

**Complementary actions of docosahexaenoic acid and genistein on COX-2, PGE<sub>2</sub> and  
invasiveness in MDA-MB-231 breast cancer cells**

Eva Horia and Bruce A. Watkins\*

Center for Enhancing Foods to Protect Health, Lipid Chemistry and Molecular Biology  
Laboratory, Purdue University, West Lafayette, Indiana, 47907-2009

Running title: N-3 PUFA, genistein and breast cancer

\* Address correspondence to:

Bruce A. Watkins, Ph.D.  
745 Agriculture Mall Drive  
Purdue University  
West Lafayette, IN 47907-2009  
Telephone: 765-494-5802  
Fax: 765-494-7953  
E-mail: [baw@purdue.edu](mailto:baw@purdue.edu)

**List of the abbreviations:**

AA, arachidonic acid; COX, cyclooxygenase; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; IL, interleukin; LA, linoleic acid; NF $\kappa$ B, nuclear factor kappa B; PGE<sub>2</sub>, prostaglandin E<sub>2</sub>; PPAR, peroxisome proliferator activated receptor; PPRE, peroxisome proliferator receptor element; LC-PUFA, long chain polyunsaturated fatty acids; Q-PCR, quantitative polymerase chain reaction; TNF $\alpha$ , tumor necrosis factor- $\alpha$ .

## ABSTRACT

N-3 polyunsaturated fatty acids (PUFA) and genistein have been associated with lowered cancer risk by reducing inflammatory prostanoids, cyclooxygenase-2 (COX-2) activity, and altering cell signaling. Few studies have investigated the effect of these compounds in combination on the molecular control of the COX-2 gene. In a series of experiments we examined a potential synchronous action of n-3 PUFA and genistein in down-regulating COX-2 expression to diminish prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) production in MDA-MB-231 human breast cancer cells. Cells were treated with genistein and various PUFA including arachidonic acid (AA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA). PGE<sub>2</sub> concentrations, expression of COX-2, and cell invasiveness were determined. The n-3 PUFA and genistein alone lowered PGE<sub>2</sub> concentration, and genistein in combination with AA reversed the high level of this prostanoid in cell cultures enriched with AA. The degree of cell invasiveness was reversed by genistein in cell cultures treated with AA and further reduced in those given DHA. The n-3 PUFA, in contrast to AA, reduced COX-2 and NFκB expression. Genistein combined with AA reversed the effects of AA alone on the expression of COX-2 and NFκB. All three fatty acids increased the expression of PPARγ in the cells only when combined with genistein. Our results support the premise that DHA and genistein exert complementary actions whilst genistein is antagonistic to AA for controlling PGE<sub>2</sub> production as well as invasiveness of MDA-MB-231 cells in culture by modulating the level of NFκB expression.

**Key words:** MDA-MB-231 breast cancer cells; docosahexaenoic acid; genistein; prostaglandin E<sub>2</sub>, COX-2; cell invasiveness

## INTRODUCTION

Variation in the incidence of cancer around the world is, in part, attributed to the significant difference in dietary patterns for fats and phytochemicals such as those in the U.S. compared to Japan. Cancer is the second leading cause of death, while breast cancer is the most common type of this disease among Americans with 1 in 7 women developing breast cancer (1). In contrast, the incidence and mortality rates of breast cancer in Japanese women are only one-third of those in Americans (2). Although the rates of breast cancer are much lower in Japan, they have continually climbed in the past 30 years as the diet in Japan has become more westernized similar to that in America.

In the past four decades, Japanese have increased their consumption of animal products and calories from fat while decreased their intake of grains (3). Meat consumption has increased 7-fold and dairy (which includes conjugated linoleic acids) up to 4-fold in Japan. Since animal products are the principle sources of arachidonic acid (AA), the change in the Japanese diet has resulted in an extraordinary rise in the amount of n-6 polyunsaturated fatty acids (PUFA) thus elevating the ratio of n-6/n-3 PUFA. Regardless of this increase, in 2000 the estimated dietary ratio of 4:1 for n-6/n-3 PUFA in Japan is still considerably lower than the 10:1 to 15:1 range in the American diet (4). The major dietary sources of n-3 PUFA (eicosapentaenoic acid EPA and docosahexaenoic acid DHA) in the Japanese diet include fish, shellfish, and seaweed.

In addition to the high n-3 PUFA intake, Japanese consume several soy containing food products. Individually, n-3 PUFA or soy components have been implicated in epidemiological (5,6), cell culture, and animal studies (7,8) to play a role in reducing the risk of breast cancer. However, few studies (only two) have investigated the combined effects of n-3 PUFA and soy

genistein on breast cancer (9,10). Considering that these food components, n-3 PUFA and soy genistein, are usually consumed as part of the daily Japanese diet, often together in the same meal, they may provide additive or synergistic beneficial effects to protect against chronic diseases.

The most prominent mechanism for the chemopreventive action of n-3 PUFA is their suppressive effect on the production of AA-derived prostanoids, particularly prostaglandin E<sub>2</sub> (PGE<sub>2</sub>). This prostanoid has been implicated to play a critical role in immune response to cancer cells, inflammation, cancer cell proliferation, differentiation, apoptosis, angiogenesis, and metastasis (7). The n-3 PUFA compete with n-6 PUFA for incorporation into the membrane phospholipids (11), for the activity of elongases and desaturases involved in the conversion of 18 carbon to 20 and 22 carbon PUFA, and for cyclooxygenase (COX) catalytic sites (12). Moreover, some studies proposed that n-3 PUFA down-regulate COX-2 expression (13) by affecting nuclear transcription factors, and altering signal transduction and cell signaling (14). Importantly, EPA-derived PGE<sub>3</sub> is much less efficient compared to PGE<sub>2</sub> in inducing COX-2 expression (15) and it is a weaker inflammatory agent (16).

Genistein was reported to lower PGE<sub>2</sub> production in mesangial cells and macrophages (17,18). Genistein may lower PGE<sub>2</sub> by blocking the mitogen-activated protein kinase signaling cascades (19) that activate the transcription of the COX-2 gene, or as a potent peroxisome proliferator activated receptor gamma (PPAR $\gamma$ ) ligand increasing peroxisome proliferator response element (PPRE) transcriptional activity at concentrations of  $\geq 5$   $\mu\text{mol/L}$  (20). Genistein has also been demonstrated to activate PPRE transcriptional activity through PPAR $\alpha$  in HeoG2 human hepatoma cells (21). Activation of PPRE has been associated with the inhibition of nuclear factor kappa B (NF $\kappa$ B) activity and a consequential reduction in COX-2 expression (22).

In addition, genistein can act via a PPAR $\gamma$ -independent mechanism to inhibit NF $\kappa$ B activation and the binding of NF $\kappa$ B to DNA (23,24). Therefore, genistein can potentially reduce the level of COX-2 protein and PGE<sub>2</sub> production by altering NF $\kappa$ B signaling as demonstrated in macrophages (18).

In the present investigation, n-3 PUFA and genistein were hypothesized to synergistically suppress AA-derived PGE<sub>2</sub> production and COX-2 expression in MDA-MB-231 cancer cells to decrease cell invasiveness. MDA-MB-231 is a highly invasive cancer cell line that overexpresses COX-2. The effects of n-6 PUFA compared to n-3 PUFA alone and in combination with genistein were studied on PGE<sub>2</sub> production and COX-2 expression. Levels of NF $\kappa$ B and PPAR $\gamma$ , the nuclear factors involved in the transcription of COX-2 gene, and the invasive capacity of the cells were also examined.

## MATERIALS AND METHODS

**Cells and reagents.** The MDA-MB-231 human breast cancer cell line was purchased from American Type Culture Collection (ATCC, Manassas, VA). Iscove's Modified Dulbecco Medium (IMDM) and fetal bovine serum (FBS) were obtained from Sigma (St. Louis, MO) and antibiotic-antimycotic solution from Invitrogen (Carlsbad, CA).

The following were also purchased: AA, EPA, and DHA ( $\geq 99\%$  purity) from Nu-Chek-Prep (Elysian, MN); fatty acid free-bovine serum albumin (BSA), dimethyl sulphoxide (DMSO), formalin solution (4% formaldehyde), and methylene blue from Sigma; genistein (5,7,4'-trihydroxyisoflavone,  $>99\%$  pure) from Indofine Chemical Company, Inc. (Hillsborough, NJ); tetradecanoyl phorbol acetate (TPA,  $\geq 99\%$  purity) from Calbiochem (San Diego, CA); and

ethanol from AAPER Alcohol and Chemical Co. (Shelbyville, KY). All other chemicals, unless noted, were purchased from Sigma.

STAT-Prostaglandin E<sub>2</sub> assay kits were purchased from Cayman Chemical (Ann Arbor, MI), Matrigel and filter inserts from BD Biosciences (Bedford, MA), RNAqueous<sup>®</sup>-4PCR kit from Ambion (Austin, TX), iScript<sup>™</sup> cDNA Synthesis kit from Bio-Rad (Hercules, CA), SYBR<sup>®</sup> Green PCR master mix from Applied Biosystems (Warrington, UK) and all primers from Sigma-Genosys (Woodlands, TX).

***Cell culture.*** MDA-MB-231 cells were routinely maintained in IMDM supplemented with 10% FBS and 1% antibiotic-antimycotic solution at 37°C in 5% CO<sub>2</sub>. Cells from passages 5 to 40 were used for the experiments.

***Fatty acid enrichment and genistein treatment.*** Treatment media with fatty acids were prepared by addition of fatty acids dissolved in ethanol (< 0.1% ethanol) into IMDM at a ratio of BSA to fatty acids of 2:1 (500 µmol/L BSA) as described previously (25). For treatments with genistein, the medium was prepared by addition of genistein dissolved in DMSO to the medium immediately prior to use, maintaining a 0.1% concentration of DMSO in the medium. The media for the control cell cultures contained only vehicle (BSA, DMSO, or BSA plus DMSO).

***PGE<sub>2</sub> assay.*** MDA-MB-231 cells were seeded at 30,000 cells/well in 12-well plates for 3 days until 90% confluent. After 24 hr of treatment with fatty acid and/or genistein supplemented medium, cells were washed with PBS then treated with IMDM containing 10% FBS and 10 nmol/L TPA for 24 hr. TPA was dissolved in DMSO (not to exceed 0.1% in the medium).

Samples of cell culture media were collected and PGE<sub>2</sub> concentrations analyzed with a competitive enzyme immunoassay kit (STAT-Prostaglandin E<sub>2</sub>).

***Invasion assay.*** The invasion capacity of MDA-MB-231 cells was examined using a modified Boyden chamber Matrigel invasion assay (26). Cells were grown to ~90% confluency, serum starved for 24 hr, followed by 24 hr treatment with PUFA and/or genistein. At the end of the treatment period,  $2 \times 10^5$  cells suspended in fresh treatment medium were added to the upper compartment of the Boyden chamber and treatment medium containing 10% FBS was added to the lower chamber. Boyden chambers were prepared by coating the upper surface of track-etched polyethylene terephthalate 8  $\mu\text{m}$ -pore size filter inserts with 85  $\mu\text{g}/\text{cm}^2$  Matrigel. After cells were incubated for 18 hr at 37°C, the invaded cells on the lower side of the membrane were fixed with formalin solution (4% w/v formaldehyde - 10% neutral buffered AFIP formulation) and stained with 0.2% methylene blue. The filters were examined by microscopy and results were expressed as percentage of invaded cells in the treatment group compared to those in the control group.

***Quantitative real-time PCR.*** Cells were cultured in 6-well plates until 90% confluent followed by treatment with fatty acid and/or genistein supplemented media for the times selected. Total RNA was isolated using an RNAqueous<sup>®</sup>-4PCR kit. The yield and quality of the RNA were assessed by UV absorbance at 260 and 280 nm, respectively. First strand cDNA for COX-2, NF $\kappa$ B, PPAR $\gamma$ , and  $\beta$ -actin were synthesized from 1  $\mu\text{g}$  RNA using an iScript<sup>™</sup> cDNA Synthesis kit. Quantitative real-time PCR was performed in 96-well optical plates using the ABI Prism 7700 Sequence Detection System (Applied Biosystems, Warrington, UK).

Briefly, 1  $\mu$ l of the cDNA product, 12.5  $\mu$ l of SYBR<sup>®</sup> Green PCR master mix, 9.5  $\mu$ l nuclease-free water, and 1  $\mu$ l (25 pmole/ $\mu$ l) each of the forward and reverse primers, were added to each well to a final volume of 25  $\mu$ l. All primers were designed using Primer Express<sup>®</sup> Software v2.0 (Applied Biosystems). Primer sequences for the genes were as follows: COX-2 forward: GAATCATTACCAGGCAAATTG, COX-2 reverse: TCTGTACTGCGGGTGAACA, NF $\kappa$ B forward: GGCTACACCGAAGCAATTGAA, NF $\kappa$ B reverse: CAGCGAGTGGGCCTGAGA, PPAR $\gamma$  forward: GGCTTCATGACAAGGGAGTTTC, PPAR $\gamma$  reverse: AA ACTCAA ACTTGGGCTCCATAAA,  $\beta$ -actin forward: CCTGGCACCCAGCACAAT,  $\beta$ -actin reverse: GCCGATCCACACGGAGTACT. The thermal settings for PCR were 50°C for 2 min, 95°C for 10 min, 95°C for 15 sec, and 59°C for 1 min [40 X]. Additional steps at 95°C for 15 sec, 59°C for 20 sec, and 19 min 59 sec temperature ramp to reach 95°C and held for 15 sec were performed to construct thermal dissociation curves to confirm the absence of nonspecific amplification.

***Statistical analyses.*** Data were analyzed by either a Student's t-test or one-way ANOVA. For ANOVA analysis, where significant differences were found, a Tukey's multiple comparison test was performed at a probability of  $P < 0.05$  (SAS software, SAS Institute Inc., Cary, NC). All data are presented as means  $\pm$  SD or as standardized differences calculated from the difference between values of treatment and control divided by the pooled SEM.

## RESULTS

***PGE<sub>2</sub> biosynthesis.*** To determine whether PUFA act alone or in combination with genistein to affect PGE<sub>2</sub> synthesis, MDA-MB-231 cells were treated for 24 hr and subsequently treated with TPA for an additional 24 hr. First, the effect of genistein alone was characterized on PGE<sub>2</sub> production. Genistein dose-dependently reduced the amount of PGE<sub>2</sub> produced by MDA-MB-231 cells compared to the vehicle control (Figure 1 panel A). The suppression observed in cells treated with genistein was 11% at 1.0  $\mu\text{mol/L}$  and 13% at 2.5  $\mu\text{mol/L}$  compared to the vehicle control. The effect of DHA on PGE<sub>2</sub> synthesis was also determined. DHA showed dose-dependent reduction of PGE<sub>2</sub> synthesis in MDA-MB-231 cells compared to the vehicle control (Figure 1 panel B). The production of PGE<sub>2</sub> in cells treated with AA was 57-fold higher compared to the vehicle control (Figure 1 panel C). The addition of genistein to cells enriched with AA reduced the amount of PGE<sub>2</sub> by 26% compared to the treatment of AA alone. Both EPA and DHA treatments [long chain (LC) n-3 PUFA] with and without genistein resulted in significantly lower amounts of PGE<sub>2</sub> in cells compared to the AA treatment. Importantly, the treatment of DHA with genistein resulted in a 37% lower concentration of PGE<sub>2</sub> compared to the vehicle control (Figure 1 panel C, insert).

***Invasion assay.*** The Matrigel invasion assay was performed to examine whether the changes in PGE<sub>2</sub> concentrations resulting from PUFA and genistein treatments correlated with the invasive phenotype of the MDA-MB-231 cells. Genistein alone at 10  $\mu\text{mol/L}$  significantly reduced the number of cells invading the membrane by 40% compared to the vehicle control (Figure 2). At concentrations lower than 10  $\mu\text{mol/L}$ , genistein did not significantly affect the

invasive capacity of MDA-MB-231 cells (data not shown). Treatment of cells with AA increased invasion by 40% compared to the vehicle control. However, simultaneous addition of genistein with AA abolished the effect of AA on cancer cell invasiveness. Thus, genistein treatment with AA attenuated the cancer promoting effect of this n-6 PUFA on breast cancer cells. In contrast, DHA significantly reduced invasion by 27%, and with the addition of genistein a further decline in the number of invaded cells was observed (a decrease of 61% compared to the vehicle control). EPA alone did not affect the invasiveness of the cells when compared to those in the vehicle control. These findings indicate that genistein blunted the invasiveness of breast cancer cells subjected to AA and markedly reduced invasiveness when treated simultaneously with DHA.

***Expression of COX-2, PPAR $\gamma$ , and NF $\kappa$ B genes.*** The effect of genistein at concentrations ranging from 0.1 to 2.5  $\mu$ M on the expression of COX-2 gene in MDA-MB-231 cells was determined after 24 hr treatment (Figure 3). No significant change in the level of COX-2 mRNA was detected using quantitative real-time PCR method. The effects of 24 hr treatments with PUFA at 50  $\mu$ mol/L (with and without genistein, 0 and 2.5  $\mu$ mol/L) on the transcription rate of COX-2, PPAR $\gamma$ , and NF $\kappa$ B were also investigated using quantitative real-time PCR in MDA-MB-231 cells. The EPA and DHA treatments including those with genistein led to reduced COX-2 mRNA levels compared to the vehicle control, but AA did not show a suppressive action on COX-2 transcription unless combined with genistein (Figure 4 panel A). Importantly, genistein alone did not affect the level of COX-2 mRNA (as also shown in Figure 3) and hence the suppression observed in the AA plus genistein treatment was an effect exclusive to the combination of these compounds. This finding suggests that genistein has a protective

effect by reducing the action of AA on COX-2 at the transcription level to the same extent achieved by LC n-3 PUFA treatments. To further study how PUFA and genistein impact the mechanism involved in the molecular control of the COX-2 gene expression, the effects of these dietary components on PPAR $\gamma$  and NF $\kappa$ B genes expression were also examined. Genistein treatment alone at the concentration used had no significant effect on mRNA levels for both PPAR $\gamma$  (Figure 4 panel B) and NF $\kappa$ B (Figure 4 panel C). The combination of all PUFA treatments with genistein resulted in a higher level of PPAR $\gamma$  mRNA, however, EPA alone reduced this transcription factor in these cells. Both LC n-3 PUFA independent of genistein addition significantly reduced the levels of NF $\kappa$ B mRNA. In contrast, the addition of genistein was necessary to lower NF $\kappa$ B mRNA in cells treated with AA (Figure 4 panel C). Looking at the expression pattern of COX-2, PPAR $\gamma$ , and NF $\kappa$ B, it can be deduced that suppression in COX-2 gene transcription concurred with the increase in PPAR $\gamma$  expression and the decrease in NF $\kappa$ B expression. The changes in mRNA levels for PPAR $\gamma$  and NF $\kappa$ B involved in the molecular control of the COX-2 gene with treatment of LC n-3 PUFA and genistein might suggest a dietary means to attenuate COX-2 protein amplification associated with the invasive phenotype of the MDA-MB-231 human breast cancer cells. Our study also found that the changes in the gene expression levels of PPAR $\gamma$  and NF $\kappa$ B did not take place at the 2 hr and 8 hr treatment durations although the changes in the expression of COX-2 gene was observed at 8 hr (data not presented). This finding may suggest that physiological concentrations of n-3 PUFA and genistein maintained for long duration are effective to sustain COX-2 gene suppression through their actions on PPAR $\gamma$  and NF $\kappa$ B.

## DISCUSSION

The chemopreventive capacity of LC n-3 PUFA has been documented in the past two decades. Evidence suggests that LC n-3 PUFA antagonize AA-derived prostanoid formation through mechanisms involving substrate replacement, enzyme competition, signal transduction, and modulation of gene expression (7,27). Genistein has also been reported to have chemopreventive actions in cancer cells (28,29), and to inhibit COX-2 expression and PGE<sub>2</sub> production (18,30).

The present investigation demonstrated that genistein reduced the synthesis of PGE<sub>2</sub> in MDA-MB-231 human breast cancer cells that overexpress the COX-2 gene. Genistein, at a physiological concentration (2.5 μmol/L) reduced the production of PGE<sub>2</sub> by 13%. This significant finding indicates that genistein suppressed PGE<sub>2</sub> production at a much lower concentration (which can be achieved by diet) than previously reported (18). Plasma concentrations of genistein were found to be as high as 6 μmol/L in subjects receiving dietary intervention (31) while the serum concentration of genistein in Japanese male subjects not receiving any dietary intervention was approximately 0.5 μmol/L (32). Therefore, the 2.5 μmol/L genistein used in the present study is relevant to an achievable dietary level and validates the findings on prostanoid synthesis in breast cancer cells.

When MDA-MB-231 cells were exposed to AA and genistein, the level of PGE<sub>2</sub> was reduced compared with those treated with AA alone. In cells treated with EPA, a prostanoid precursor, the level of PGE<sub>2</sub> produced was substantially lower than that in cells treated with AA. However, nearly a 50% higher PGE<sub>2</sub> concentration was observed in EPA-treated cells compared to the vehicle control. Since the antibody for PGE<sub>2</sub> used in this assay had a 43% cross-reactivity with PGE<sub>3</sub>, the apparent increase in PGE<sub>2</sub> concentration observed in EPA-treated cells

(compared to the vehicle control) was likely due to an increase in EPA-derived PGE<sub>3</sub>, not PGE<sub>2</sub>. Indeed, treatment of A549 human lung cancer cells with 50 μM EPA was shown to boost PGE<sub>3</sub> synthesis and increase the ratio of PGE<sub>3</sub> to PGE<sub>2</sub> level 10-fold by the preferential action of COX-2 over COX-1 enzyme (33). Moreover, it has been established that the MDA-MB-231 cells express a low level of COX-1 and that prostanoid synthesis in these cells is catalyzed mostly by the constitutively high level of COX-2 (34). When cells were treated with DHA, a non-PG precursor, we demonstrated that DHA dose-dependently reduced the synthesis of PGE<sub>2</sub>. In a separate experiment to determine the effect of DHA in combination with genistein on PGE<sub>2</sub> production, treatment with DHA alone tended to lower the PGE<sub>2</sub> concentration (although not significant), and cells treated with DHA and genistein in combination had the level of PGE<sub>2</sub> further lowered. Hence, our data provide evidence for an additive effect of DHA plus genistein in suppressing the endogenous production of PGE<sub>2</sub> in MDA-MB-231 cells.

In our study, treatments with LC n-3 PUFA reduced COX-2 mRNA level independent of the addition of genistein. This is not surprising since both DHA and EPA were reported to lower COX-2 expression by blocking the toll-like receptor-mediated pathway thereby inhibiting NFκB activation (35). Treatments with AA, on the other hand, showed reduction in COX-2 mRNA level only when combined with genistein. We observed that the reduction in COX-2 expression coincided with increased PPARγ expression and lowered NFκB expression. Our observation is consistent with another study in which cervical cancer cells treated with PPARγ ligand had upregulated PPARγ expression, suppressed binding activity of NFκB, and reduced expression of COX-2 gene (36). Genistein has been shown to activate PPARγ (20), and to inactivate NFκB or prevent NFκB binding to DNA (37). We propose that LC n-3 PUFA act complementarily with genistein to increase the transcription of PPARγ and decrease the transcription of NFκB to

suppress the expression of COX-2. Since NFκB has two binding sites on the COX-2 promoter (37), it is feasible that the addition of genistein to cells enriched with AA led to inactivation of NFκB, mediated through higher expression of PPARγ or by direct effect on NFκB, to reduce the transcription of the COX-2 gene. When genistein was in combination with EPA or DHA, genistein did not enhance the suppression of COX-2 gene expression but worked through another mechanism (likely to also involve PPARγ) to lower PGE<sub>2</sub> production and the invasive phenotype of the cancer cells. It is also noteworthy that activation of PPRE may directly induce the COX-2 gene promoter (38), however, since there are two sites for NFκB and only one for PPRE which is located much further upstream from the start site of transcription compared to the NFκB sites, it seems that suppression of NFκB may override activation of PPRE.

From our investigation, the chemopreventive effect of LC n-3 PUFA and genistein may be three-fold. First, we observed that treatment with DHA and genistein reduced the synthesis of PGE<sub>2</sub>, a compound implicated in carcinogenesis and inflammation. Second, EPA and DHA lowered the expression of COX-2 and NFκB to decrease the production of PGE<sub>2</sub>. The lowering effect of EPA and DHA on NFκB transcription in the same breast cancer cells was observed in our laboratory with stearidonic acid (18:4n-3) enrichment (25). Third, genistein blocked the actions of AA on PGE<sub>2</sub>, cell invasiveness, and COX-2 and NFκB expression.

Simultaneous targeting of COX-2 and PPARγ has been suggested as a powerful mechanism to lower the risk of cancer (39). We observed that LC n-3 PUFA effectively downregulated PGE<sub>2</sub> production, along with DHA being a potential ligand for RXRα (40) and genistein as a PPARγ ligand, they appear to work together to activate the PPRE trans-suppression of pro-inflammatory and pro-carcinogenic genes (e.g. NFκB). In breast cancer cells, including the MDA-MB-231 cell line used in our study, treatment with 15-d-PGJ<sub>2</sub> was reported

to induce apoptosis (41) and inhibit proliferation (42). Therefore, our study is the first to evoke a complementary nature of DHA and genistein that would alter or decrease the flux through prostanoid pathways.

Intakes of foods containing LC n-3 PUFA and genistein may be an effective strategy to reduce the risk of breast cancer by downregulating the production of pro-inflammatory cytokines and invasiveness of cancer cells. Importantly, this study found that in AA-treated human cancer cells, genistein effectively lowered PGE<sub>2</sub> as well as the expression of COX-2 and NFκB, suggesting a potential cancer protective effect of soy products in Japanese populations that recently began consume increasing amount of dietary n-6 PUFA (arachidonic acid). Figure 5 illustrates possible targets for the proposed antagonistic effect of genistein on AA and its complementary actions with DHA on prostanoid synthesis. Genistein antagonized the effect of AA but complemented those of EPA and DHA on molecular and biochemical controls for PGE<sub>2</sub> production. We found in this study that genistein in combination with EPA and DHA affected the expression of COX-2; however, additional research must confirm changes in the transcriptional activity of the COX-2 gene by various transcription factors and the dietary factors used in this investigation.

### **ACKNOWLEDGMENTS**

The assistance of Dr. Sophie Lelievre with the invasion assay is appreciated. This work was supported by a 21<sup>st</sup> Century Research and Technology grant awarded to the Center for Enhancing Foods to Protect Health (<http://www.efph.purdue.edu>) at Purdue University.

Figure 1

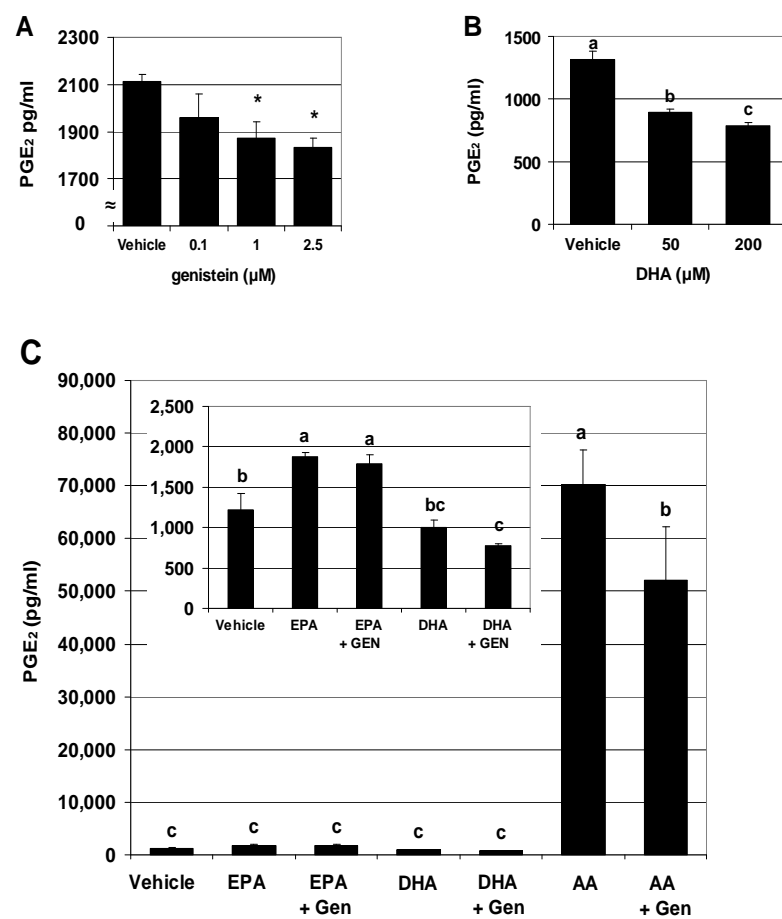


Figure 2

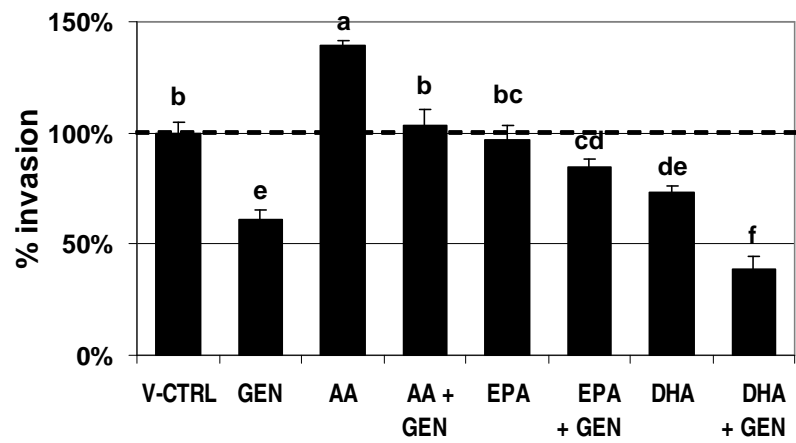


Figure 3

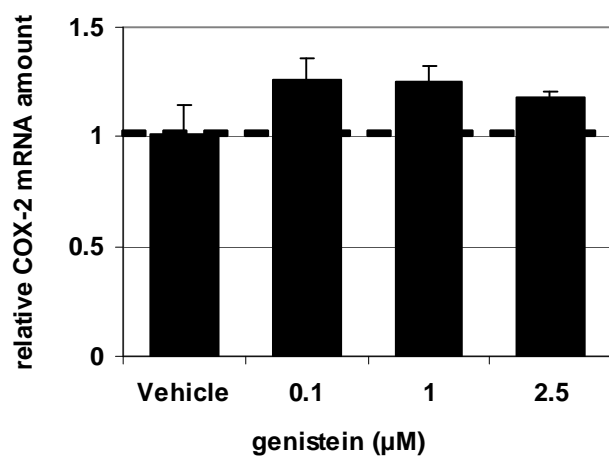


Figure 4

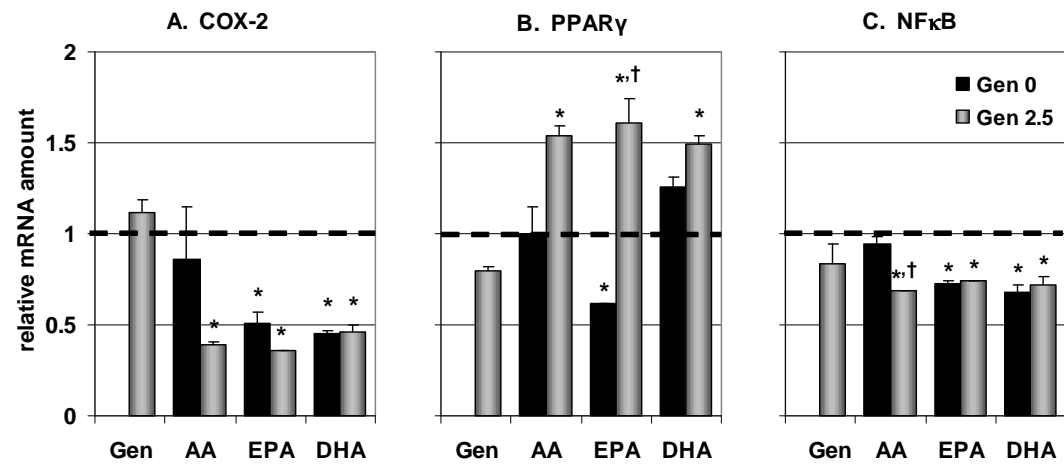
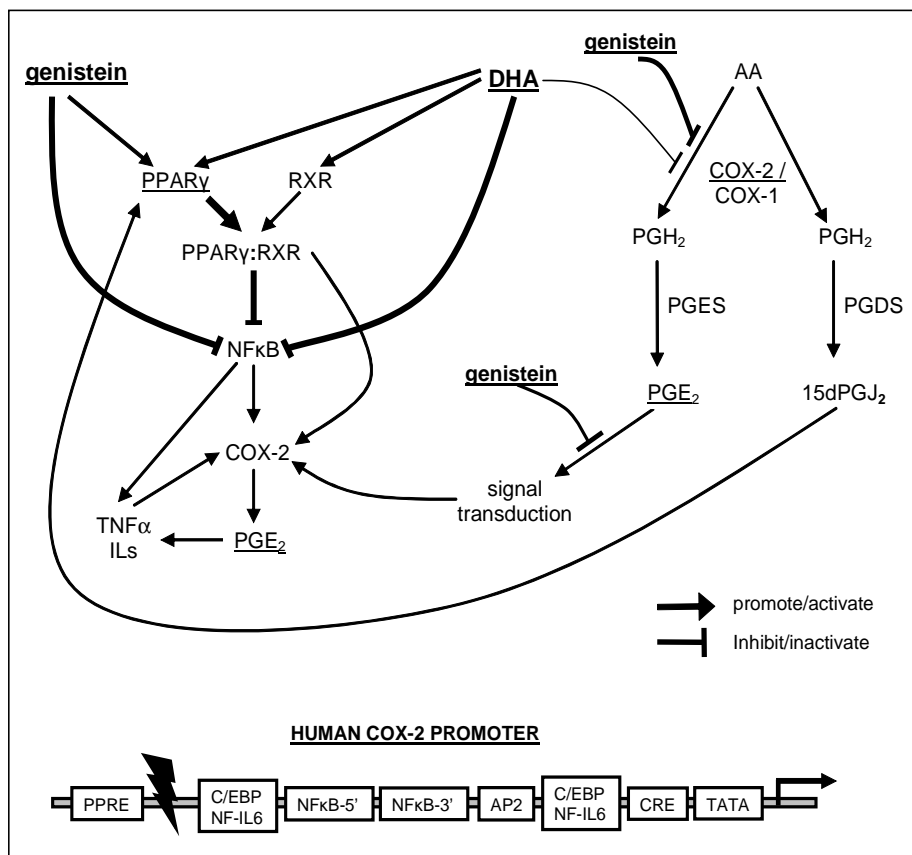


Figure 5



**Fig. 1.** The effects of PUFA and genistein on the production of PGE<sub>2</sub>. In panel A, genistein dose-dependently reduced PGE<sub>2</sub> in MDA-MB-231 cells. Subconfluent cells were treated with genistein for 24 hr and were subsequently induced with 10 nmol/L TPA for an additional 24 hr. Vehicle control cells were treated with 0.1% DMSO. Asterisks on bars indicate significant difference from control ( $P < 0.05$ ) by two-tailed Student's t-test ( $n = 2$ ). In panel B, DHA dose-dependently reduced PGE<sub>2</sub> synthesis. Subconfluent cells were treated with DHA for 24 hr and were subsequently induced with 10 nmol/L TPA for an additional 24 hr. Vehicle control cells were treated with BSA. Letters on bars indicate significant difference ( $P < 0.05$ ) by Tukey's multiple comparison test ( $n=3$ ). In panel C, n-3 PUFA and genistein in combination reduced the synthesis of PGE<sub>2</sub>. Subconfluent cells were treated with 200  $\mu\text{mol/L}$  PUFA and 2.5  $\mu\text{mol/L}$  genistein for 24 hr and were subsequently induced with 10 nmol/L TPA for an additional 24 hr. Vehicle control cells were treated with 100  $\mu\text{mol/L}$  BSA and 0.1% DMSO vehicle. Letters on bars indicate significant difference ( $P < 0.05$ ) by Tukey's multiple comparison test ( $n = 3$ ). The data shown is indicative of three separate experiments.

**Fig. 2.** The effects of PUFA and genistein on Matrigel invasion capacity of MDA-MB-231 cells. N-3 PUFA and genistein in combination reduced cell invasiveness. Subconfluent cells were serum-starved for 24 hr followed by treatment with vehicle control (100  $\mu\text{mol/L}$  BSA + 0.1% DMSO), PUFA (200  $\mu\text{mol/L}$ ) and/or genistein (10  $\mu\text{mol/L}$ ) for an additional 24 hr. Cells were subsequently placed into the Boyden chambers with fresh treatment media for 18 hr. Results are expressed as % invasion compared to the vehicle control cells (V-CTRL, 100% invasion) represented by the dotted line. Letters on bars indicate a significant difference ( $P < 0.05$ ) by Tukey's multiple comparison test ( $n = 4$ ). The data shown is indicative of two separate experiments.

**Fig. 3.** Genistein did not affect the expression of COX-2 gene. Subconfluent MDA-MB-231 cells were treated with genistein at concentrations 0.1, 1.0, and 2.5  $\mu\text{mol/L}$  in serum-free media for 24 hr. The value from vehicle control cells treated with 0.1% DMSO was set at 1 and represented by the dotted line. For each sample, the  $C_T$  (threshold concentration) values for the COX-2 gene was adjusted to the  $C_T$  for the control gene  $\beta$ -actin ( $\Delta C_T = C_{T_{\text{cox-2}}} - C_{T_{\text{actin}}}$ ). The  $\Delta C_T$  values were further normalized to the  $\Delta C_T$  of the vehicle control ( $\Delta \Delta C_T = \Delta C_{T_{\text{treatment}}} - \Delta C_{T_{\text{control}}}$ ). The relative quantity of the gene in a treatment group compared to the control was calculated by  $2^{-\Delta \Delta C_T}$ . Bars represent mean values ( $n = 2$ ). Significant difference from control (\*) was calculated by a two-tailed Student's t-test ( $P < 0.05$ ).

**Fig. 4.** The effects of PUFA and genistein on COX-2 (panel A), PPAR $\gamma$  (panel B), and NF $\kappa$ B (panel C) genes expression. Subconfluent MDA-MB-231 cells were treated with 50  $\mu$ mol/L PUFA and/or 2.5  $\mu$ mol/L genistein in serum-free media for 24 hr. The values from vehicle control cells treated with 25  $\mu$ mol/L BSA and 0.1% DMSO were set at 1 and represented by the dotted line. For each sample, the  $C_T$  (threshold concentration) values for the gene of interest was adjusted to the  $C_T$  for the control gene  $\beta$ -actin ( $\Delta C_T = C_{T_{\text{gene}}} - C_{T_{\text{actin}}}$ ). The  $\Delta C_T$  values were further normalized to the  $\Delta C_T$  of the vehicle control ( $\Delta \Delta C_T = \Delta C_{T_{\text{treatment}}} - \Delta C_{T_{\text{control}}}$ ). The relative quantity of the gene in a treatment group compared to the control was calculated by  $2^{-\Delta \Delta C_T}$ . Bars represent mean values ( $n = 2$ ) and are indicative of two experiments. Significant difference from control (\*) and from PUFA only treatment (†) is calculated by a two-tailed Student's t-test ( $P < 0.05$ ).

**Fig. 5.** In the present study, the combination of DHA and genistein antagonized the effect of AA on prostanoid production. Genistein and DHA can inhibit the activation of NF $\kappa$ B by PPAR $\gamma$ -dependent and -independent mechanisms (20,23,24,35,40), leading to downregulation of the COX-2 gene, PGE $_2$  production, and synthesis of NF $\kappa$ B-regulated proinflammatory cytokines (18,22,43). DHA and genistein can also suppress the production of PGE $_2$  by altering the flux through the COX-2 enzyme (11,12). Additionally, genistein can potentially interfere with signal transduction involved in the elevation of cAMP levels (17,44), hence it prevents the inducing effect of PGE $_2$  on COX-2 gene transcription.

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