Biomarkers for Personalizing Omega-3 Fatty Acid Dosing

Yan Jiang1, Zora Djuric2, Ananda Sen2, Jianwei Ren2, Dmitry Kuklev3, Ian Waters2, Lili Zhao4, Charis L. Uhlson5, Yu H. Hong3, Robert C. Murphy5, Daniel P. Normolle6, William L. Smith3, and Dean E. Brenner1,7,8

Abstract

Prostaglandin E\(_2\) (PGE\(_2\)) has been linked to a higher risk of colorectal cancer. PGE\(_2\) in colon tissue can be reduced by increasing dietary eicosapentaenoic acid (EPA). The dose-dependent relationships between dietary EPA, serum EPA:arachidonate (AA) ratio, urinary PGE\(_2\) metabolites, and colonic eicosanoids were evaluated to develop biomarkers for prediction of colonic PGE\(_2\). Male rats were fed diets containing EPA:w\(_6\) fatty acid ratios of 0, 0.1, 0.2, 0.4, or 0.6 for 5 weeks. Increasing the dietary EPA:w\(_6\) fatty acid ratio increased EPA:AA ratios in serum and in the proximal, transverse, and distal colon (\(P < 0.001\)). The urinary PGE\(_2\) metabolite was reduced (\(P = 0.006\)). EPA-rich diets reduced colonic tissue PGE\(_2\) concentrations by 58% to 66% and increased PGE\(_3\) by 19- to 28-fold. Other AA-derived eicosanoids were reduced by 35% to 83%. The changes were not linear, with the largest changes in eicosanoids observed with the lower doses. A mathematical model predicts colonic tissue eicosanoids from the EPA:AA ratio in serum and the EPA dose. Every 10% increase in serum EPA:AA was associated with a 2% decrease in the (geometric) mean of PGE\(_2\) in the distal colon. These mathematical relationships can now be applied to individualized EPA dosing in clinical trials. Cancer Prev Res; 7(10): 1011–22. ©2014 AACR.

Introduction

Large, population-based studies have indicated that NSAIDs can reduce the risk of colorectal adenocarcinoma by approximately 50% (1, 2). This effect of NSAIDs may be mediated by inhibition of the cyclooxygenase (COX) activity of prostaglandin H synthases 1 and 2, also commonly referred to as COX-1 and COX-2. Unfortunately, NSAIDs have unacceptable toxicity profiles that prevent daily use in otherwise healthy populations (3–6). COX is not the only target of NSAIDs for prevention of colorectal and other cancers. NSAIDs indirectly modulate lipoxygenase (LOX) pathways that can affect tissue concentrations of hydroxyeicosatetraenoic acids (HETE), leukotriene (LT), and hydroxyoctadecadienoic acids (HODE). Lipoxygenases (5-LOX and 12-LOX) and their downstream products have been associated with enhanced carcinogenesis in multiple epithelia (7, 8). 15-LOX expression correlates with increased colonic crypt differentiation, and its main downstream product, 13-S-HODE, decreases during the colonic carcinogenesis process (9, 10).

An alternative approach to pharmacologic inhibition of COX and LOX may lie in a nutraceutical approach—modulation of the pool of phospholipid-derived fatty acid substrates. The \(\omega\)3 fatty acid eicosapentaenoic acid (EPA) from fish oil is an alternative substrate for COX (11), yielding eicosanoids that are less proinflammatory than the products from \(\omega\)6 fatty acids (12, 13). EPA inhibits COX-2 expression, prostaglandin E\(_2\) (PGE\(_2\)) production, and cytokine production in the colon (12–14). Both EPA and docosahexaenoic acids (DHA) from fish oil inhibit the growth of experimental tumors in tissue culture and in animals, including xenografts of human tumors in nude mice (15), yet only EPA can serve as an efficient substrate for COX-2 (16, 17). Although some studies report that DHA can potentially alter the activity of COX-2 (18, 19) and lipoxygenases (20), these effects are via nonmetabolic substrate–enzyme competitive interactions. Our \textit{in vitro} data suggest that the EPA is an effective competitive inhibitor of arachidonic acid (AA) for COX-1 (11, 16, 17). Because COX-1 is the source of most prostaglandins in normal mucosa, our data \textit{in vitro} suggest that reduction of local colorectal mucosal PGE\(_2\) production \textit{in vivo} would be more...
contingent upon increasing the colorectal mucosal EPA:ω6 fatty acid ratios as opposed to DHA.

The goal of the current study was to identify mathematical relationships between serum biomarkers that could predict colonic PGE2 concentrations in a rat model with the goal of translating this information to humans. We focused on distal colon. Proximal and distal colon cancers differ in their etiology and biology (21, 22). Chronic inflammation appears to be relatively more important for distal versus proximal colon cancer (23, 24). Here, we document the relationships between dietary dosing, serum EPA:AA, EPA:AA in the distal colon, urinary PGE2 metabolites, and distal colorectal tissue PGE2 concentrations in an F344 rat model.

Materials and Methods

Animals and diets

All animal protocols for this experiment were approved by the University Committee on Use and Care of Animals at the University of Michigan. Male F344 rats (5 weeks old) were purchased from Harlan Laboratories (Haslett, MI). The pelleted low-fatAIN93-G, high-fat control diet (EPA:ω6 fatty acid ratio = 0), and high-fat fish oil diets (EPA:ω6 fatty acid ratio = 0.1, 0.2, 0.4, 0.6) were prepared by Dyets Inc. Supplementary Table S1 shows the composition of the experimental diets. The Western blend oil contained coconut oil (45% by weight), olive oil (30% by weight), corn oil (15% by weight), and soybean oil (10% by weight). The Western blend oil was mixed with menhaden oil to achieve the desired EPA:ω6 ratios.

To minimize lipid oxidation, OmegaPure brand menhaden oil from omega protein was stabilized against oxidation with a combination of mixed tocopherols and tert-butylhydroquinone (TBHQ). This menhaden oil was then blended with the "Western" blend oil that was also stabilized with TBHQ. The fat blend was then added to the diet and mixed in a planetary mixer in cold conditions with an average temperature change less than 3°C. After pelleting, the diet was air dried (25°C) for 24 hours. During visual inspection before packaging, any dark- or mottled-looking pellets that indicated the fat had oxidized were discarded and remade. We stored the diets frozen in a −80°C freezer. Small amounts were thawed in the refrigerator every 2 days for feeding. We replaced the food in the animal cage on a daily basis to minimize lipid oxidation. The rats were given water ad libitum throughout the experiment. The rats were maintained on a 12-hour light/dark cycle.

Sample collection and the experimental design

Rats were acclimated to the AIN93-G diet for 1 week. Sixty rats used for this experiment were randomly divided into five groups (12/group) and provided with one of the five diets: control diet or one of four fish oil diets for five consecutive weeks. Body weight was recorded weekly. During the last week of feeding, the rats were individually housed in metabolic cages for 24 hours for urine collection. At the end of the study, animals were euthanized by isoflurane inhalation and decapitation. Blood was collected from the neck, and serum was separated and stored at −80°C. The animals were not fasted before necropsy to preserve the colon biology. The colon was immediately removed from the animal and rinsed with cold PBS containing indomethacin (5.6 μg/mL). The colon was cut into three equal sections horizontally, which were denoted as the "proximal" (cecal end), "transverse," and "distal" (rectal end) colon. The colonic sections were snap frozen in liquid nitrogen and stored at −80°C before processing. Frozen colonic tissue samples were pulverized in liquid nitrogen, and then 1 mL of cold PBS (with indomethacin) was added to the tissue powder to make a tissue homogenate. The suspension was sonicated in ice water for 3 minutes (20-second sonication, 20-second cooling cycle) and stored in a −80°C freezer until further analysis. An aliquot of the homogenate from each sample was used to determine protein concentrations using the Bradford assay (Bio-Rad Laboratories Inc.).

The assays were carried out in batches in a balanced manner. The design conformed to a completely randomized block design. There were six batches in all with each batch yielding fatty acid and eicosanoid measurements from all three locations of twelve rats. Two rats from each diet dose group were analyzed in every batch, making an even dose distribution within batch.

Synthesis of d5-PGE3

The internal standard d5-EPA was synthesized as described in a recent report (25). This material was used to synthesize d5-PGE3 using human recombinant COX-2, as described previously (16), making a 800 μg/mL solution in 50 mmol/L PBS. Human microsomal recombinant prostaglandin E synthase-1 (mPGES-1) was from Cayman Chemical. Buffer A (prepared immediately before the reaction) contained 0.5 mol/L TrisHCl, pH 8.3 (200 mL), deionized water (800 mL), 1 mmol/L phenol (10 mL), and 5 mmol/L hemin in DMSO (1 mL). Buffer B contained NaH1PO4.H2O (436 mg), Na2HPO4 (971 mg), reduced glutathione (77 mg), EDTA2Na2.H2O (37 mg), Triton X-100 (320 mg), and 98 mL of water. The d5-EPA (10 μL of a 6 mmol/L solution in ethanol) was added to Buffer A (900 μL) and the sample was mixed well. Recombinant human COX-2 was then added (30 μL of a 0.8 mg/mL solution in 50 mmol/L phosphate-buffered saline). The sample was mixed vigorously for 120 seconds. Buffer B (180 μL) was added with mixing and then mPGES-1 (6 μL of a 20 mg/mL solution in Buffer B) was added, the sample was mixed well, and left at room temperature for 10 minutes. The reaction was quenched with 450 μL of acetonitrile/0.2 mol/L citric acid (4:1, v/v). The reaction mixture was transferred into plastic tubes and centrifuged at 1,300 × g for 5 minutes to remove precipitated protein.

For purification, the supernatant (750 μL) was injected onto an HPLC (high-performance liquid chromatograph) system equipped with C18-RP-HPLC column (Shodex RSpack DE-413L; 250 mm × 4.6 mm, 5 mm) operated at
1 mL/min with a binary gradient of solvent A with solvent B. Solvent A was acetonitrile/H2O/acetic acid (0:70:0.1, v/v/v). Solvent B was acetonitrile/H2O/acetic acid (90:10:0.1, v/v/v). The gradient was solvent A-100% (5 min), which was gradually switched to solvent B-100% for 15 minutes, solvent B-100% for 8 minutes, solvent A-100% for 6 minutes. The peak d5-PGE3 was collected between 14.5 and 15.5 minutes. The separation was performed twice to yield 2 mL of the eluate, which was evaporated under vacuum and redissolved in ethanol. HPLC-MS (ESI, negative ion mode), m/z (1%) (26): 354.2 (100%) [M (deuterated-d5) - H]-.

Eicosanoid analysis by chiral LC-MS/MS

The d5-PGE3 and d5-EPA used in the assays were synthesized in our laboratory (25). Other eicosanoid standards were obtained from Cayman Chemical. The LC-MS/MS method for eicosanoid analysis was adapted from the literature as previously described (27, 28). For extraction of eicosanoids, 300 µL of the colonic homogenate was added to 12 × 75 mm glass tubes on ice, along with 1 N citric acid (20 µL), 30 mmol/L disodium EDTA (20 µL), and deuterated internal standard. The resulting solution was then extracted twice with 2 mL hexane:ethyl acetate [1:1 v/v, containing 0.1% BHT (w/v)]. The pooled extracts were evaporated and reconstituted with 100 µL of HPLC mobile phase. Aliquots (20 µL) were injected for analysis by LC-MS/MS.

HPLC separation was performed on a Waters 2695 separations module, using a Chiral-Pak AD-RH analytical column (2.1 × 150 mm, 5-µm particle size; Chiral Technologies). The column was maintained at 40°C. Mobile phase A was 10 mmol/L ammonium acetate pH 4.1 and mobile phase B was acetonitrile. The flow rate was 0.2 mL/min. The linear gradient program was as follows: 30% to 50% B (0–5 minutes), 50% to 100% B (5–24 minutes), 100% B (25–30 minutes), and return to 30% B (30–37 minutes). The effluent was introduced into a Finnigan TSQ Quantum Ultra triple quadrupole mass spectrometer by electrospray ionization (ESI), and detection of negative ions was done as previously described (27). Eicosanoid concentrations in colonic tissue were normalized to protein concentrations.

Urinary metabolite analysis by LC-MS/MS

Rat urine samples (1 mL) were processed following the method of Murphey and colleagues (26). Briefly, LC was performed on a Zorbax Eclipse XDB-C18 column (Agilent Technologies) attached to a Surveyor MS Pump (Thermo-Finnigan). Detection was done with a Thermo-Finnigan TSQ Quantum triple quadrupole mass spectrometer operating in the selected reaction monitoring mode. Quantification of urinary metabolite of PGE2, tetrنانor PGE-M (9,15-dioxo-11α-hydroxy-2,3,4,5-tetranor-prostan-1,20-dic acid, PGE-M) was done using an isotopically labeled internal standard (using m/z 336 and 339 ions). Urinary 2,3-dinor-6-keto-PGF1α, a metabolite product of PGF2α, was also determined in the urine samples following procedures documented previously (29).

Urinary creatinine was measured using an Autoanalyzer (Technicon). Data were expressed as nanograms per milligram creatinine.

Fatty acid analysis by GC-MS

For quantitation of fatty acids, 10 µL of internal standard (17:0, 1 mg/mL in hexane) was added to 150 µL of colon homogenate or serum. The samples were then extracted with 1.5 mL of Folch reagent (chloroform:methanol, 2:1), vortexed for 2 minutes, and centrifuged (200 × g for 5 minutes). The organic layer was removed to a 10 × 75 mm glass tube and dried in a SpeedVac. The samples were solubilized in 150 µL of hexane:chloroform (1:1) and vortexed. Fatty acid methyl esters (FAME) were prepared by adding 10 µL of METH-PREP II derivatization reagent (0.2 mol/L methanolic (m-trifluoromethylphenyl) trimethylammonium hydroxide; Alltech). GC-MS was conducted as previously described using selected ion monitoring (30).

Data analysis

Descriptive plots and summary measures (mean and SD) were calculated for fatty acids percentages and eicosanoid concentrations obtained in serum, as well as in different segments (transverse, proximal, and distal) of the colon. The primary statistical framework adopted was that of a linear mixed-effect regression model that properly accounted for the clustering within the sections of the same colonic tissue. In the first set of such models, eicosanoids and EPA:AA ratios were compared across the different sections in analysis of covariance models with section as a 3-level factor, and a linear and quadratic dose effect as covariates. The model was further adjusted for variation across analytical batches by using batch as a categorical variable. A random subject effect incorporated clustering within the different sections of the colon. Mathematically, the model for a given measure assumed the analytical form

\[ y_{ijk} = \mu + \alpha_i + \beta_j + \gamma_k + \delta d_i + \epsilon_{ijk} \]  

where \( y_{ijk} \) was the natural logarithm of the outcome measure (eicosanoid) from the \( i \)th section of the colon of \( j \)th animal in the \( k \)th batch, \( i = 1, \ldots, 10; j = 1, \ldots, 6; k = 1, 2, 3 \). In the model, \( \mu \) denotes the overall mean, \( \alpha_i \) denotes the \( i \)th subject effect, \( \beta_j \) denotes the \( j \)th batch effect, and \( \gamma_k \) denotes the \( k \)th section effect. Furthermore, \( d_i \) indicates the dose level for the \( i \)th subject in the \( j \)th batch, and \( \delta_1 \) and \( \delta_2 \) reflect the linear and quadratic dose effects. The random error \( \epsilon_{ijk} \) and the subject effect \( \alpha_i \) were both assumed to have normal distributions with mean zero and constant variance, independently of each other. Post hoc pairwise comparison between sections was carried out with Bonferroni adjustment for multiple comparisons. In a separate set of analogous analysis, the association between eicosanoids and, the PGD2, PGE3 ratio was investigated after controlling for the batch and section effects. A random subject effect was used in the models as before. Logarithmic transformation was used for all the eicosanoid measures based on the behavior of the residuals and other model selection criteria such as Akaike information criterion and Bayesian information criterion.
To graphically assess the goodness of fit, plots of fitted mean curve of PGE\textsubscript{2} and PGE\textsubscript{3} as a function of dietary EPA:ω6 ratio were superimposed on the observed values at the proximal and distal sections of colon. A similar plot depicted the relationship between PGE\textsubscript{2} and PGE\textsubscript{3} and EPA: AA in serum. Each plot was further accompanied by 95% point wise confidence intervals of the regression function. We illustrate the construction of the confidence interval by means of the dose–response curve that was explained by a regression function of the form
\[ y = a + b \times \text{dose} + c \times \text{dose}^2, \]
where \( y \) was the response [\( \log(\text{PGE}_2) \) or \( \log(\text{PGE}_3) \)] at a given colonic section, and \( a, b, \) and \( c \) were fitted regression coefficients. At a given dose \( d \), the lower and upper confidence limits of the regression function were calculated as
\[ y(d) \pm 1.96 \times \text{s.e.} \{ y(d) \} \]
with the standard error (s.e.) \( y(d) \) given by
\[ \text{s.e.} \{ y(d) \} = \sqrt{\text{var}(a) + d^2\text{var}(b) + d^2\text{var}(c) + 2d\text{cov}(a,b) + 2d\text{cov}(a,c) + 2d^2\text{cov}(b,c)}. \]

where var and cov denote variance and covariance terms, respectively. The confidence limits for PGE\textsubscript{2} or PGE\textsubscript{3} were obtained by exponentiating the expressions in Equation (1).


**Results**

**Diets and body weight**

The modified AIN93-G diet contained EPA to ω6 fatty acids at ratios of 0, 0.1, 0.2, 0.4, and 0.6, while keeping total fat content constant at 34% of calories using a Western blend fat (Supplementary Table S1). The fatty acid composition of the diets given in Supplementary Table S1 was independently verified by GC-MS (data not shown). Rats consumed these experimental diets for 5 weeks. The body weight of the rats was measured weekly. No differences were found in body weight among the means of the five rat groups (Supplementary Fig. S1).

**Colonic and serum fatty acid concentrations**

Total fatty acids were assayed in the colonic sections and in the serum from the rats without separation of lipid subfractions (Supplementary Table S2). Because blood was obtained in the nonfasting state and fatty acids were extracted directly from serum, one limitation of the study is that much of the serum fatty acid would have been from chylomicrons. We found that percentages of AA in total fatty acids varied in different anatomic colonic segments; but, the percentages of EPA did not differ among colonic segments (Fig. 1A and B). Distal colon had a higher percentage of AA than the proximal or transverse colon. The percentage of AA in the serum reflected that of the colon. The percentage of EPA in the serum was higher than in the three colonic tissue sections in rats fed with EPA-containing diets.

The percentage of EPA in the total fatty acid pool increased in all three colonic segments as well as in the serum when the dietary EPA:ω6 ratio dose was increased (Fig. 1B). We also observed a dose-dependent increase in docosahexaenoic acid (DHA, 22:6) and a decrease in lauric acid (12:0; Supplementary Table S2). EPA:AA ratios for the tissues and sera were calculated, and there was a consistent increase in the EPA:AA ratio upon increasing dietary EPA with changes in serum being the largest and changes in distal colon being the smallest (Table 1).

Similar to AA, increasing the dose of dietary EPA caused no change in total ω6 polyunsaturated fatty acids (PUFA) in any of the colonic sections or in the serum (Fig. 1C). Dietary EPA caused a dose-dependent increase of ω3 PUFAs in both colon and sera (Fig. 1D). Although serum ω6 PUFAs were similar to colonic tissue ω6 PUFAs levels, ω3 PUFAs were consistently higher in serum than in the three colonic tissue segments in the rats fed EPA-enriched diets (Fig. 1C and D). The average EPA:AA ratios were quite similar in proximal and transverse sections, whereas the EPA:AA ratio in the distal section was significantly lower than either of the other two sections (Fig. 1E).

**Colonic prostaglandin concentrations**

The proximal colonic PGE\textsubscript{2} concentrations were three times higher than the distal PGE\textsubscript{3} concentrations, regardless of the dietary EPA:ω6 fed (Supplementary Table S3); the same relative relationship was detected for PGE\textsubscript{3}. Statistical analyses showed that adjusting for the dose and batch effects, both PGE\textsubscript{2} and PGE\textsubscript{3} exhibited the following significant pattern decreasing the average concentration: proximal > transverse > distal (Supplementary Table S3). Interestingly, even though the highest EPA dose was well above most human dietary intakes, the PGE\textsubscript{3} concentrations in the rat colon were less than 10% of the PGE\textsubscript{2} concentrations. The major PGE (and eicosanoid) product in colonic tissue was PGE\textsubscript{2} despite large increases in dietary ω3 fatty acids.

Increased dietary EPA caused a nonlinear, dose-dependent reduction in colonic tissue PGE\textsubscript{2} concentrations. The reduction in PGE\textsubscript{2} concentrations was maximal with an EPA:ω6 diet ratio of 0.4 but small incremental reductions occurred with the 0.6 diet in proximal, transverse, and distal colon (Fig. 2A). PGE\textsubscript{2} concentrations decreased 66% in the proximal colon, 58% in the transverse, and 61% in the distal colon with the 0.4 EPA:ω6 diet relative to the diet with no EPA (Supplementary Table S3). Colonic PGE\textsubscript{3} concentrations increased with increasing dietary EPA:ω6 fatty acid ratios \( P < 0.0001 \) in all three colonic sections (Fig. 2B and Supplementary Table S3). The highest-dose EPA diet increased the concentrations of PGE\textsubscript{3} by 19-fold in...
the proximal colon, 28-fold in the transverse colon, and 19-fold in the distal colon as compared with rats fed no EPA.

**Colonic concentrations of HETEs and HODEs**

We also measured the concentrations of LOX products in colonic tissues from rats fed the different diets. The proximal colon consistently had the highest concentrations of 5-\(\text{S}\)-HETE and 15-\(\text{S}\)-HETE (Fig. 2C and D; Supplementary Table S3). The concentrations of 12-\(\text{S}\)-HETE and 13-\(\text{S}\)-HODE did not differ among the three colonic segments from rats in any dietary group (Fig. 2E and F; Supplementary Table S3).

For the 5-\(\text{S}\)-HETE, 12-\(\text{S}\)-HETE, and 15-\(\text{S}\)-HETE, the pattern of significant differences among the colonic sections was not consistent (Supplementary Table S3). Among the four HETEs and HODE, increased dietary EPA:\(\text{w}\_6\) fatty acid ratios caused the largest reduction in 12-\(\text{S}\)-HETE, by more than 80% in all colonic segments (Fig. 2E and Supplemental Table S3). Similarly, consistent decreases by...
dietary EPA were found in all three colonic segments for 15-S-HETE (Fig. 2D and Supplementary Table S3), and a trend of decrease was found in 13-S-HODE (Fig. 2F and Supplementary Table S3). The largest reduction for both 12-S-HETE and 15-S-HETE occurred with dietary EPA:ω6 fatty acid ratios between 0 and 0.1. Further increases in dietary EPA:ω6 fatty acid ratios did not cause changes in 5-S-HETE in the three colonic segments. In rats fed the control diet, the relative amount of ω3 to ω6 isomers of 5-HETE was 1.2 in the proximal colon, 1.9 in the transverse colon, and 1.7 in the distal colon; for 12-HETE, it was 0.1 in the proximal colon, 0.06 in the transverse colon, and 0.06 in the distal colon; for 15-HETE, it was 0.4; and for 13-HODE, it was 0.2 in all three anatomic colon segments. Increased dietary EPA:ω6 fatty acid ratios did not cause changes in concentrations of the R-enantiomers for any of these eicosanoids in the colonic samples (Supplementary Table S3).

**Urinary metabolites**

Increasing the dietary EPA:ω6 fatty acid ratio from 0 to 0.4 caused a dose-dependent decline in urinary PGE-M with little further change at the highest EPA:ω6 dose (Fig. 3 and Table 1). A maximal 43% reduction was seen in urinary PGE-M in rats fed the 0.6 EPA:ω6 diet (Fig. 3A). The concentration of the urinary metabolite of prostacyclin (PGI2-M), 2,3-dinor-6-keto-PGF1α, was not altered by increased dietary EPA (Fig. 3B).

**Mathematical relationship between fatty acids and eicosanoids**

Both the linear and the quadratic terms corresponding to EPA dose in the diet were significant for EPA:AA, PGE2, and PGE3 as well as their ratio (Table 1). Because of the existence of the quadratic term, the rate of change in the eicosanoids was dose dependent. This is consistent with the plateauing pattern demonstrated in Fig. 2A. Although the coefficient in Table 1 corresponding to dose indicated the initial change in the eicosanoid measurement per unit dose, the dose-squared coefficient contributed to the plateauing effect.

Dose was significantly associated with urinary PGE-M but not 2,3-dinor-6-keto-PGF1α. For the HETE’s and HODE’s, the patterns of associations were mixed. The linear dose effect was significantly associated with both 12-S-HETE and 15-S-HETE, whereas the association was not significant for 5-S-HETE or 13-S-HODE. On the other hand, 12-S-HETE was the only monohydroxy acid that exhibited any significant association with the quadratic effect of dose.

Key fatty acid percentages (EPA, ω3HUFA, ω3PUFA, ω6HUFA, and ω3PUFA:ω6PUFA) demonstrated significant associations with PGE2 and PGE3 (Table 2) both in the colonic tissue and the serum. The coefficients corresponding from the linear regression on the log–log scale can be translated into an interpretation of percent change. For the PGE2 and serum EPA:AA relationship (adjusting for batch and location effect), the relationship reduced to the equation

\[
\frac{[PGE_2]_I}{[PGE_2]_II} = \left(\frac{[EPA:AA]_I}{[EPA:AA]_II}\right)^{-0.2},
\]

where the ratios of both sides of Equation (5) represent fold changes between dose levels I and II. Thus, to achieve a
10% increase in EPA:AA, we set the right-hand side of Equation (5) to 1.1, which yields (PGE$_2$)$_{II}$ = 0.98(PGE$_2$)$_I$, or equivalently a 2% decrease in the (geometric) mean of PGE$_2$, the association being significant with $P < 0.0001$. Similarly, a 10% increase in EPA:AA corresponds to a (1.1)$^{0.74} / C_0$ value significantly different from the control diet ($P < 0.01$). Detailed results of the statistical analyses are presented in Supplementary Table S3.

The dose–response curves in Fig. 4A–D tracked the observed data quite well both for PGE$_2$ and PGE$_3$. The absolute amount of R isomers (data not shown) did not change appreciably with fish oil feeding. Two-way ANOVA was conducted for each eicosanoid. Diet was a significant factor for all the eicosanoids except 5-S-HETE, 5-R-HETE, and 15-R-HETE, and PGE$_3$/PGE$_2$ ratio. *, values significantly different from the control diet ($P < 0.0001$). Similar interpretations apply to other coefficients.

The dose–response curves in Fig. 4A–D tracked the observed data quite well both for PGE$_2$ and PGE$_3$. A dose close to 0.4 seemed optimal in any colon section. The curves for PGE$_2$ showed a tighter cluster than that for PGE$_3$. The fit...
through the scatter of serum EPA:AA and PGE_2 showed close conformity to the data (Fig. 4E–H). By contrast, there seemed to be high variability in PGE_3 values over a short range of EPA:AA and the fitted curve was driven by a few outlying observations.

Discussion

Omega-3 fatty acids in the form of dietary fish oil appear to have cancer-preventive effects; however, all studies do not agree, and taking high doses can be problematic in terms of both compliance and toxicity (31, 32). Beneficial effects of fish oil may be limited by dietary intakes of other fats (33, 34). Individualized dosing is an attractive approach for optimizing the beneficial, preventive effects of ω3 fatty acid formulations. Development of mechanism-based, rapidly assayed, accurate, analytically cost-effective biomarkers will facilitate such individualized dosing to allow anticarcinogenic efficacy while minimizing toxicity.

We translated our in vitro data demonstrating that the ω3 fatty acids inhibit the catalytic activity of COX-1 (11, 16, 35). Both EPA and DHA can serve as substrates for ovine and human COX-1 (16, 35); but, they are much poorer substrates than AA. Both ω3 fatty acids can inhibit the oxygenation of AA by COX-1 and COX-2 (16). In a nonneoplastic epithelium, COX-1 is the primary cyclooxygenase form present and is the likely target for reducing PGE_2. Both DHA and EPA were present in the diet and were incorporated into colon tissue with similar dose-dependent kinetics. EPA is oxygenated by COX-1 but at 10% of the efficiency of AA (35). EPA competes directly with AA for the catalytic site of COX-1 (36). DHA is a poor substrate and modest inhibitor of AA oxygenation by COX-1 (35, 37). We therefore chose to model PGE_2 reduction in colon using the EPA:AA fatty acid ratio.

Although less relevant to the current model, EPA is metabolized by COX-2 to form PGE_3, which has antiproliferative effects in cancer (38). The small amounts of PGE_3 found in the colon are likely due to EPA metabolism via COX-2.

We were able to establish in F344 rats a quantitative, statistically valid relationship in vivo between serum fatty acids using the EPA:AA ratio, urinary PG metabolites, and colonic tissue concentrations of PGE_2 (Table 2). Dietary intake of EPA reduced colonic tissue PGE_2 concentrations in a log-linear relationship while increasing PGE_3 concentrations. Previously published data suggest that an increased dietary ω3:ω6 ratio is more important than the total amount of ω3 fatty acid intake in altering eicosanoid production in rodents (39) and in reducing human colonic epithelial proliferation (33). The doses of EPA or fish oil used in human studies for cancer prevention ranged from over 7 g/day to as low as 2 g/day, but none of these trials employed a dose–response with biomarker approach to individualize human dosing (33, 40–44). The data presented here provide a working model that predicts the relationship between dietary ω3 fatty acids, serum and tissue fatty acid concentrations, and resulting eicosanoids in an epithelial target the distal colon. Such relationships, based upon known enzymatic substrate–product relationships, were translated to in vivo rodent model systems in preparation for future human applications (11, 16, 17). Our data support a mathematical model that may be useful for designing an individualized human dosing strategy. Currently, our model only incorporates EPA supplementation in healthy individuals without known colonic inflammatory stress because of our data suggesting the EPA inhibit COX-1 metabolism of AA more efficiently than DHA. We recognize that DHA has other anticarcinogenic effects and may have a role in cancer risk reductions with ω3 fatty acids (15, 45). In future studies, we would need to model DHA modulation of cytochrome metabolism to epoxides and COX-2 interactions (19, 20).

Human metabolism may differ from rodents. Published data to date suggest that fatty acids in both rodents and humans after feeding ω3 fatty acids correlate with intake but few direct comparisons with comparable doses are known. For example, Stark assayed bioavailability of ω3 fatty acids in rats, pigs, and humans, but none were dosed equivalently (46). Previous data suggest a pharmacodynamic relationship between fatty acid concentrations in blood or tissue compartments and eicosanoids in rodents (13) and in...
humans (41); but a direct comparison with pharmacodynamic models using rat data and applying it to humans has not been published. The data presented here provide a rodent-based pharmacodynamic model that we are currently testing in a human clinical trial of EPA supplementation in humans using a colon eicosanoid endpoint. Such a pharmacodynamic trial will permit rodent to human comparisons in the future.

A diet high in EPA from fish oil reduced colonic concentrations of PGE₂ by at least 50% in all three regions of colon, but did not alter percentages of AA or 6 PUFA, as opposed to substituting for them (47). The present data demonstrate that incremental increases in the dietary intake of EPA reduced colonic PGE₂ by 60%, an effect that is likely to be reproduced in other epithelial tissues.

In addition to the potential utility of serum fatty acids as biomarkers of colon PGE₂, urinary PGE-M has been reported to be associated with colorectal cancer risk in a large prospective cohort study (48). In our study, dietary EPA induced a dose-dependent reduction of PGE-M; however, neither dietary EPA nor PGE-M were as highly correlated with tissue eicosanoid concentrations as the EPA:AA ratio in tissue and serum. Changes in urinary PGE-M represent not only colonic COXs but also systemic metabolism. Kakutani and colleagues (49) demonstrated that an AA-enriched diet did not increase urinary PGE-M concentrations and did not modulate PGE₂ in blood in humans. However, our data suggest that urinary PGE-M may be a useful biomarker for epithelial proinflammatory state. We observed that an ω3 fatty acid diet did not modulate urinary 2,3-dinor-6-keto PGF₁α similar to what Kakutani and colleagues reported (49).

Previously, our group developed a chiral LC/MS-MS method to quantify HETEs and HODEs in the colonic tissue of rodents (27). The technology permits more efficient interrogation of eicosanoid products beyond COX products. Among all the AA-derived eicosanoids measured, colonic PGE₂ was the most abundant form followed by 12-S-HETE, and 5-S-HETE was the lowest. The ω3 fatty acid competition for eicosanoid-metabolizing proteins extends beyond COX. 5-LOX and 12-LOX products have mitogenic effects, inducing DNA synthesis while inhibiting cell-cycle arrest and caspase release, and promoting angiogenesis (7). The role of 15-LOX-1 and 15-LOX-2 metabolites appears less clear with the predominance of data supporting antiinflammatory and anticarcinogenesis mechanisms via PPARγ-induced apoptosis and DNA-dependent protein kinase activity activation of p53 (7). Our data suggest that ω3 fatty acid supplementation will have useful anticarcinogenic activity as much via LOX pathway modulation as with cyclooxygenase pathway modulation.

Table 2. Effect of colon and serum fatty acid variables on colon prostaglandins

<table>
<thead>
<tr>
<th>Samples</th>
<th>Variables</th>
<th>Coefficient</th>
<th>SE</th>
<th>P</th>
<th>Coefficient</th>
<th>SE</th>
<th>P</th>
<th>Coefficient</th>
<th>SE</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colonic tissue</td>
<td>Log AA</td>
<td>0.055</td>
<td>0.139</td>
<td>0.692</td>
<td>-0.043</td>
<td>0.051</td>
<td>0.395</td>
<td>-0.062</td>
<td>0.096</td>
<td>0.515</td>
</tr>
<tr>
<td></td>
<td>Log ω6HUF A</td>
<td>-2.456</td>
<td>0.525</td>
<td>&lt;0.0001</td>
<td>0.671</td>
<td>0.163</td>
<td>&lt;0.0001</td>
<td>-1.909</td>
<td>0.364</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>Log ω6PUFA</td>
<td>0.073</td>
<td>0.282</td>
<td>0.797</td>
<td>-0.077</td>
<td>0.103</td>
<td>0.454</td>
<td>-0.152</td>
<td>0.194</td>
<td>0.435</td>
</tr>
<tr>
<td></td>
<td>Log EPA</td>
<td>0.670</td>
<td>0.099</td>
<td>&lt;0.0001</td>
<td>-0.150</td>
<td>0.032</td>
<td>&lt;0.0001</td>
<td>0.485</td>
<td>0.070</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>Log ω3HUF A</td>
<td>2.327</td>
<td>0.174</td>
<td>&lt;0.0001</td>
<td>-0.386</td>
<td>0.068</td>
<td>&lt;0.0001</td>
<td>1.608</td>
<td>0.135</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>Log ω3PUFA</td>
<td>0.738</td>
<td>0.162</td>
<td>&lt;0.0001</td>
<td>-0.218</td>
<td>0.052</td>
<td>&lt;0.0001</td>
<td>0.526</td>
<td>0.112</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>Log (EPA:AA)</td>
<td>0.911</td>
<td>0.098</td>
<td>&lt;0.0001</td>
<td>-0.173</td>
<td>0.033</td>
<td>&lt;0.0001</td>
<td>0.709</td>
<td>0.068</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>Log (ω3:ω6PUFA)</td>
<td>1.509</td>
<td>0.172</td>
<td>&lt;0.0001</td>
<td>-0.309</td>
<td>0.057</td>
<td>&lt;0.0001</td>
<td>1.084</td>
<td>0.123</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Serum</td>
<td>Log AA</td>
<td>0.382</td>
<td>0.908</td>
<td>0.674</td>
<td>0.045</td>
<td>0.230</td>
<td>0.844</td>
<td>0.575</td>
<td>0.657</td>
<td>0.384</td>
</tr>
<tr>
<td></td>
<td>Log ω6HUF A</td>
<td>-4.064</td>
<td>0.352</td>
<td>&lt;0.0001</td>
<td>0.829</td>
<td>0.122</td>
<td>&lt;0.0001</td>
<td>-2.870</td>
<td>0.275</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>Log ω6PUFA</td>
<td>-0.773</td>
<td>1.494</td>
<td>0.606</td>
<td>0.423</td>
<td>0.374</td>
<td>0.261</td>
<td>-0.219</td>
<td>1.090</td>
<td>0.842</td>
</tr>
<tr>
<td></td>
<td>Log EPA</td>
<td>0.961</td>
<td>0.079</td>
<td>&lt;0.0001</td>
<td>-0.183</td>
<td>0.030</td>
<td>&lt;0.0001</td>
<td>0.709</td>
<td>0.055</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>Log ω3HUF A</td>
<td>2.711</td>
<td>0.189</td>
<td>&lt;0.0001</td>
<td>-0.534</td>
<td>0.076</td>
<td>&lt;0.0001</td>
<td>1.955</td>
<td>0.143</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>Log ω3PUFA</td>
<td>1.773</td>
<td>0.124</td>
<td>&lt;0.0001</td>
<td>-0.338</td>
<td>0.051</td>
<td>&lt;0.0001</td>
<td>1.298</td>
<td>0.089</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>Log (EPA:AA)</td>
<td>1.021</td>
<td>0.076</td>
<td>&lt;0.0001</td>
<td>-0.198</td>
<td>0.030</td>
<td>&lt;0.0001</td>
<td>0.744</td>
<td>0.055</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>Log (ω3:ω6PUFA)</td>
<td>1.786</td>
<td>0.118</td>
<td>&lt;0.0001</td>
<td>-0.350</td>
<td>0.049</td>
<td>&lt;0.0001</td>
<td>1.296</td>
<td>0.088</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

NOTE: Data are shown as regression coefficients, SE, and statistical significance (P value).

ªCoefficients (with SE and P) are from linear mixed-effect regression models adjusted for analytical batch and, if appropriate, colon section.

ªCoefficients represent rate of change per 0.1 unit of dose. All outcome variables were transformed using natural logarithm.
Disclosure of Potential Conflicts of Interest

W.L. Smith is a consultant/advisory board member for Cayman Chemical Co. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions

Conception and design: Y. Jiang, Z. Djuric, A. Sen, I. Waters, D.P. Normolle, W.L. Smith, D.E. Brenner
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Y. Jiang, J. Ren, I. Waters, C.L. Uhlson, Y.H. Hong, R.C. Murphy, W.L. Smith
Analysis and interpretation of data (e.g., statistical analysis, bio-statistics, computational analysis): Y. Jiang, A. Sen, I. Waters, L. Zhao, Y.H. Hong, R.C. Murphy, W.L. Smith, D.E. Brenner
Writing, review, and/or revision of the manuscript: Y. Jiang, Z. Djuric, A. Sen, I. Waters, R.C. Murphy, W.L. Smith, D.E. Brenner
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): Y. Jiang, J. Ren, D.E. Brenner
Study supervision: Y. Jiang, W.L. Smith, D.E. Brenner

Acknowledgments

The authors thank the University of Michigan Unit for Laboratory Animal Medicine team for assistance with special diet feeding. They also thank students, Curtis Austin and Gretchen Bentz, for help with the animal study.

Grant Support

This study was financially supported by NIH GM068848 (to W.L. Smith), NIH HL117798 and NIH AT002782 (to R.C. Murphy), and NIH CA130810 and VA GRECC (to D.E. Brenner).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received April 21, 2014; revised July 16, 2014; accepted August 1, 2014; published OnlineFirst August 19, 2014.

References


Personalized Omega-3 Fatty Acid Dosing


34. Broughton KS, Whelan J, Hardardottir I, Kinsella JE. Effect of increasing the dietary (n-3) to (n-6) polyunsaturated fatty acid ratio on murine liver and peritoneal cell fatty acids and eicosanoid formation. J Nutr 1991;121:155–64.


46. Stark KD. The percentage of n-3 highly unsaturated fatty acids in total HUFA as a biomarker for omega-3 fatty acid status in tissues. Lipids 2008;43:45–53.