

DHA blocks TPA-induced cell invasion by inhibiting MMP-9 expression via suppression of the PPAR- γ /NF- κ B pathway in MCF-7 cells

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Abstract. Docosahexaenoic acid (DHA) is an omega-3 fatty acid that is considered to have applications in cancer prevention and treatment. The beneficial effects of DHA against cancer metastasis are well established; however, the mechanisms underlying these effects in breast cancer are not clear. Cell invasion is critical for neoplastic metastasis, and involves the degradation of the extracellular matrix by matrix metalloproteinase (MMP)-9. The present study investigated the inhibitory effect of DHA on MMP-9 expression and cell invasion induced by 12-*O*-tetradecanoylphorbol-13-acetate (TPA) in the MCF-7 breast cancer cell line. DHA inhibited the TPA-induced activation of mitogen-activated protein kinase (MAPK) and the transcription of nuclear factor (NF)- κ B, but did not inhibit the transcription of activator protein-1. DHA increased the activity of peroxisome proliferator-activated receptor (PPAR)- γ , an effect that was reversed by the application

of the PPAR- γ antagonist GW9662. In addition, combined treatment with GW9662 and DHA increased NF- κ B-related protein expression. These results indicate that DHA regulates MMP-9 expression and cell invasion via modulation of the MAPK signaling pathway and PPAR- γ /NF- κ B activity. This suggests that DHA could be a potential therapeutic agent for the prevention of breast cancer metastasis.

Introduction

Docosahexaenoic acid (DHA) is an omega (ω)-3 fatty acid (22:6 Δ 4,7,10,13,16,19) and a member of a family of compounds known to possess multiple benefits for human health, including anticancer properties (1). Epidemiological evidence strongly links fish oil, which is rich in DHA and eicosapentaenoic acid (EPA), with a reduced incidence of several types of cancer, including breast cancer (2,3). Epidemiological studies, dietary studies in mice and humans, and tissue culture studies have substantiated the beneficial role of DHA in breast cancer prevention (4-7).

Cancer treatment failure largely occurs due to cancer cell proliferation, invasion and metastasis, which ultimately lead to mortality. Invasion and metastasis are the major causes of morbidity and mortality in breast cancer patients (3). Metastasis involves the penetration of cancer cells into the extracellular basement membrane by proteolytic degradation of components of the extracellular matrix (ECM) (8), including type IV collagen, laminin, heparin sulfate proteoglycan, nidogen and fibronectin (9), which normally provide biochemical and mechanical barriers to cell movement (10).

ECM degradation requires extracellular proteinases such as matrix metalloproteinases (MMPs), a group of zinc and calcium-dependent endopeptidases that can be divided into different subclasses (including collagenases, gelatinases and stromelysins) based on their substrate (8). MMP-9 is directly

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associated with invasion, metastasis and poor prognosis in breast cancer (11,12). MMP-9 is stimulated by growth factors (including fibroblast growth factor 2, epidermal growth factor and hepatocyte growth factor), cytokines (such as tumor necrosis factor α), oncogenes (such as Ras) and 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (13-17). TPA is a selective activator of protein kinase C (14). TPA can stimulate MMP-9 synthesis and secretion during breast cancer cell invasion (18,19). Cytokine and TPA treatment induce MMP-9 expression via activation of transcription factors such as nuclear factor (NF)- κ B and activator protein (AP)-1 (20-22), since the MMP-9 gene promoter contains binding sites for both factors (23). Mitogen-activated protein kinase (MAPK) signaling is important for AP-1 and NF- κ B activation, and requires nuclear factor of κ light polypeptide gene enhancer in B cells inhibitor (I κ B) kinase, extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) or p38 MAPK, depending on the cell type (17,24,25). Thus, inhibiting MMP-9 expression and/or its upstream regulatory pathways may aid in the treatment of malignant tumors, including breast carcinoma.

Peroxisome proliferator-activated receptors (PPARs) belong to the nuclear receptor superfamily of ligand-activated transcription factors. Three types of PPARs [- α , - β and - δ (β)] have been identified thus far (26). PPAR- γ is expressed in various cell types and tissues, and is important in the regulation of inflammatory (27) and immune reactions (28), as well as in NF- κ B activation (29). In a previous study, agents that inhibit MMP-9 expression in MCF-7 cells were described (30-32). The present study reports for the first time that increased PPAR- γ expression inhibits breast cancer metastasis via regulation of NF- κ B activation. These results provide an insight into the anti-cancer actions of an ω -3 fatty acid, which could aid in the development of new cancer therapeutic strategies involving the use of fish oil as a dietary supplement.

Materials and methods

Cells and reagents. MCF-7 cells were obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics at 37°C in a 5% CO₂ incubator. TPA, *cis*-4,7,10,13,16,19-DHA, MTT, GW9662 (a PPAR- γ antagonist) and anti- β -actin antibody (catalog no. A5441) were obtained from Sigma-Aldrich (Merck Millipore, Darmstadt, Germany). Antibodies against p38 (catalog no. 9212), phosphorylated (p)-p38 (catalog no. 9211), JNK (catalog no. 9252), p-JNK (catalog no. 9251), ERK (catalog no. 9102) and p-ERK (catalog no. 9101) were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Antibodies against PPAR- γ (catalog no. 7169), MMP-9 (catalog no. 12759), p50 (catalog no. 7178), p65 (catalog no. 372) and proliferating cell nuclear antigen (catalog no. 7907), and horseradish peroxidase (HRP)-conjugated immunoglobulin (Ig)G (catalog no. 2004, 2005) were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). [γ -³²P] adenosine triphosphate was obtained from GE Healthcare Life Sciences (Chalfont, UK). High glucose DMEM, FBS and PBS were acquired from Gibco (Thermo Fisher Scientific, Inc., Waltham, MA, USA).

Cell viability assay. The effect of DHA on MCF-7 cell viability was determined by MTT assay. A stock solution of 200 mM DHA was prepared in dimethylsulfoxide (DMSO) and diluted with DMEM prior to the experiments. Briefly, 3x10⁴ cells/well were seeded in 96-well plates and incubated at 37°C for 24 h to allow attachment. Cells were then left untreated, or were treated with 50, 100 and 200 μ M DHA for 24 h at 37°C. Cells were next washed with PBS prior to the addition of MTT (0.5 mg/ml in PBS), and incubated at 37°C for 30 min. Formazan crystals were dissolved with DMSO (100 μ l/well), and absorbance was measured at 570 nm using a Model 3550 microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Western blot analysis. MCF-7 cells (5x10⁵) were pre-treated with 50 and 100 μ M DHA for 1 h, and then incubated with TPA for 24 h at 37°C. Cells were lysed with ice-cold M-PER[®] Mammalian Protein Extraction Reagent (Pierce; Thermo Fisher Scientific, Inc.). The protein concentration in the lysate was determined by the Bradford method (33). Samples (20 μ g) were separated by 10% SDS-PAGE and transferred to Hybond polyvinylidene difluoride membranes (GE Healthcare Life Sciences). Membranes were blocked for 2 h with 2% bovine serum albumin (Sigma-Aldrich; Merck Millipore) or 5% skimmed milk, and then incubated overnight at 4°C with primary antibodies at 1:2,000 dilution, followed by incubation with HRP-conjugated IgG at 1:2,000 dilution for 2 h at 4°C. Protein expression levels were measured by signal analysis using an image analyzer (Fujifilm, Tokyo, Japan) and specific immunoreactive signals were visualized with an enhanced chemiluminescence kit (GE Healthcare Life Sciences).

Gelatin zymography assay. Conditioned media were collected after 24 h of cell stimulation, mixed with non-reducing sample buffer and resolved by PAGE containing 0.1% (w/v) gelatin. The gel was washed at room temperature for 30 min with 2.5% Triton X-100 solution, and incubated at 37°C for 16 h in 5 mM CaCl₂, 0.02% Brij (Sigma-Aldrich; Merck Millipore) 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 (Fujifilm). Proteolysis was imaged as a white zone in a dark blue field. Densitometric analysis was performed using MultiGauge image analysis software (version 3.0; Fujifilm).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from cells using a FastPure RNA kit (Takara Bio, Inc., Otsu, Japan). The RNA concentration and purity were assessed by measuring the absorbance value at 260/280 nm. Complementary DNA was synthesized from 1 μ g total RNA using a PrimeScript RT reagent kit (Takara Bio, Inc.) at 37°C for 15 min and 85°C for 5 sec. MMP-9 and GAPDH messenger (m)RNA expression were determined by qPCR using the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems; Thermo Fisher Scientific, Inc.) and SYBR Green PCR Master Mix (Applied Biosystems; Thermo Fisher Scientific, Inc.) with the following sense and antisense primer sets: MMP-9 (NM_004994), 5'-CCT GGA GAC CTG AGA ACC

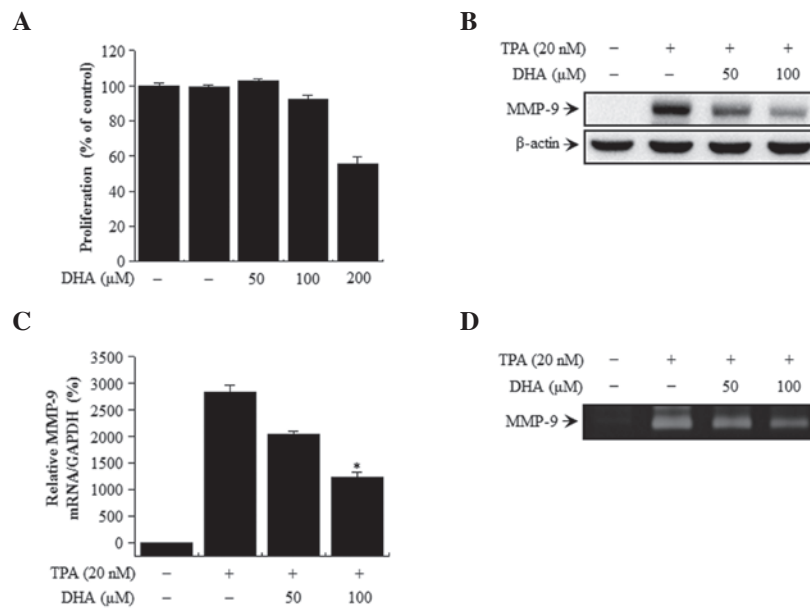


Figure 1. Effect of DHA on cell viability and TPA-induced MMP-9 expression in MCF-7 cells. (A) The cytotoxicity of DHA was assessed using the MTT assay in cells exposed to the indicated concentrations of DHA for 24 h. The optical density value of control cells was considered as 100%. (B) MMP-9 expression was detected in cell lysates by western blotting, with β -actin used as a loading control. (C) MMP-9 mRNA level was analyzed by reverse transcription-quantitative polymerase chain reaction relative to the level of GAPDH. (D) MMP-9 activity was evaluated in conditioned medium by gelatin zymography. Values represent the mean \pm standard error of the mean of three independent experiments. * $P < 0.01$ vs. TPA. TPA, 12-*O*-tetradecanoylphorbol-13-acetate; DHA, docosahexaenoic acid; MMP, matrix metalloproteinase; mRNA, messenger RNA.

AATCT-3' (forward) and 5'-CCA CCC GAG TGT AAC CAT AGC-3' (reverse), and GAPDH (NM_002046), 5'-ATG GAA ATC CCA TCA CCA TCTT-3' (forward) and 5'-CGC CCC ACT TGA TTT TGG-3' (reverse). To control for variation in mRNA concentration, the results were normalized to the level of GAPDH. Relative quantitation was performed using the comparative $2^{-\Delta\Delta C_t}$ method (34), according to the manufacturer's protocol.

Preparation of nuclear extract. MCF-7 cells (2×10^6) were treated with DHA in the presence or absence of TPA for 4 h. Cells were immediately washed twice, scraped into 1.5 ml ice-cold PBS (pH 7.5) and pelleted at $1,500 \times g$ for 3 min. Cytoplasmic and nuclear extracts were prepared from cells using the NE-PER[®] Nuclear and Cytoplasmic Extraction kit (Pierce; Thermo Fisher Scientific, Inc.).

Electrophoretic mobility shift assay (EMSA). NF- κ B activation was evaluated with a gel mobility shift assay using nuclear extracts. Oligonucleotides containing a binding site for the κ chain (κ B, 5'-CCG GTT AAC AGA GGG GGC TTT CCGAG-3') or AP-1 (5'-CGC TTG ATG AGT CAG CCG GAA-3') binding site was synthesized and used as a probe (Promega, Madison, WI, USA). The two complementary strands were annealed and labeled with [α -³²P] deoxycytidine triphosphate. Labeled oligonucleotides (10,000 cpm) were combined with 10 μ g 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) (Roche, Basel, Switzerland) and 1 mM dithiothreitol], and incubated for 30 min at room temperature in a final volume of 20 μ l. The reaction products were analyzed by 4% PAGE in 0.5X Tris-borate buffer. The gels were then dried and examined by autoradiography. A

50-fold excess of cold κ B oligonucleotide was used as a control to confirm binding specificity.

Invasion assay. The invasion assay was conducted in 24-well chambers (8- μ m pore size) coated with 20 μ l Matrigel diluted in DMEM. The Matrigel coating was re-hydrated in 0.5 ml DMEM for 30 min immediately prior to the experiment. Cells (2×10^5) were added to the upper chamber, with the chemoattractant in the bottom well. Conditioned medium (0.5 ml) was added to the lower compartment of the invasion chamber, followed by incubation for 24 h. Subsequently, cells on the upper side of the chamber were removed using cotton swabs, while those that had migrated were fixed and stained with Toluidine Blue solution. Invading cells were counted in five random areas of the membrane under a light microscope. Data from three individual experiments performed in triplicate were analyzed and presented as the mean \pm standard error of the mean.

Statistical analysis. Data were evaluated by analysis of variance and Duncan's test using the Microsoft 2010 Excel program (Microsoft Corporation, Redmond, WA, USA). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

MCF-7 cell viability is unaffected by DHA treatment. The cytotoxicity of DHA on MCF-7 cells was evaluated by MTT assay. There were no changes in cell viability or morphology upon treatment with the indicated concentrations of DHA for 24 h (Fig. 1A). Therefore, the subsequent experiments were performed at optimal, non-toxic DHA concentrations of 50 and 100 μ M.

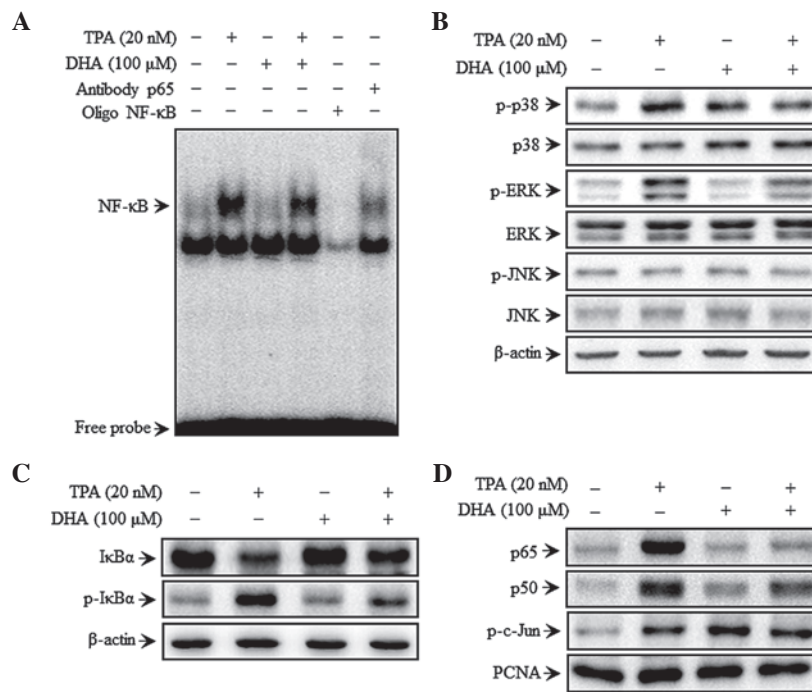


Figure 2. DHA inhibits TPA-induced transcriptional activation of matrix metalloproteinase-9 and p38 phosphorylation in MCF-7 cells. Cells were treated with DHA in the presence of TPA for 4 h, followed by nuclear extraction. (A) NF- κ B DNA binding was analyzed by electrophoretic mobility shift assay. (B) Cells were pretreated with TPA for 30 min in the presence or absence of DHA, and cell lysates were prepared for western blotting to detect p-p38, p38, p-JNK, JNK, p-ERK and ERK expression. (C) Cytoplasmic levels of NF- κ B subunits, I κ B α and p-I κ B α , were determined by western blotting. (D) Nuclear levels of NF κ B (p50 and p65) and activator protein 1 (p-c-Jun) subunits were determined by western blotting. TPA, 12-*O*-tetradecanoylphorbol-13-acetate; DHA, docosahexaenoic acid; NF, nuclear factor; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; I κ B, nuclear factor of κ light polypeptide gene enhancer in B cells inhibitor; p, phosphorylated; PCNA, proliferating cell nuclear antigen.

DHA suppresses TPA-induced MMP-9 activation in MCF-7 cells. The effect of DHA on TPA-induced MMP-9 expression in MCF-7 cells was examined by western blot analysis, RT-qPCR and gelatin zymography. DHA treatment blocked the upregulation of MMP-9 protein expression induced by TPA, as determined by western blotting (Fig. 1B). Accordingly, RT-qPCR analysis revealed that the increase in MMP-9 expression induced by TPA treatment was abrogated by DHA in a dose-dependent manner (Fig. 1C). MMP-9 secretion was stimulated by TPA, but this effect was abrogated by treatment with DHA, as determined by zymography (Fig. 1D). These results indicate that DHA potently inhibits the TPA-induced increase in MMP-9 levels in MCF-7 cells.

DHA inhibits TPA-induced NF- κ B but not AP-1 DNA binding activity, as well as MAPK signaling. To investigate the mechanism of DHA-mediated inhibition of MMP-9 expression, the effect of DHA on TPA-induced NF- κ B activation was evaluated by EMSA. TPA increased the NF- κ B binding activity, whereas pre-treatment with DHA abolished this effect for NF- κ B (Fig. 2A) but not for AP-1 (data not shown). These results suggest that DHA specifically blocks NF- κ B activation in MCF-7 cells. DHA inhibited the phosphorylation of p38 and ERK, but not that of JNK, 30 min after TPA treatment (Fig. 2B). Additionally, TPA induced the phosphorylation of cytoplasmic I κ B α and the consequent nuclear translocation of the NF- κ B subunits p50 and p65, as determined by western blot analysis. In addition, c-Jun but not c-Fos expression was upregulated upon TPA treatment. Additionally, TPA also increased p-I κ B α expression, as well as p65 and p50

translocation, which was suppressed by treatment with DHA (Fig. 2C and D). However, the TPA induced phosphorylation of c-Jun (a major subunit of AP-1) was unaffected by DHA (Fig. 2D). These results suggest that the effect of DHA on the regulation of TPA-induced MMP-9 expression is via modulation of MAPK signaling.

DHA increases PPAR- γ expression. DHA treatment increased PPAR- γ expression in a dose-dependent manner in MCF-7 cells (Fig. 3A). To verify whether MMP-9 expression is regulated by PPAR- γ , TPA-treated cells were treated concurrently with DHA in the presence or absence of GW9662. MMP-9 expression was higher upon treatment in the presence of GW9662 than following treatment with DHA alone (Fig. 3B). GW9662 also rescued the expression of p65 and p50 that was abolished by DHA treatment (Fig. 3C). These results indicate that DHA inhibits MAPK signaling and MMP-9 expression by increasing PPAR- γ expression.

DHA treatment inhibits TPA-induced MCF-7 cell invasion. The upregulation of MMP-9 expression contributes to cancer cell invasion (10,27). Therefore, the effect of DHA on the invasive potential of MCF-7 cells was investigated by Matrigel assay. TPA treatment stimulated MCF-7 cell invasion 10-fold more, compared with untreated control cells. Treatment with DHA decreased TPA-induced cell invasion by 60%. However, a decrease of only 30% was observed in the presence of both GW9662 and DHA (Fig. 4), indicating that DHA suppresses the invasive potential of breast cancer cells via PPAR- γ activation.

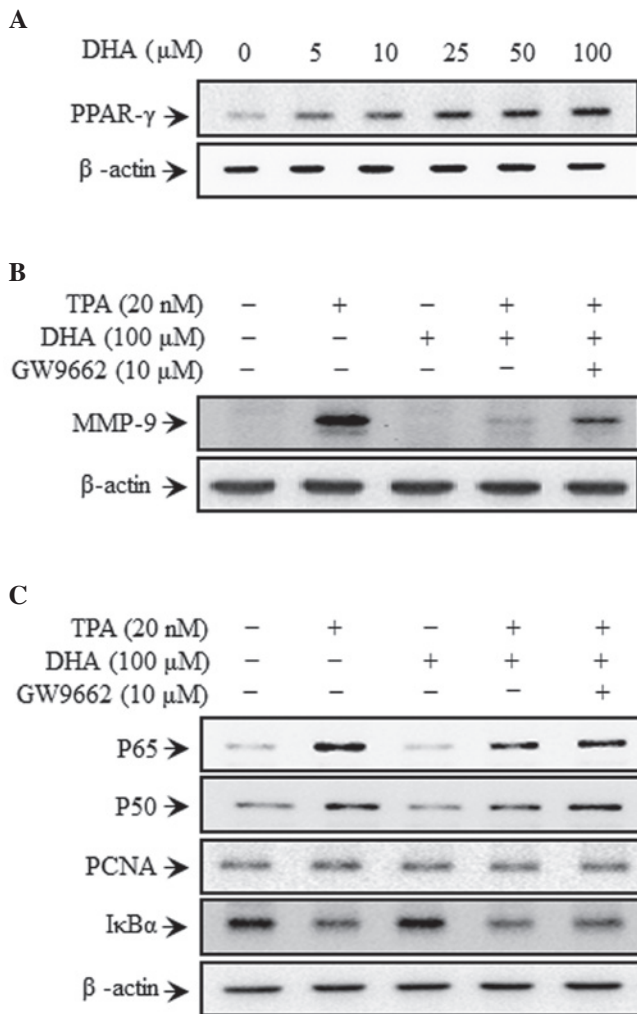


Figure 3. DHA induces upregulation of PPAR- γ in MCF-7 cells. Cells were treated with DHA and GW9662 in the presence of TPA for 24 h. Subsequently, cell lysates were prepared for western blotting to detect (A) PPAR- γ , (B) MMP-9 and (C) p50, p65 and I κ B α expression. TPA, 12-*O*-tetradecanoylphorbol-13-acetate; DHA, docosahexaenoic acid; I κ B, nuclear factor of κ light polypeptide gene enhancer in B cells inhibitor; PCNA, proliferating cell nuclear antigen; PPAR, peroxisome proliferator-activated receptor.

Discussion

The protective effects of fish oil are attributable to ω -3 polyunsaturated fatty acids (PUFAs), including EPA (20:5 ω -3) and DHA (22:6 n-3), which are converted by fish from α -linolenic acid (LNA; 18:3 ω -3) present in ingested cold water vegetation (1). A study of metastatic mouse mammary carcinoma reported that a diet containing α -LNA-rich linseed oil effectively arrested tumor progression (12). Furthermore, tumor growth and metastasis were inhibited by diets containing fish oil, EPA or DHA (27,35). ω -3 PUFAs may affect carcinogenesis by altering transcription factor activity, gene expression and/or signal transduction (7,36). DHA is a particularly potent enhancer of tumor cell chemosensitivity (37). However, there is little information on the anti-metastatic effects of DHA in human breast cancer cells.

Metastasis is the primary cause of breast cancer mortality, and involves cell proliferation, ECM degradation, cell migration and tumor growth at secondary sites (19,20). Tumor cell

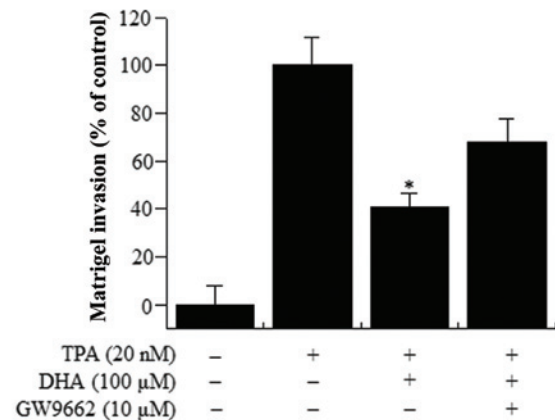
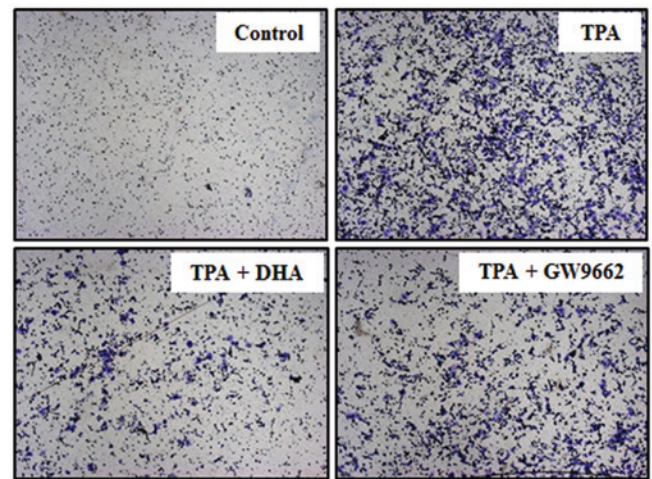


Figure 4. Effect of DHA on the TPA-induced invasion of MCF-7 cells. Cell invasion was determined by Matrigel assay. Cells were seeded in the upper chamber and drugs were placed in the well. After 24 h of incubation, invaded cells on the bottom of the filter were fixed, stained and counted ($\times 100$ magnification). Values represent the mean + standard error of the mean of three independent experiments. * $P < 0.01$ vs. TPA. TPA, 12-*O*-tetradecanoylphorbol-13-acetate; DHA, docosahexaenoic acid.

invasion is an early step in this process, representing the transition from a benign state to malignancy (11). Tumor invasion is morphologically distinguished by a distortion of the primary tumor edge where individual or cohorts of tumor cells actively invade the surrounding ECM (38). MMP-9 is a critical molecule in tumor invasion and metastasis, and its activation is associated with the progression of mammary tumors (39). Inflammatory cytokines, growth factors or phorbol esters stimulate MMP-9 by activating different intracellular signaling pathways in breast cancer cells (40-42). Thus, inhibiting the expression and/or activity of MMP-9 may be an important strategy for slowing or preventing tumor metastasis.

AP-1 belongs to the basic region/leucine zipper motif group of DNA-binding proteins, and homo or heterodimerizes in response to signaling events to indirectly or directly activate c-Jun and c-Fos expression (43-46). NF- κ B is a member of a family of inducible transcription factors that regulate host inflammatory and immune responses (47) as a result of MAPK signaling, which requires cell type-specific I κ B kinase expression (17,24,25,48,49). Both AP-1 and NF- κ B have been implicated in TPA-mediated MMP-9 gene induction (20,21). The present results demonstrate that DHA inhibits

TPA-induced MMP-9 expression via activation of NF- κ B, but not of AP-1, in MCF-7 breast cancer cells.

PPAR- γ is a member of the nuclear receptor and ligand-activated steroid hormone receptor-regulated transcription factor superfamily (26). ω -3 PUFAs are natural ligands of nuclear receptors such as PPAR- α and - γ , and can upregulate PPAR- γ expression (50). PPAR- γ has been reported to inhibit the NF- κ B signaling pathway by reducing NF- κ B binding activity and physically interacting with both p65 and p50 (51,26). The present study confirmed that PPAR- γ expression was increased by treatment with DHA, which consequently led to a decrease in p65 and p50 levels, and a decrease in MMP-9 expression. NF- κ B inhibition by DHA was reversed by treatment with the PPAR- γ -specific antagonist GW9662, demonstrating that TPA-induced tumor cell invasion was suppressed by DHA through a PPAR- γ -dependent mechanism.

In conclusion, DHA is a potent inhibitor of TPA-induced MMP-9 expression, and blocks breast carcinoma cell invasion by modulating the NF- κ B signaling pathway via PPAR- γ upregulation. These findings suggest that DHA may be an effective therapeutic agent for preventing breast tumor invasion and metastasis.

Acknowledgements

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