Complementary actions of docosahexaenoic acid and genistein on COX-2, PGE\textsubscript{2} and invasiveness in MDA-MB-231 breast cancer cells

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N-3 polyunsaturated fatty acids (PUFA) and genistein have been associated with lowered cancer risk by reducing inflammatory prostanooids, 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\textsubscript{2} (PGE\textsubscript{2}) 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\textsubscript{2} concentrations, expression of COX-2, and cell invasiveness were determined. The n-3 PUFA and genistein alone lowered PGE\textsubscript{2} concentration, and genistein in combination with AA reversed the high level of this prostanooid 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 NF\textsubscript{κ}B expression. Genistein combined with AA reversed the effects of AA alone on the expression of COX-2 and NF\textsubscript{κ}B. All three fatty acids increased the expression of PPAR\textgamma in the cells only when combined with genistein. Our results support the premise that DHA and genistein exert complementary actions whilst genistein is antagonist to AA for controlling PGE\textsubscript{2} production as well as invasiveness of MDA-MB-231 cells in culture by modulating the level of NF\textsubscript{κ}B expression.

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 USA compared to Japan. Cancer is the second leading cause of death, while breast cancer is the most common type of 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 the year 2000 the estimated dietary ratio of 4:1 for n-6/n-3 PUFA in Japan is still considerably lower than the 10:1–15:1 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 prostanooids, particularly prostaglandin E\textsubscript{2} (PGE\textsubscript{2}). This prostanooid 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\textsubscript{3} is much less efficient compared to PGE\textsubscript{2} in inducing COX-2 expression (15) and it is a weaker inflammatory agent (16).

Abbreviations: AA, arachidonic acid; COX, cyclooxygenase; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; IL, interleukin; LA, linoleic acid; NF\textsubscript{κ}B, nuclear factor kappa B; PGE\textsubscript{2}, prostaglandin E\textsubscript{2}; PPAR, peroxisome proliferator activated receptor; PPRE, peroxisome proliferator receptor element; LC-PUFA, long-chain polyunsaturated fatty acids; Q-PCR, quantitative polymerase chain reaction; TNFa, tumor necrosis factor-α.

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Genistein was reported to lower PGE₂ production in mesangial cells and macrophages (17,18). Genistein may lower PGE₂ 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 (PPARγ) ligand increasing peroxisome proliferator response element (PPRE) transcriptional activity at concentrations of ≥5 μmol/l (20). Genistein has also been demonstrated to activate PPRE transcriptional activity through PPARα in HeoG2 human hepatoma cells (21).

Activation of PPRE has been associated with the inhibition of nuclear factor kappa B (NFκB) (20). In addition, genistein can act via a PPARγ-independent mechanism to inhibit NFκB activation and the binding of NFκB to DNA (23,24). Therefore, genistein can potentially reduce the level of COX-2 protein and PGE₂ production by altering NFκB signaling as demonstrated in macrophages (18).

In the present investigation, n-3 PUFA and genistein were hypothesized to synergistically suppress AA-derived PGE₂ 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 with n-3 PUFA alone and in combination with genistein were studied on PGE₂ production and COX-2 expression. Levels of NFκB and PPARγ, 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). Isovce’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 (>99% purity) from Nu-Chek-Prep (Elkay, MN); fatty acid-free serum albumin (BSA), dimethyl sulfoxide (DMSO), formalin solution (4% formaldehyde), and methylene blue from Sigma; genistein (5,7,4',5-trihydroxyisoflavone, >99% pure) from Indofine Chemical Company, Inc. (Hillsborough, NJ); tetradecanoyl phorbol acetate (TPA, >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-PGE₂ assay kits were purchased from Cayman Chemical (Ann Arbor, MI), Matrigel and filter inserts from BD Biosciences (Bedford, MA), Modified Dulbecco Medium (IMDM) and fetal bovine serum (FBS) were dissolved in ethanol (0.1% ethanol) into IMDM at a ratio of BSA to fatty acid and/or genistein-superplemented medium, cells were washed with PBS then treated with IMDM containing 10% FBS and 10 nmol/l TPA for 24 h. TPA was dissolved in DMSO (not to exceed 0.1% in the medium). Samples of cell culture media were collected and PGE₂ concentrations analyzed with a competitive enzyme immunoassay kit (STAT-PGE₂).

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 h, followed by 24 h treatment with PUFA and/or genistein. At the end of the treatment period, 2 × 10⁵ 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 μm-pore size filter inserts with 85 μg/cm² Matrigel. After cells were incubated for 18 h 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®-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, NFκB, PPARγ and β-actin were synthesized from 1 μg RNA using an iScript™ 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 μl of the cDNA product, 12.5 μl of SYBR® Green PCR master mix, 9.5 μl nuclelease-free water and 1 μl (25 pmol) each of the forward and reverse primers, were added to each well to a final volume of 25 μl. All primers were designed using Primer Express® Software v2.0 (Applied Biosystems). Primer sequences for the genes were as follows: COX-2 forward: GAATCTATCAGGCAAAT-TG, COX-2 reverse: TCTGTACTGGGTTGAACA, NFκB forward: GG-CTACACCGAAGCAATTGA, NFκB reverse: CAGCGGATGAGCGTGA-GA, PPARY forward: GGCTTCTAGACAGAAAGGATTTC, PPARY reverse: AAATCACAATCTGGGTCATTAAA, β-actin forward: CCTGGACCC-AGCACAAT, β-actin reverse: GCCGATCCACGAGGTACT. The thermal settings for PCR were 50°C for 2 min, 95°C for 10 min, 95°C for 15 s, and 59°C for 1 min (40x). Additional steps at 95°C for 15 s, 59°C for 20 s and 19 min 59 s temperature ramp to reach 95°C and held for 15 s 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 ± SD or as standardized differences calculated from the difference between values of treatment and control divided by the pooled SEM.

Results

PGE₂ biosynthesis

To determine whether PUFA act alone or in combination with genistein to affect PGE₂ synthesis, MDA-MB-231 cells were treated for 24 h and subsequently treated with TPA for an additional 24 h. First, the effect of genistein alone was characterized on PGE₂ production. Genistein dose-dependently reduced the amount of PGE₂ 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 μmol/l and 13% at 2.5 μmol/l compared with the vehicle control. The effect of DHA on PGE₂ synthesis was also determined. DHA showed dose-dependent reduction of PGE₂ synthesis in MDA-MB-231 cells compared with the vehicle control (Figure 1 panel B).

The production of PGE₂ in cells treated with AA was 57-fold...
higher compared with the vehicle control (Figure 1 panel C). The addition of genistein to cells enriched with AA reduced the amount of PGE2 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 PGE2 in cells compared with the AA treatment. Importantly, the treatment of DHA with genistein resulted in a 37% lower concentration of PGE2 compared with the vehicle control (Figure 1 panel C, inset).

Invasion assay

The Matrigel invasion assay was performed to examine whether the changes in PGE2 concentrations resulting from PUFA and genistein treatments correlated with the invasive phenotype of the MDA-MB-231 cells. Genistein alone at 10 μmol/l significantly reduced the number of cells invading the membrane by 40% compared to the vehicle control (Figure 2). At concentrations <10 μmol/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 with 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 with 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, PPARγ and NFκB genes

The effect of genistein at concentrations ranging from 0.1 to 2.5 μM on the expression of COX-2 gene in MDA-MB-231 cells was determined after 24 h 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 h treatments with PUFA at 50 μmol/l (with and without genistein, 0 and 2.5 μmol/l) on the transcription rate of COX-2, PPARγ and NFκB 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 PPARγ and NFκB genes expression were also examined. Genistein treatment alone at the concentration used had no significant effect on mRNA levels for both PPARγ (Figure 4 panel B) and NFκB (Figure 4 panel C). The combination of all PUFA treatments with genistein resulted in a higher level of PPARγ 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 NFκB mRNA. In contrast, the addition of genistein was necessary to lower NFκB mRNA in cells treated with AA (Figure 4 panel C). Looking at the expression pattern of COX-2, PPARγ, and NFκB, it can be deduced that suppression in COX-2 gene transcription concurred with the increase in PPARγ expression and the decrease in NFκB expression. The changes in mRNA levels for PPARγ and NFκB 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 PPARγ and NFκB did not take place at the 2 and 8 h treatment durations although the changes in the expression of COX-2 gene was observed at 8 h (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 PPARγ and NFκB.

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 PGE2 production (18,30).

The present investigation demonstrated that genistein reduced the synthesis of PGE2 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 PGE2 by 13%. This significant finding indicates that genistein suppressed PGE2 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 ~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$_2$ was reduced compared with those treated with AA alone. In cells treated with EPA, a prostanoid precursor, the level of PGE$_2$ produced was substantially lower than that in cells treated with AA. However, nearly a 50% higher PGE$_2$ concentration was observed in EPA-treated cells compared to the vehicle control. Since the antibody for PGE$_2$ used in this assay had a 43% cross-reactivity with PGE$_3$, the apparent increase in PGE$_2$ concentration observed in EPA-treated cells (compared to the vehicle control) was likely due to an increase in EPA-derived PGE$_3$, not PGE$_2$. Indeed, treatment of A549 human lung cancer cells with 50 μM EPA was shown to boost PGE$_3$ synthesis and increase the ratio of PGE$_1$–PGE$_2$ level 10-fold by the preferential action of COX-2 over COX-1 (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$_2$. In a separate experiment to determine the effect of DHA in combination with genistein on PGE$_2$ production, treatment with DHA alone tended to lower the PGE$_2$ concentration (although not significant), and cells treated with DHA and genistein in combination had the level of PGE$_2$ further lowered. Hence, our data provide evidence for an additive effect of DHA plus genistein in suppressing the endogenous production of PGE$_2$ 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 NFkB 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 NFkB 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 NFkB, and reduced expression of COX-2 gene (36).

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 NFkB by PPARγ-dependent and -independent mechanisms (20,23,24,35,40), leading to down-regulation of the COX-2 gene, PGE$_2$ production, and synthesis of NFkB-regulated pro-inflammatory 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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Genistein has been shown to activate PPARγ (20), and to inactivate NFkB or prevent NFkB 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 NFkB to suppress the expression of COX-2. Since NFkB 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 NFkB, mediated through higher expression of PPARγ or by direct effect on NFkB, 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$_2$ 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 NFkB and only one for PPRE which is located much further upstream from the start site of transcription compared with the NFkB sites, it seems that suppression of NFkB may override activation of PPRE.

From our investigation, the chemopreventive effect of LC n-3 PUFA and genistein may be 3-fold. First, we observed that treatment with DHA and genistein reduced the synthesis of PGE$_2$, a compound implicated in carcinogenesis and inflammation. Second, EPA and DHA lowered the expression of COX-2 and NFkB to decrease the production of PGE$_2$. The lowering effect of EPA and DHA on NFkB transcription in the same breast cancer cells was observed in our laboratory with stearidonic acid (18:4 n-3) enrichment (25). Third, genistein blocked the actions of AA on PGE$_2$, cell invasiveness, and COX-2 and NFkB 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 down-regulated PGE$_2$ 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. NFkB). In breast cancer cells, including the MDA-MB-231 cell line used in our study, treatment with 15-d-PGJ$_2$ 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 prostanoi pathways.

Intakes of foods containing LC n-3 PUFA and genistein may be an effective strategy to reduce the risk of breast cancer by down-regulating 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$_2$ as well as the expression of COX-2 and NFkB, suggesting a potential cancer protective effect of soy products in Japanese populations that recently began to consume increasing amounts of dietary n-6 PUFA (AA). 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$_2$ 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.

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References


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