCF-7 cell viability was determined using an MTT assay. After incubation, the cells were treated with MTT (0.5 mg/ml) solution for 30 min. The dark blue formazan crystals were solubilized with DMSO (100 μl/well), and detected at 570 nm using a microplate reader (Model 3550, BIO-RAD, Richmond, CA, USA).

Western blot analysis
After treatment, cells were collected and washed with PBS. The protein concentration in the lysate was determined using the Bradford method (40). The samples (20 μg) of total cell lysates or cytosolic fractions/nuclear fractions were separated by 10% SDS-PAGE. The PVDF membranes were blocked with 5% bovine serum albumin or 5% skim milk, and then incubated overnight with 1 μg/ml primary antibodies. 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
The enzyme activity of MMP-9 was determined by gelatin zymography. Conditioned media were collected after 24 h stimulation, mixed with non-reducing sample buffer, and electrophoresed using 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 assessed based on the white zone in a dark blue field.

Quantitative real-time PCR assay
Total RNA was isolated with a FastPure™ RNA Kit (TaKaRa, Shiga, Japan). RNA concentration and purity were determined by absorbance at 260/280 nm. 1 μg total RNA was converted to cDNA 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, CCTGGAG ACCTGAGAACCAATCT; antisense, CCACCCGAG TGTA ACCATAGC and GAPDH (NM 002046) sense, ATGGA AATCCCATCACCATCTT; antisense, CGCCCCACTTG ATTTT GG. To control for variation in mRNA concentration, all results were normalized to the GAPDH, a housekeeping gene. Relative quantitation was performed using the comparative $\Delta\Delta C_t$ method, according to the manufacturer's instructions.

Preparation of nuclear extract
Cytoplasmic and nuclear extracts were prepared using the NE-PER® Nuclear and Cytoplasmic Extraction Reagents (Pierce Biotechnology, Rockford, IL), according to the manufacturer's instructions.

Electrophoretic mobility shift assay (EMSA)
Labeled [α-^{32}P]dCTP 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. The oligonucleotide sequences of NF-κB and AP-1 probe were those from human MMP-9 promoter; NF-κB, 5'-CCGGTTAACAGAGGGGGCTTTCCGAG-3' and AP-1, 5'-C GCTTGATGAGTCAGCCGGAA-3'. The oligomers were synthesized, and used as a probe in the gel retardation assay. Specific binding was controlled by competition, with a 50-fold excess of cold κB and AP-1 oligonucleotide.

Invasion assay
Cells (2 × 10^5) were added to an inner cup of a 24-well chamber (BD Biosciences) that had been coated with 20 μl matrigel (BD Biosciences, Franklin Lakes, NJ) 1 : 6 dilution in serum-free medium). TPA and sulforaphane were placed in the bottom well. 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, in each case, the mean $\pm$ SE from three individual experiments performed in triplicate.

Statistical analysis
Statistical data analysis was performed using ANOVA. Differences with a $P < 0.05$ were considered statistically significant.

Acknowledgements

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