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Comparison of stearidonic acid and α-linolenic acid on PGE_2 production and COX-2 protein levels in MDA-MB-231 breast cancer cell cultures☆

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Abstract

The bioactivity of stearidonic acid (SDA, 18:4n-3) and α-linolenic acid (LNA, 18:3n-3) on cyclooxygenase-2 (COX-2) enzyme expression and prostaglandin E_2 (PGE_2) production has not been evaluated. This investigation examined the effects of SDA and LNA on PGE_2 biosynthesis and COX-2 protein and mRNA levels in MDA-MB-231 human breast cancer cells. Cells were supplemented with SDA, LNA, linoleic acid and arachidonic acid (AA) at concentrations ranging from 10 to 200 μM. At 50 and 200 μM, both SDA and LNA treatments and their combinations reduced PGE_2 production as compared with AA. At 50 μM, SDA treatment also lowered the COX-2 protein level as compared with the vehicle, but this reduction was not observed with the LNA treatment. Gas chromatographic analysis of fatty acids in cellular lipids of breast cancer cells revealed that SDA led to significantly greater concentrations of 20:5n-3 and other long-chain (LC) n-3 polyunsaturated fatty acids (PUFAs) (20:4n-3, 22:4n-3 and 22:5n-3) as compared with the LNA treatment. Both SDA and LNA reduced the level of 20:4n-6; however, SDA was more effective than LNA in decreasing the ratio of n-6/n-3 PUFAs in cells. In addition, SDA was more potent than LNA in suppressing the expression of the COX-2 gene, which was associated with the reduction in the levels of nuclear factor kappa B and peroxisome proliferator-activated receptor gamma mRNA. This study showed that although PGE_2 production in MDA-MB-231 breast cancer cells was not significantly different between the SDA and LNA treatments, SDA was more effective than LNA in converting into LC n-3 PUFAs and in reducing COX-2 protein and mRNA levels.

Keywords: Stearidonic acid; α-Linolenic acid; Breast cancer cells; n-3 PUFA; COX-2; Prostaglandin E_2

1. Introduction

Diets of high fat content have been associated with a greater incidence of cancer [1]. Recently, concern in the role that dietary fat plays in carcinogenesis has shifted from the amount consumed to the type of fat, that is, n-3 vs. n-6 polyunsaturated fatty acids (PUFAs). Epidemiological studies suggest a significant inverse relationship between the consumption of fish oil rich in long-chain (LC) n-3 PUFAs and breast cancer rates [2,3]. In the past decade, the LC n-3 PUFA eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3) have been the focus of cancer research for understanding the role of dietary fatty acids in cell cultures, animal models and clinical studies [4,5]. These investigations suggest a positive effect of LC n-3 PUFAs in decreasing the risk of mammary carcinogenesis, while the opposite effect was demonstrated by n-6 PUFAs such as arachidonic acid (AA; 20:4n-6) [4–8]. No study, however, has evaluated the effects of stearidonic acid (SDA; 18:4n-3) in cancer cells.

SDA is the desaturation product of α-linolenic acid (LNA; 18:3n-3). In rats fed with a diet consisting of 1% SDA or 1% LNA for 3 weeks, SDA increased the molar ratio of EPA more effectively than did LNA [9]. The group fed with the SDA-supplemented diet had only trace amounts of SDA in tissue lipids, suggesting that the fatty acid was immediately and almost completely converted to LC n-3 PUFAs. In humans, SDA was four times more effective than

Abbreviations: AA, Arachidonic acid; COX, Cyclooxygenase; DHA, Docosahexaenoic acid; EPA, Eicosapentaenoic acid; IMDM, Iscove's modified Dulbecco's medium; LA, Linoleic acid; LNA, α-linolenic acid; NFκB, Nuclear factor kappa B; PGE_2, Prostaglandin E_2; PPAR, Peroxisome proliferator-activated receptor; PUFA, Polyunsaturated fatty acid; SDA, Stearidonic acid; TPA, Tetradecanoyl phorbol acetate.

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LNA in increasing the level of EPA in erythrocytes and plasma phospholipids [10]. In this study, the concentration of DHA was not elevated by supplementation with either SDA or LNA, although the amount of 22:5n-3 was significantly increased by SDA. These data are consistent with the premise that Δ^6 desaturation is the rate-limiting step in the conversion of LNA to EPA and that SDA has a biochemical advantage over LNA in elevating the levels of LC n-3 PUFAs in tissues.

One of the proposed mechanisms for the anticarcinogenic effects of LC n-3 PUFAs is modulating the biosynthesis of prostanoids. Previous investigations demonstrated that prostanoids derived from n-6 PUFAs [e.g., prostaglandin E_2 (PGE_2)] may augment the risk of breast tumor development in humans [11]. Moreover, cyclooxygenase-2 (COX-2), the inducible enzyme that catalyzes prostaglandin production, has been used as a biomarker for human breast cancer. COX-2 was detected in human breast cancers but not in normal breast tissue [12], and the expression of this protein is positively associated with breast tumor cell density [13–15]. Moreover, PGE_2 levels were positively correlated with increased tumorigenic and metastatic potentials of human breast cancer cells [13]. The suppressive effect of EPA and DHA on the growth and metastasis of MDA-MB-435 human breast cancer cells in athymic nude mice was accompanied by reduced cellular AA concentration and PGE_2 production [8]. Therefore, based on these findings, we propose that SDA is more potent than LNA in exerting EPA- and DHA-like effects on antagonizing the production of PGE_2 from AA since SDA is more efficiently converted to LC n-3 PUFAs.

The present investigation examined how SDA and LNA influence fatty acid biochemistry and COX-2 protein in a human breast cancer cell line (MDA-MB-231) that is a highly invasive, estrogen-independent cell that overexpresses COX-2. The effects of SDA on cell growth, cellular fatty acid composition, PGE_2 production, COX-2 protein and mRNA levels for COX-2, peroxisome proliferator-activated receptor gamma (PPARγ) and nuclear factor kappa B (NFκB) were compared with those of LNA in MDA-MB-231 cell cultures.

2. Methods and materials

2.1. Materials

The MDA-MB-231 human breast cancer cell line was purchased from the American Type Culture Collection (Manassas, VA, USA). Ethyl linoleate, ethyl linolenate and ethyl arachidonate (≥99% purity) were purchased from Nu-Chek-Prep (Elysian, MN, USA); fatty acid-free BSA was from Sigma (St. Louis, MO, USA). An oil containing 70% ethyl SDA was obtained from K.D. Pharma (Bexbach, Germany) and purified using an aminopropyl silica solid-phase extraction column (Phenomenex, Torrance, CA, USA) [16] before use in cell culture.

2.2. Cell culture

Cells were maintained in Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic–antimycotic solution (Gibco Invitrogen, Carlsbad, CA, USA) at 37°C in 5% CO_2. Cells from passages 5 to 40 were selected for the experiments.

2.3. Fatty acid enrichment

Cells were seeded at an initial density of 9×10^4 cells in 60-mm plates. At 90% confluency, the medium was changed to IMDM supplemented with fatty acid ethyl esters or control medium for 24 h in the presence of serum. The stock fatty acid medium was prepared by addition of fatty acid ethyl esters dissolved in ethanol into IMDM containing 500 μM BSA for a concentration of 1 mM PUFA. The medium was flushed with N_2 gas and incubated at 37°C. The stock fatty acid medium was diluted with the basal medium to obtain various fatty acid concentrations while maintaining a 2:1 fatty acid/BSA ratio (<0.1% ethanol). The medium contained only BSA as the vehicle for the control cell cultures.

2.4. Cell proliferation assay

Cells were seeded in 96-well plates at a density of 1500 cells/well. At 90% confluency, the medium was changed to fatty acid-supplemented medium for 24 h and cell viability was determined using a CellTiter 96 AQ_{ueous} One Solution Cell Proliferation assay kit (Promega, Madison, WI, USA). The assay is based on the reduction of tetrazolium salt into soluble colored formazan, which can then be measured by spectrophotometric absorbance at 490 nm.

2.5. Cellular fatty acid analysis

At the end of the 24-h enrichment period, cells were washed with PBS, scraped, centrifuged at $900\times g$ for 10 min and stored at −20°C until analyzed. Total lipids in cell pellets were extracted with chloroform/methanol (2:1, v/v) and subsequently converted to fatty acid methyl esters (FAME) by BF_3-catalyzed transesterification [17]. A gas chromatograph (HP 6890 series, autosampler 7683, GC 3365 ChemStation Rev.A.08.03, Agilent Technologies, Palo Alto, CA, USA) with a DB-23 column (30 m, 0.53 mm i.d., 0.5-μm film thickness, Agilent Technologies) was used in the analysis of FAME. The GC analysis of FAME was performed at 100°C for 2 min, temperature programmed 4°C/min to 150°C and held for 5 min, 3°C/min to 165°C and held for 13.5 min, 2°C/min to 185°C and held for 10 min and 10°C/min to 200°C and held for 5.5 min. The temperatures of the injector and detector were 225°C and 250°C, respectively.

2.6. PGE_2 assay

Cells were seeded at 30,000 cells/well in 12-well plates for 3 days until 90% confluent. After 24 h of treatment with fatty acid-supplemented medium, cells were washed with

PBS then treated with IMDM containing 10% FBS and 10 nM tetradecanoyl phorbol acetate (TPA; Calbiochem, San Diego, CA, USA) for 24 h. TPA was dissolved in DMSO (not to exceed 0.1% in the medium). Media were collected and PGE_2 concentrations were determined with a competitive enzyme immunoassay kit (STAT-Prostaglandin E_2, Cayman Chemical, Ann Arbor, MI, USA).

2.7. Western blotting

Cells were grown as described for the PGE_2 assay experiment and treated for 24 h with serum-free fatty acid-supplemented medium followed by 1-h induction with 10 nM TPA. Total protein was extracted from cells and resolved by electrophoresis on 10% Tris–HCl polyacrylamide gel (5 μg/lane). Proteins were transferred to polyvinylidene fluoride membranes that were probed with a 1:4000 dilution of polyclonal rabbit antihuman COX-2 antibody (Oxford Biomedical Research, Oxford, MI, USA) and a 1:50,000 dilution of mouse anti-β-actin antibody (Sigma). Secondary antibodies included donkey antirabbit (COX-2) and sheep antimouse (β-actin) linked to horseradish peroxidase at dilutions of 1:5000 and 1:40,000, respectively (Amersham, Piscataway, NJ, USA). Detection was performed using an enhanced-chemiluminescence detection system (Amersham). The chemiluminescent emission from the membranes was captured on X-ray films and the bands detected were analyzed by KODAK 1D Image Analysis Software (Eastman Kodak Company, New Haven, CT, USA).

2.8. Quantitative real-time polymerase chain reaction

Cells were cultured in 6-well plates until 90% confluent in IMDM supplemented with 10% FBS and 1% antibiotic–antimycotic solution and switched to serum-free fatty acid-supplemented media for 24 h. Subsequently, TPA at a final concentration of 10 nM was added and cells were incubated for an additional hour. Total RNA was isolated using an RNAqueous-4PCR kit (Ambion, Austin, TX, USA). The yield and quality of the RNA were assessed by UV absorbance at 260 and 280 nm. First-strand cDNAs for COX-2, NFκB, PPARγ and β-actin were synthesized from 1 μg RNA using an iScript cDNA Synthesis kit (Bio-Rad, Hercules, CA, USA). Quantitative real-time polymerase chain reaction (PCR) was performed in 96-well optical plates using an 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 (Applied Biosystems), 9.5 μl nuclease-free water and 1 μl (25 pmol/μl) each of the forward and reverse primers were added to each well to a final volume of 25 μl. All primers (Sigma-Genosys, Woodlands, TX, USA) were designed using Primer Express software v2.0 (Applied Biosystems). Primer sequences for the genes were as follows: COX-2 forward, GAATCATTCACCAGGCAAATTG; COX-2 reverse, TCTGTACTGCGGGTGGAACA; NFκB forward, GGCTACACCGAAGCAATTGAA; NFκB reverse, CAGCGAGTGGGCCTGAGA; PPARγ forward, GGCTTCATGACAAGGGAGTTTC; PPARγ reverse, AAACTCAAACTTGGGCTCCATAAA; β-actin forward, CCTGGCACCCAGCACAAT; β-actin reverse, GCCGATCCACACGGAGTACT. 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 (40×). Additional steps at 95°C for 15 s, 59°C for 20 s and 19 min 59 s temperature ramp to reach 95°C for 15 s were performed to construct thermal dissociation curves to confirm the absence of nonspecific amplification.

2.9. Statistical analyses

Data were analyzed by Student's *t* test or one-way analysis of variance (ANOVA), and where significant differences were found, a Student–Newman–Keuls (SNK) or Tukey's multiple comparison test was performed at a probability of $P<.05$ (SAS software, SAS Institute Inc., Cary, NC, USA). All data are presented as means±S.D., except for the quantitative real-time PCR values, which are standardized differences calculated from the difference between values of treatment and control, divided by the pooled S.E.M.

3. Results

3.1. Cell proliferation assay

The effects of SDA, LNA and AA concentrations on cell growth were compared with the no-fatty acid vehicle BSA control following 24 h of treatment (Fig. 1). The assay indirectly measures cell growth by determining the number of viable cells, according to the amount of tetrazolium salt bioreduced by metabolically active cells, after a period allowed for proliferation. Based on the absorbance measure-

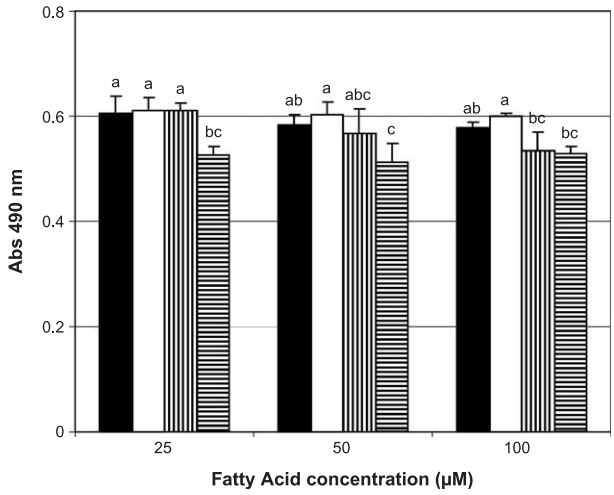

Fig. 1. MDA-MB-231 cell proliferation assay. Subconfluent cells were cultured in medium containing vehicle BSA control (■) or treated with either AA (□), SDA (▥) or LNA (▤) at concentrations of 25, 50 and 100 μM for 24 h. Data are presented as mean absorbance at 490 nm±S.D. ($n=4$). Values within treatment groups having different letters are significantly different by one-way ANOVA and SNK multiple comparison test ($P<.05$).

Table 1
Fatty acid composition (wt.%) of MDA-MB-231 breast cancer cells enriched with 50 μM fatty acids for 24 h[1]

FA	Fatty acid treatments[2]				Pooled S.D.	ANOVA *P* value
	Vehicle	SDA	1:1 SDA/LNA	LNA		
14:0	1.3	1.3	1.3	1.3	0.03	n.s.
16:0	14.9	15.0	14.8	14.7	0.2	n.s.
16:1t	2.1[a]	1.9[b]	1.9[b]	2.0[b]	0.03	.001
16:1n-7	1.3[a]	1.3[a,b]	1.3[b]	1.3[a,b]	0.01	.04
17:0	0.5	0.5	0.5	0.5	0.01	n.s.
18:0	17.5	16.5	16.6	16.8	0.3	n.s.
18:1n-9	25.6[a]	23.9[b]	24.1[b]	24.7[a,b]	0.4	.02
18:1n-7	5.0[a]	4.7[b]	4.9[a,b]	5.0[a]	0.1	.048
18:2n-6	2.1[a]	1.9[b]	1.9[b]	2.1[a,b]	0.1	.02
18:3n-3	0.3	0.3	0.4	0.5	0.1	n.s.
18:4n-3	n.d.	n.d.	n.d.	n.d.		–
18:4n-1	n.d.[c]	0.2[a]	0.2[b]	n.d.[c]	0.01	.0001
20:0	0.4	0.4	0.3	0.2	0.1	n.s.
20:1n-9	1.1	1.4	1.7	1.7	0.5	n.s.
20:2n-6	1.5	1.4	1.4	1.5	0.1	n.s.
20:3n-6	1.8[a]	1.8[a]	1.7[b]	1.7[b]	0.04	.007
20:4n-6	6.2[a]	5.8[b]	5.5[b]	5.7[b]	0.1	.01
20:3n-3	n.d.	n.d.	n.d.	0.2	0.2	n.s.
20:4n-3	n.d.[d]	0.5[a]	0.4[b]	0.3[c]	0.03	.0001
20:5n-3	0.7[d]	2.1[a]	1.6[b]	1.4[c]	0.05	.0001
22:0	0.3	0.3	0.2	0.1	0.1	n.s.
22:1n-9	n.d.	n.d.	0.2	0.1	0.1	n.s.
22:2n-6	0.3	0.3	0.3	0.3	0.04	n.s.
22:4n-6	0.9[a]	0.8[b]	0.8[b]	0.8[b]	0.02	.006
22:4n-3	n.d.	n.d.	n.d.	n.d.		n.s.
22:5n-3	2.9[c]	3.7[a]	3.3[b]	3.2[b]	0.1	.0002
22:6n-3	4.3[a]	4.2[a,b]	3.9[b]	4.1[a,b]	0.1	.045
24:1	0.3[a,b]	0.4[a]	0.3[a,b]	0.1[b]	0.1	.04
SAT	34.8	34.0	33.8	33.6	0.6	n.s.
MONO	35.4[a]	33.5[b]	34.3[a,b]	34.8[a]	0.4	.02
PUFAs	21.1[b]	23.2[a]	21.4[b]	21.7[b]	0.5	.01
n-6 PUFAs	12.9[a]	12.1[b]	11.6[b]	12.1[b]	0.3	.01
n-3 PUFAs	8.2[c]	10.9[a]	9.6[b]	9.7[b]	0.3	.0006
n-6/n-3	1.6[a]	1.1[c]	1.2[b]	1.3[b]	0.03	.0001

SAT indicates total saturated fatty acids; MONO, total monounsaturated fatty acids; PUFAs, total polyunsaturated fatty acids; n.d., not detected (peak detection at 10 ng); n.s., not significant.

[1] Mean values for the fatty acid level ($n=2$ for control and SDA; $n=3$ for the other two treatments) within a row having different superscripts are significantly different by one-way ANOVA and SNK multiple range test ($P<.05$).

[2] Treatment media include: vehicle (25 μM BSA), SDA (50 μM fatty acids containing 70% ethyl stearidonate), 1:1 SDA/LNA (25 μM fatty acids containing 70% ethyl stearidonate plus 25 μM ethyl linolenate) and LNA (50 μM ethyl linolenate).

ments, all fatty acid treatments tended to support growth moderately (or did not affect growth); however, at 25 and 50 μM, LNA treatments lowered the cell numbers. The SDA treatment was not statistically different from the control (vehicle BSA) for all concentrations tested. The 50 μM fatty acid treatment was selected for the subsequent experiments since the enrichment period was only 24 h and this level of fatty acids can be achieved in vivo [18]. No cytotoxic effect was demonstrated by any of the PUFAs at 50 μM, and the cell numbers are generally similar between all treatments.

3.2. Cellular fatty acid profiles

Treatment of MDA-MB-231 cells with 50 μM SDA compared with LNA demonstrated a significant increase in the concentrations of 20:4n-3, 20:5n-3, 22:5n-3, total PUFAs and total n-3 PUFAs in the cellular lipids (Table 1). SDA tripled the amount of EPA compared with the vehicle BSA control, while LNA doubled it, and the 1:1 mixture of SDA/LNA increased EPA by 1.3-fold. The difference in the LC n-3 PUFA formation efficiency between SDA and LNA was magnified with a higher amount of SDA treatment, as demonstrated in the experiment using 200 μM SDA that increased the level of EPA by 5-fold compared with a 2.6-fold increase with LNA treatment (data not shown).

Both SDA and LNA treatments resulted in a similar concentration of 22:6n-3 in cultured cells compared with the vehicle BSA control. The concentration of AA was similarly lowered by the SDA, LNA and combination treatment (SDA/LNA) compared with the vehicle control cells. Consistently, the 1:1 SDA/LNA combination treatment resulted in concentrations of the aforementioned n-3 PUFAs that were intermediate for the individual SDA and LNA treatments. SDA was not detected in any of the fatty acid

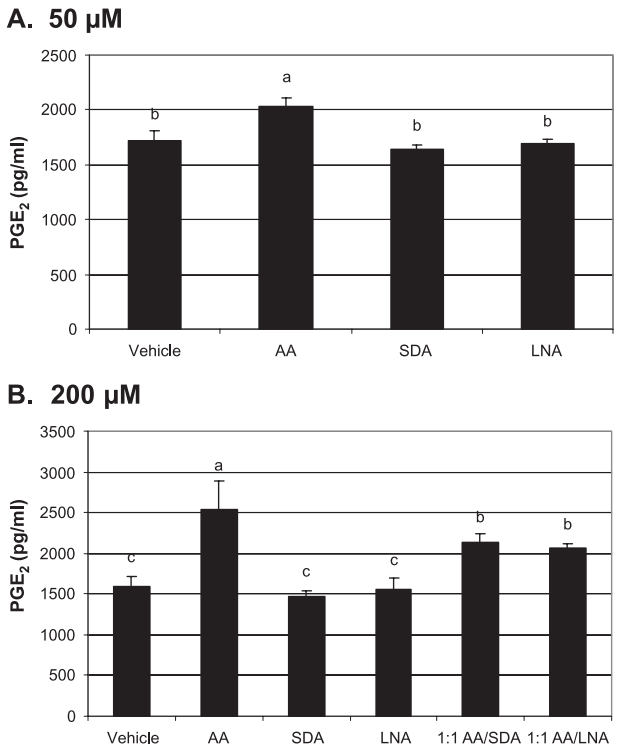

Fig. 2. Effects of AA, SDA and LNA on PGE_2 production in MDA-MB-231 cells. Subconfluent cells were supplemented with 50 (A) or 200 (B) μM of fatty acids with vehicle (BSA 25 or 100 μM) for 24 h. The cells were washed with PBS and treated with basal medium containing 10% FBS and 10 nM TPA for 24 h, and the medium was assayed using a STAT PGE_2 EIA kit. Data are presented as mean values±S.D. ($n=3$). Values with different letters are significantly different by one-way ANOVA and SNK multiple comparison test ($P<.05$).

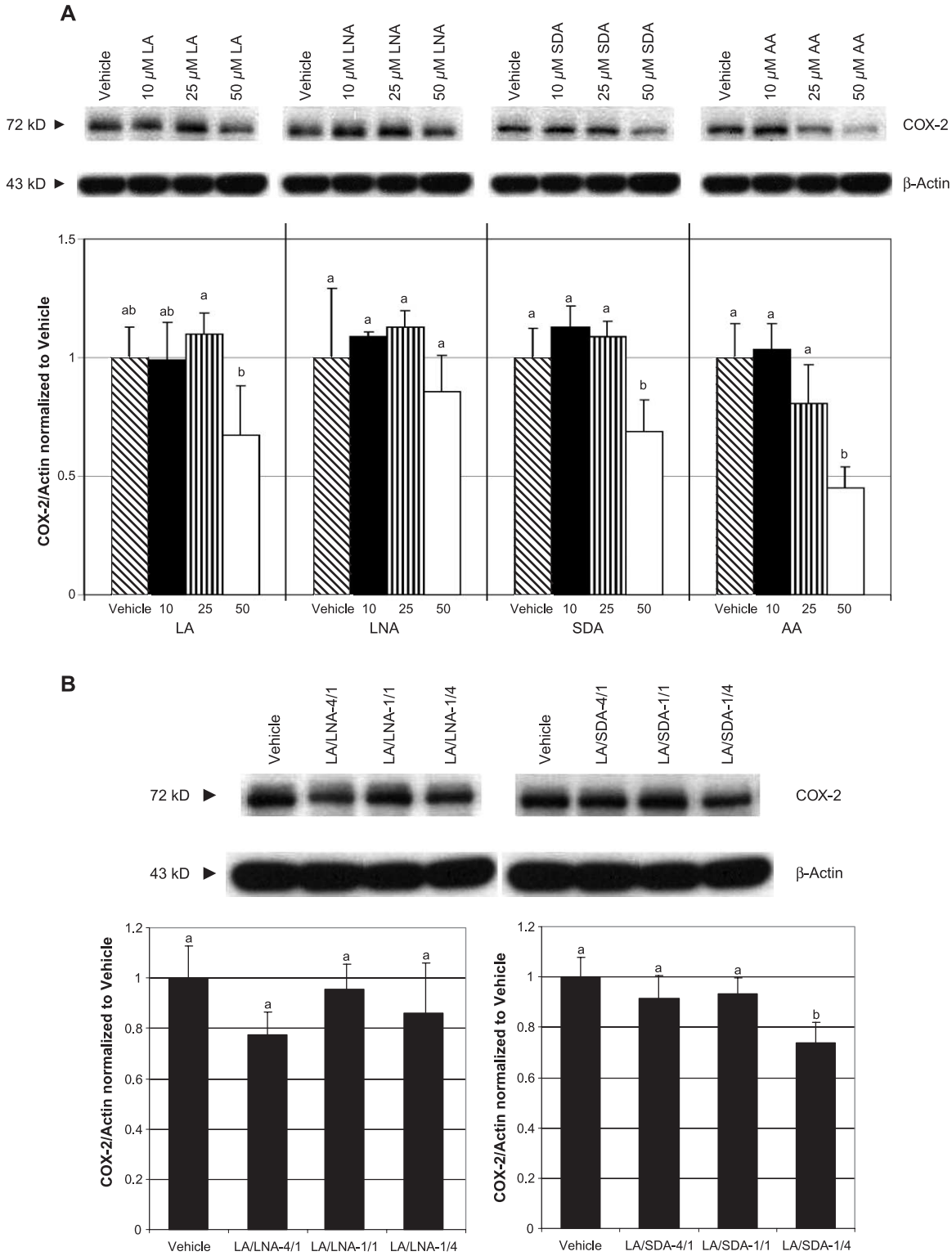

Fig. 3. (A) The effects of LA, LNA, SDA and AA on COX-2 protein level in MDA-MB-231 cells supplemented with fatty acids (10, 25 and 50 μM). (B) The effect of cosupplementation of LA with LNA or SDA at ratios of 4:1, 1:1 and 1:4 (total fatty acid concentration of 50 μM) on COX-2 protein level in MDA-MB-231 cells. Subconfluent MDA-MB-231 cells were treated for 24 h in the absence of serum prior to 1-h induction with 10 nM TPA. Cellular protein extracts were analyzed by SDS-PAGE and Western blotting, with protein extract of vehicle control (BSA) on each gel. The values for COX-2/β-actin ratio of treatments were normalized to the ratio of the control. Data are presented as mean values for the ratio±S.D. ($n=3$). Figure of Western blot is the representative of the triplicate samples. Values with different letters are significantly different within the respective fatty acid group by one-way ANOVA and Tukey's multiple comparison test ($P<.05$). Two-way ANOVA indicated significant differences both among fatty acid types and concentrations at $P<.05$.

treated cells, while LNA was found at low levels in all groups. The experiments on enriching cells with SDA compared with LNA indicated that SDA is biochemically more potent in increasing the individual 20 and 22 carbon n-3 PUFAs.

3.3. PGE_2 assay

When MDA-MB-231 cells were induced with 10 nM TPA following the fatty acid enrichment (24 h), AA-enriched cultures demonstrated a higher production of PGE_2 compared with those treated with SDA and LNA (Fig. 2A). Cells treated with SDA and LNA had PGE_2 levels similar to the vehicle control. A mixture of AA with either SDA or LNA at a 1:1 ratio resulted in a lower production of PGE_2 compared with the AA treatment, but the values were higher than the SDA or LNA treatments alone (Fig. 2B). In this case, both SDA and LNA attenuated the production of PGE_2 in the presence of AA in MDA-MB-231 cells.

3.4. Western blotting

In a series of experiments, MDA-MB-231 cells were treated with varying concentrations of fatty acids and combinations of linoleic acid (LA) with SDA or LNA to test their effects on COX-2 protein levels by Western blotting. Supplementing these cells with SDA, LA and AA for 24 h showed that all treatments, except for LNA, significantly lowered the level of COX-2 protein at 50 μM compared with the vehicle control (Fig. 3A). Statistical analysis by two-way ANOVA indicated significant differences both by fatty acid types and concentrations at $P<.05$. No effect on COX-2 protein level was observed at fatty acid concentrations below 50 μM. To examine the effects of SDA on cellular COX-2 protein levels, combinations with the 18 carbon n-6 fatty acid 18:2n-6 were used to eliminate the effects of a direct prostanoid precursor (e.g., AA). Hence, SDA and LNA were combined with LA and were included in the treatments. In these experiments, SDA demonstrated a significant effect on lowering the COX-2 protein level in a ratio of 1:4 (LA/SDA) (Fig. 3B) with a total fatty acid concentration of 50 μM. In contrast, LNA in combination with LA had no effect on the level of COX-2 protein compared with the vehicle BSA control (Fig. 3B).

3.5. Quantitative real-time PCR

Analysis of mRNA levels by quantitative real-time PCR indicated a trend in lowering COX-2 expression by n-3 PUFAs (Fig. 4A). SDA-treated cells had the greatest effect on lowering COX-2 transcription followed by the LNA treatment. The addition of LA to SDA or LNA diminished the lowering effect exerted by the n-3 PUFAs. These results suggest that both SDA and LNA in combination with LA were not effective in reducing COX-2 mRNA for the period tested.

To determine if the n-3 PUFA effect on COX-2 was mediated by changes in nuclear factors, the levels of mRNA for NFκB and PPARγ were determined in cells treated with PUFAs. Although not statistically different (due to the small

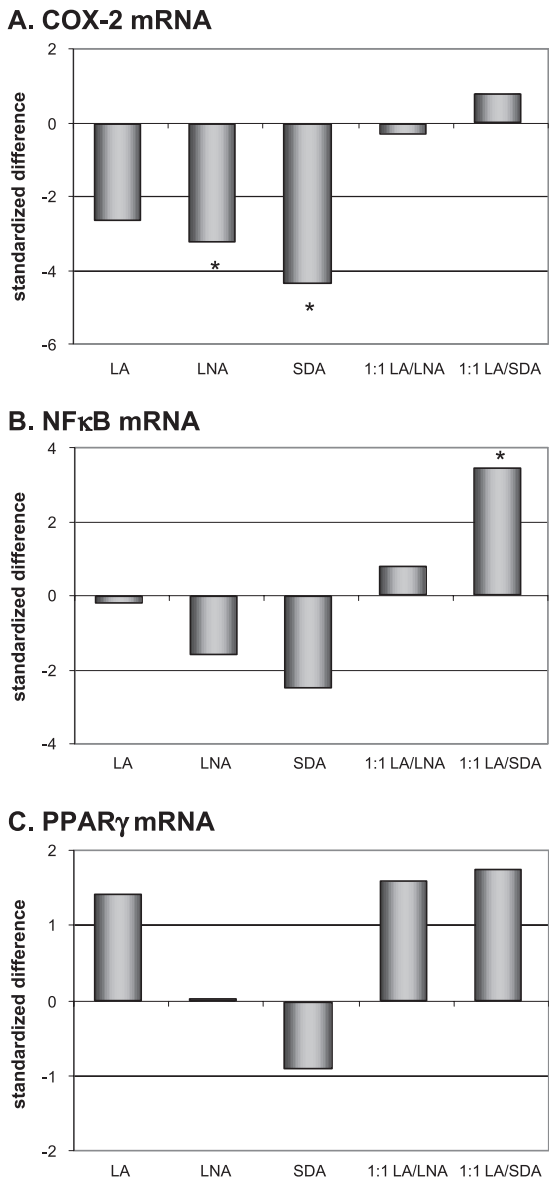

Fig. 4. The effects of LA, LNA, SDA and 1:1 combinations of LA/LNA and LA/SDA on the mRNA level of COX-2, NFκB and PPARγ in MDA-MB-231 cells supplemented with 50 μM total fatty acids. Subconfluent MDA-MB-231 cells were treated for 24 h in serum free fatty acid-supplemented media prior to 1-h induction with 10 nM TPA. Total RNA was extracted using an RNAqueous-4PCR kit and reverse transcription using an iScript cDNA synthesis kit. Quantitative real-time PCR was performed using an ABI Prism 7700 in 96-well optical plates. Control cells contained media with 25 μM BSA and no fatty acids. For each sample, the C_T (threshold concentration) values for each gene of interest were adjusted to the C_T for the control gene β-actin ($\Delta C_T = C_{T_{gene}} - C_{T_{actin}}$). The ΔC_T values were further normalized to the ΔC_T of the control ($\Delta\Delta C_T = \Delta C_{T_{treatment}} - \Delta C_{T_{control}}$). The relative quantity of a gene in a treatment group compared with the control is calculated by $2^{-\Delta\Delta CT}$. Values are presented as standardized difference calculated from the difference between $2^{-\Delta\Delta CT}$ values of treatment and control and divided by the pooled S.E.M. Bars are the mean values of $n=2$. An asterisk indicates significant difference from control at $P<.05$ by two-tailed Student's t test.

sample size), SDA tended to be more potent in suppressing the transcription of NFκB and PPARγ compared with LNA in the MDA-MB-231 cells (Fig. 4B and C). Cells treated with LA demonstrated the highest levels of PPARγ mRNA compared with the SDA and LNA treatments. The addition of LA with SDA to the cell cultures prevented the lowering effect of SDA on PPARγ and NFκB mRNA (Fig. 4B and C).

4. Discussion

The results from the enrichment experiments in MDA-MB-231 cells showed that SDA was more effective than LNA in elevating the amount of LC n-3 PUFAs in the cells. SDA, unlike LNA, was not detected in cellular lipids, indicating that SDA was rapidly metabolized or converted to LC n-3 PUFAs. These results are expected since SDA is a downstream metabolite of Δ^6-desaturase, that is, conversion of LNA to SDA, which would lead to greater amounts of LC n-3 PUFAs when compared with enrichment with LNA. However, neither SDA nor LNA treatment increased the concentration of 22:6n-3 in MDA-MB-231 cells. Our finding corroborates previous reports indicating that both SDA and LNA supplementation failed to increase the level of 22:6n-3 in the T47D breast cancer cell line [19] and in blood of subjects in a recent clinical study [10].

In our investigation, AA-treated cells produced a higher level of PGE_2 but a reduced amount of COX-2 protein, suggesting a possible feedback inhibition in which PGE_2 down-regulated COX-2 protein. Our results are consistent with a report on the actions of PGE_2 in this cell line [20].

TPA was used in the cell cultures to induce the release of AA from the membrane phospholipids [21] and facilitate the production of PGE_2. Clearly, enrichment with AA compared with 18 carbon n-3 PUFAs resulted in a higher amount of PGE_2 in these cells. One explanation for the lack of difference observed in the concentration of PGE_2 in SDA- and LNA-enriched cells was that the antibody used in the PGE_2 assay kit had a cross-reactivity of 43% with PGE_3. Since SDA-treated cells contained 50% more cellular EPA compared with LNA-treated cells, the SDA-enriched cells could potentially produce a lower yield of PGE_2 but a significantly higher level of PGE_3. Therefore, any difference in PGE_2 production between the SDA- and LNA-treated cells when tested with the EIA assay kit could not be determined since the antibody cross-reacted with PGE_3.

SDA was more potent than LNA in suppressing the transcription of the COX-2 gene, which may be indicative of a unique action imposed by SDA at the gene level. To further study the actions involved in the suppression of COX-2 gene transcription by SDA, the mRNA levels of NFκB and PPARγ were measured. Both of these transcription factors are involved in COX-2 gene expression as the promoter of COX-2 contains two sites for NFκB binding [22] and a peroxisome proliferator response element (PPRE) site for the binding of the PPARγ heterodimer [23] in human mammary epithelial cells. PPARγ is a ligand-dependent transcription factor that, when activated, forms a heterodimer with the retinoid X receptor (RXR), and together they bind to the promoter region of the target genes. The PPARγ/RXR heterodimer can be activated by the binding of a ligand to either receptor, but simultaneous binding of both ligands produces a synergistic effect on the activation of PPRE [24]. Previous investigations have demonstrated that PPARγ can be activated by LC PUFAs, eicosanoids, phytochemicals and several synthetic ligands currently used as antidiabetic drugs [25]. The n-6 PUFAs (LA and AA) have been shown in MDA-MB-231 cells to induce PPARγ transcriptional activity, while n-3 PUFAs such as EPA and LNA tended to inhibit the transactivation of PPARγ [26]. In human colon cancer cells (HCT116), 100 μM of DHA was found to be a potent inhibitor of PPRE transactivation, and its action was associated with the suppression of PPARγ binding to DNA [27].

Although activation of PPARγ is hypothesized to produce anticarcinogenic effects due to its anti-inflammatory, antiproliferative, proapoptotic and differentiation properties [28], increases in NFκB and PPARγ levels have been associated with greater transcription of COX-2 genes [23,29–31]. In our study, SDA appeared to suppress COX-2 expression via a reduction in the levels of both NFκB and PPARγ mRNA. For example, SDA compared with LA and LNA was the more potent fatty acid that exerted an inhibition of COX-2 expression and the transcription factors NFκB and PPARγ. Addition of LA to the LNA or SDA treatment abolished the suppressive effect of these n-3 PUFAs on COX-2, NFκB and PPARγ genes. The reduction in the magnitude of COX-2 expression observed in cells treated with SDA is likely due to the change in the NFκB and PPARγ levels, which is consistent with the lower mRNA levels of all three genes. When LA was combined with SDA, the effect of SDA on PPARγ mRNA was abolished. LA has been shown to be a more potent activator for PPARγ than LNA [26], and activation of PPARγ has also been demonstrated to increase the amounts of PPARγ mRNA [32] and protein [33]. Interestingly, the LA/SDA mixture resulted in an increase of NFκB mRNA in cells when compared with cells treated with SDA. It is unclear if LA interacts with SDA or its desaturation/elongation products as a transcription factor ligand to alter the expression of NFκB and PPARγ.

COX-2 and prostaglandins play a crucial role in the initiation and development of cancer, as demonstrated by experiments using PUFAs, COX-2 inhibitors and treatments that alter COX-2 expression. In a mouse model with spontaneous mammary tumors, a COX-2 inhibitor was reported to hinder the development and metastasis of tumors [34]. In a different mouse model, overexpression of the COX-2 gene in the human mammary gland induced tumorigenesis [35]. Supplementation of mice with EPA or DHA was associated with delayed tumor growth and metastasis as well as significant reductions in tissue concentrations of AA and PGE_2 compared with those given LA [8]. Moreover, both

EPA and DHA effectively attenuated new tumor formation more so than tumor cell growth, as indicated by reduced tumor number, not tumor size, when supplemented to a chemically induced rat model of mammary tumorigenesis [36]. In our experiments, we observed that SDA reduced the amounts of AA and PGE_2 in MDA-MB-231 breast cancer cells compared with the vehicle control and AA-treated cells, respectively.

A study utilizing a mouse model for intestinal tumorigenesis showed that tumor multiplicity was lowered by 50% in mice fed with SDA or EPA and that SDA was as potent as EPA in lowering PGE_2 production [37]. This study also showed that SDA or EPA diets did not increase DHA concentration in the tumor phospholipids, although EPA was dramatically increased. A similar observation in our study revealed that SDA and LNA supplementation elevated the concentration of EPA but not of DHA. These actions suggest that the mechanism involved in SDA- and EPA-mediated tumor reductions is independent of their conversion to DHA and possibly involves modulation of signal transduction and gene expression in addition to reduced synthesis of PGE_2.

The beneficial effects of n-3 PUFAs on breast cancer are associated with higher intakes of EPA and DHA that cannot be attained by simply following the new recommendations [dietary reference intakes (DRIs)] for n-3 PUFAs [38]. The DRIs for n-3 PUFAs are centered on the consumption of LNA, with the adequate intake of 1.6 and 1.1 g/d for adult men and women, respectively, which represents 0.6% to 1.2% of total energy intake [39]. Whether the DRIs for n-3 PUFAs are adequate for chronic disease risk reduction is still uncertain; however, the common diet in Japan, a country with remarkably low rates of cancer, includes 26% of energy from fat and 4 g/d of n-3 PUFAs [40].

The present investigation demonstrated that SDA compared with LNA significantly elevated LC n-3 PUFA concentrations and lowered COX-2 protein when combined with LA in MDA-MB-231 cells. The combination of LA with LNA or SDA in our study is important because, currently, LA and LNA are the most common dietary PUFAs in the food supply and we consume a diet consisting of a mixture of these PUFAs. The effects of these different PUFAs in combination provide greater insight into the actions of dietary PUFAs on molecular targets in order to modify the biochemical markers of disease. Once understood, this knowledge will help determine the proper dietary fat recommendations. Our findings suggest that consuming the recommended level of LNA in the presence of LA may not be effective in reducing the risk of chronic diseases associated with overexpression of COX-2. SDA in this study was almost two times more effective than LNA in increasing cellular EPA concentration, and SDA may be a potential alternative to EPA (and perhaps DHA) for people who do not consume fish or other sources of LC n-3 PUFAs. It is also noteworthy that an oil high in SDA has been shown in a clinical study to exert the hypotriglyceridemic properties of fish oil, attributed to the increase in cellular EPA content [41]. Moreover, stable and inexpensive dietary sources of SDA have been recently developed by genetically engineered oilseed crops [42,43].

In conclusion, we demonstrated that SDA was superior to LNA in LC n-3 PUFA formation and in lowering the level of COX-2 protein and mRNA expression in breast cancer cells. Thus, dietary SDA may be more beneficial than LNA in reducing the risk of breast cancer by effectively antagonizing PGE_2 production from AA. Future experiments should examine the actions of SDA on transactivation of COX-2 and include studies on NFκB and PPARγ.

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