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Effect of cyclooxygenase genotype and dietary fish oil on colonic eicosanoids in mice☆

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Abstract

Dietary ω3 fatty acids can modulate substrate availability for cyclooxygenases (COXs) and lipoxygenases, thus modulating downstream eicosanoid formation. This could be an alternative approach to using nonsteroidal anti-inflammatory drugs and other COX inhibitors for limiting Prostaglandin E_2 (PGE_2) synthesis in colon cancer prevention. The aims of this study were to evaluate to what extent COX- and lipoxygenase-derived products could be modulated by dietary fish oil in normal colonic mucosa and to evaluate the role of COX-1 and COX-2 in the formation of these products. Mice (wild-type, COX-1 null or COX-2 null) were fed a diet supplying a broad mixture of fatty acids present in European/American diets, supplemented with either olive oil (oleate control diet) or menhaden (fish) oil *ad libitum* for 9–11 weeks. Colonic eicosanoid levels were measured by liquid chromatography tandem mass spectroscopy (LC-MS/MS), and proliferation was assessed by Ki67 immunohistochemistry. For the dietary alteration of colonic arachidonic acid: eicosapentaenoic ratios resulted in large shifts in formation of COX and lipoxygenase metabolites. COX-1 knockout virtually abolished PGE_2 formation, but interestingly, 12-hydroxyeicosatetraenoic (12-HETE) acid and 15-HETE formation was increased. The large changes in eicosanoid profiles were accompanied by relatively small changes in colonic crypt proliferation, but such changes in eicosanoid formation might have greater biological impact upon carcinogen challenge. These results indicate that in normal colon, inhibition of COX-2 would have little effect on reducing PGE_2 levels.

Keywords: Colon cancer; Fish oil; Cyclooxygenase; Prostaglandin E_2; Hydroxyeicosatetraenoic acids; EPA

1. Introduction

Prostaglandin E_2 (PGE_2) has been identified as a key proinflammatory signaling molecule involved in the promotion and development of sporadic colorectal cancer [1,2]. Up-regulation of cyclooxygenase-2 (COX-2) and increased PGE_2 synthesis are hallmarks of colon cancer progression [1,3–5]. Relatively less is known about the role of other arachidonic acid (AA) metabolites in colon cancer, but they do play a role in the inflammatory response and in carcinogenesis [6–8]. Despite strong preclinical and clinical data suggesting their potential efficacy as preventive agents against colon cancer, pharmacological agents that reduce colorectal PGE_2 concentrations through COX inhibition (nonsteroidal anti-inflammatory drugs) have unacceptable cardiovascular and gastrointestinal toxicity profiles for daily prescription to otherwise healthy populations [9].

Modification of substrate availability for eicosanoid synthesis is an alternative approach for colon cancer prevention. Dietary ω3 fatty acids can modulate substrate pools available to COXs and lipoxygenases (LOXs), thereby controlling downstream eicosanoid formation and subsequent receptor activation [10] (Fig. 1). The ω3 fatty acid

Abbreviations: AA, arachidonic acid; AIN, American Institute of Nutrition; COX, cyclooxygenase; DAB, 3,3′-diaminobenzidine; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; ESI, electrospray ionization; EPZ, extent of the proliferative zone; H/E, hematoxylin/eosin; HETE, hydroxyeicosatetraenoic acid; HODE, hydroxyoctadecadienoic acid; HUFA, highly unsaturated fatty acid; IHC, immunohistochemistry; LI, labeling index; LT, leukotriene; LC-MS/MS, liquid chromatography tandem mass spectrometry; NSAIDs, nonsteroidal anti-inflammatory drugs; PBS, phosphate-buffered saline; PG, prostaglandin; PUFA, polyunsaturated fatty acid; SCD, stearoyl-CoA desaturase.

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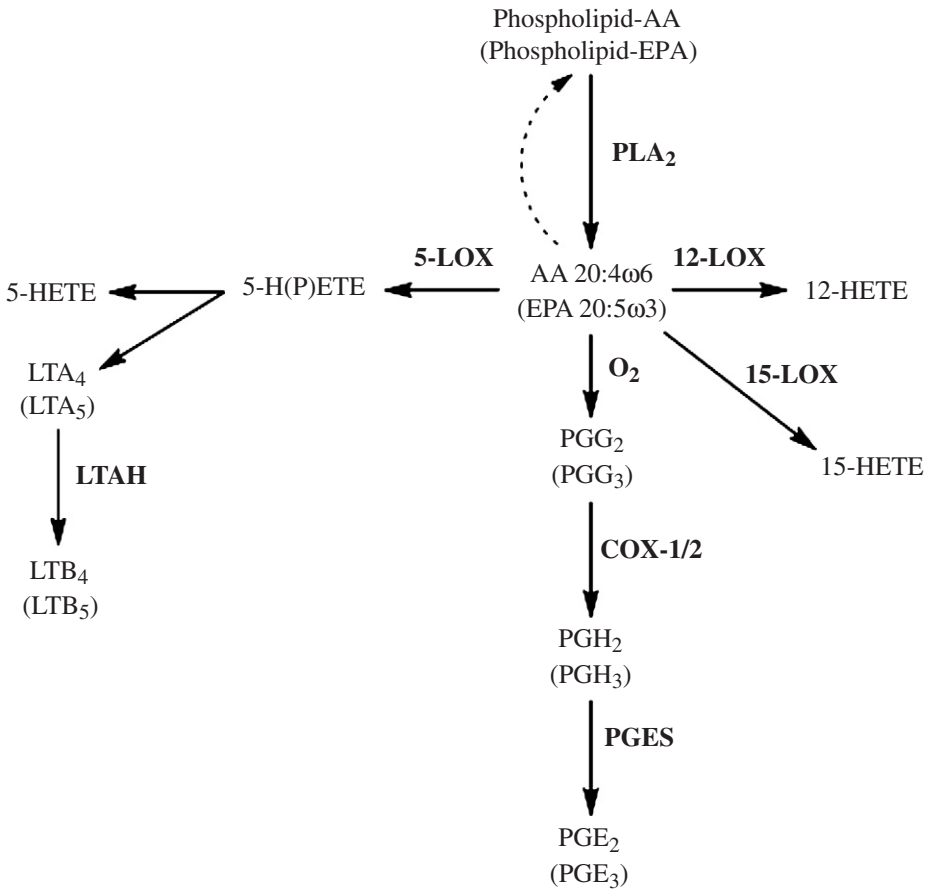

Fig. 1. Pathways of eicosanoid biosynthesis from arachidonic and EPA precursors. For each AA metabolite, the compound in parentheses is the corresponding EPA metabolite. PLA_2, phospholipase A_2; H(P)EPE, hydroperoxy EPA; H(P)ETE, hydroperoxyeicosatetraenoic acid; LTA, leukotriene A; LTAH, LTA hydrolase; LTB, leukotriene B; PGES, prostaglandin E synthase; PGHS, prostaglandin H synthase.

eicosapentaenoic acid (EPA) is a poor substrate for COXs and a reasonably good inhibitor of AA (ω6) oxygenation [11]. EPA can also serve as a substrate for eicosanoid formation, generating 3-series prostaglandins (PGs), including PGE_3, and 5-series leukotrienes (LT) such as leukotriene B_5 (LTB_5) [12,13]. The three-series PG products are generally less proinflammatory than the two-series products [13–15].

Typical North American and Western European diets contain high levels of ω6 fatty acids from plant oils. However, diets rich in ω3 fatty acids are associated with reduced risks for colorectal adenocarcinoma as well as other epithelial malignancies [16,17]. In animal models, data from multiple laboratories demonstrate that there are colon tumor-promoting effects of dietary ω6 fatty acids, saturated fatty acids and Western-style lipid profiles, whereas ω3 fatty acids and fish oil lack colon tumor-promoting effects [18,19]. Also, increasing the ω3:ω6 ratio can reduce local PGE_2 concentrations, particularly in the context of a low-fat diet when ω6 intakes are low [20]. Supplementation of human diets with fish oil enriched with EPA and docosahexaenoic acid (DHA) reduces PGE_2 synthesis and crypt proliferation in human colorectal mucosal [21–24].

Despite experimental evidence of the benefits of shifting the ω3/ω6 balance toward the ω3 fatty acids, few data on the resulting alterations to the balance of ω3- versus ω6-derived eicosanoid balance in the colon exists. The majority of studies have measured PGE_2 only. Replacement of corn oil with fish oil in the diets of rats has been shown to reduce colonic PGE_2 by 4.5-fold, producing a PGE_2/PGE_3 ratio of ~2.2:1 [25]. There is relatively less known about the potential impact of fish oil on LT production in the colon and to what extent ω6 products might be decreased when ω3 products are increased. In addition to the effects of diet, decreased activities of COX-1 and/or -2 might result in shunting of substrate to LOXs.

This study therefore aimed to profile eicosanoid products of AA and EPA in histologically normal colon of mice that did or did not carry a genetic deletion of COX-1 or COX-2. The effects of a control diet containing Western-blend fat supplemented with oleate were compared with that of a fish oil diet because modifying substrate pools presented to COX and LOXs should represent a less toxic, physiological approach for modulating colon cancer risk in normal tissues. The use of mice with genetic knockout of COX-1 or COX-2 also allowed for investigation of possible shunting of the substrate fatty acids to LOXs. We therefore profiled eicosanoid products from AA and EPA in histologically normal colon of mice that did or did not carry a genetic deletion of COX-1 or COX-2.

2. Materials and methods

2.1. 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. Six-week old female C57BL/6 mice (wild-type, COX-1 null, or COX-2 null, n=20 per group) were a generous gift from Dr. Robert Langenbach (University of North Carolina, Chapel Hill, NC, USA). Mice were fed modified AIN-93G diets (Dyets, Bethlehem, PA, USA) that comprised casein (20% by weight), cornstarch (33.7%), dyetrose (13.2%), sucrose (10%), cellulose (5%), Western fat mixture (7%), tert-butyl hydroquinone (0.0026%), salt mix (3.5%), vitamin mix (1%), L-cysteine (0.3%) and choline bitartrate (0.25%). This diet also contained either 6% by weight olive oil [oleate diet, as olive oil contains large amounts of oleic acid (18:1ω9)] or 6% by weight menhaden oil [fish oil diet, rich in EPA (20:5ω3) and DHA (22:6ω3)]. The Western fat mixture (7% of the diet by weight)

comprised a broad mixture of fatty acids present in European and American diets, composed of coconut oil (45 wt.% supplying saturated fats), olive oil (30 wt.% supplying oleate), corn oil (15 wt.% supplying linoleic acid) and soybean oil (10 wt.% supplying α-linolenic acid). Mice were fed one of these two diets (n=10/genotype/diet) *ad libitum* for 9–11 weeks. See Table 1 for the diet compositions. Mice were maintained on a 12-h light/dark cycle. At the end of the study, animals were killed by isoflurane inhalation and decapitation. Blood was collected from the neck into heparin-containing tubes, and the colon was immediately removed and immediately rinsed with ice-cold phosphate-buffered saline (PBS) containing indomethacin (5.6 μg/ml). A small portion of the lower colon was removed for histological analysis, and the remaining tissue was sliced vertically, and the mucosa was scraped off. The mucosa was immediately snap-frozen in liquid nitrogen and stored at −80°C prior to processing.

Sections of whole colon tissue were fixed for 24 h in neutral buffered formalin, before brief storage in ethanol. Sections were embedded in paraffin blocks for preparation of slides. Frozen mucosal samples (~140 mg) were pulverized using a Multisample Bio-Pulverizer (Research Products International Corp., Mt. Prospect, IL, USA) that was cooled with dry ice and liquid nitrogen, and homogenates were prepared in 500 μl of cold PBS containing 5.6 μg/ml indomethacin. The suspension was subjected to ultrasonication in ice water for 3 min (20 s sonication, 20 s cooling cycle), further diluted with 500 μl cold PBS/indomethacin, snap frozen and stored at −80°C prior to analysis of eicosanoids. A small portion (10 μl) of the homogenate was analyzed for protein content using Advanced Protein Assay (Cytoskeleton, Denver, CO, USA).

2.2. Western blotting

Western blotting was performed to confirm COX expression in our genetic models. Mucosa homogenates were centrifuged (13,000×g), and supernatants were analyzed. Supernatant proteins (10 μg) were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis on 10% Tris–HCl gels (Bio-Rad Laboratories, Hercules, CA, USA), transferred onto polyvinylidine fluoride membrane (Millipore Corporation, Billerica, MA, USA) and analyzed by immunoblotting using antibodies against rabbit antimouse COX-1 antibody (Cayman Chemical, Ann Arbor, MI, USA) and COX-2 antibody (Novus Biologicals, LLC, Littleton, CO, USA). Antibodies against β-actin (Abcam, San Francisco, CA, USA) were used to control for loading. Antigen–antibody complexes were detected using Western Blotting Lumiglo Reagent (Santa Cruz Biotechnology, Santa Cruz, CA, USA). For COX-2 blotting, a lysate of NIH3T3 mouse fibroblasts expressing COX-2 was included as a positive control. Representative COX-1 and COX-2 Western blots of colonic mucosa from COX-1 null, COX-2 null and wild-type mice are presented in Fig. 2. COX-1 was absent in the mucosa of COX-1 null mice but was detected in both wild-type and COX-2 null mice. COX-2 was not detected in the mucosa of any of the genotypes, consistent with the low expression of COX-2 in nontransformed mucosa [1,26,27].

2.3. Total fatty acid analysis by GC-MS

For extraction of total fatty acids, 10 μl of internal standard (17:0, 1 mg/ml in hexane) was added to 150 μl of mucosal homogenate. The sample was then added to 1.5 ml Folch reagent (chloroform/methanol 2:1), vortexed for 1 min and centrifuged (200×g for 5 min). The organic layer was removed to a 12×75-mm glass tube and dried in a SpeedVac. The sample was solubilized in 150 μl of hexane/chloroform (1:1) and vortexed. Fatty acid methyl esters were prepared by adding 10 μl of METH-PREP II

Table 1
Fatty acid composition of the diets

	Fatty acid	Diet	
		Oleate	Fish oil
Composition (g/kg diet)	Stearic acid (18:0)	2.7	3.3
	Oleic acid (18:1ω9)	59.3	30.2
	Linoleic acid (18:2ω6)	15.9	11.3
	Linolenic acid (18:3ω3)	1.4	1.5
	AA (20:4ω6)	0	0.7
	EPA (20:5ω3)	0	7.9
	DHA (22:6ω3)	0	5.2
	SFA	36.2	46.2
	MUFA	62.3	34.6
	PUFA	17.1	31.3
	ω3	1.1	17.5
	ω6	16.0	12.1
	ω9	61.9	34.4
Ratios	ω3/ω6	0.1	1.4
	ω9/ω6	3.9	2.9
	AA/EPA	—	0.1
	EPA/DHA	—	1.5

Mice were fed a modified AIN-93 diet containing 6% Western fat blend and either 6% olive oil (oleate diet, representing a diet high in MUFA) or 6% menhaden oil (high PUFA). SFA, saturated fatty acids, MUFA, monounsaturated fatty acids, PUFA, polyunsaturated fatty acids.

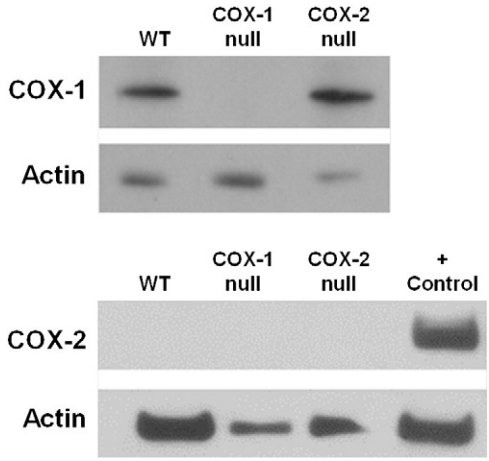

Fig. 2. Representative COX-1 and COX-2 Western blots of colonic mucosa from wild-type (WT), COX-1 null and COX-2 null mice fed the oleate diet. For the COX-2 experiment, a lysate of NIH3T3 mouse fibroblast cells known to express COX-2 was included as a positive control.

derivatization reagent [0.2 N methanolic (*m*-trifluoromethylphenyl)trimethylammonium hydroxide; Alltech, Deerfield, IL, USA); the sample was vortexed (0.5 min) and held at room temperature for 30 min. The organic layer was then removed for analysis. For quantification, standard curves were prepared using mixtures of fatty acids in varying concentrations derivatized in the same manner as tissue extracts.

GC analysis was performed on an Hewlett Packard 5890 GC with a 5971 MSD (Santa Clara CA, USA) and a SP-2330 capillary column 30 m×0.32 mm, 0.2-μm film thickness (Supelco, Bellefonte, PA, USA) as previously described [28]. The carrier gas was He with column head pressure of 10 psi. Splitless injection was at 220°C using a total flow rate of 50 ml/min. Temperature ramping was performed from 70 to 220°C as described previously. Total run time was 21.3 min, and single ion monitoring was used that was optimized for each fatty acid.

2.4. Eicosanoid analysis by liquid chromatography tandem mass spectrometry (LC-MS/MS)

For extraction of eicosanoids, 350 μl of the homogenate was added to 12×75-mm glass tubes on ice, along with 1 N citric acid (20 μl) and deuterated internal standards (50 ng/ml PGE_2-d_4, 200 ng/ml LTB_4-d_4, 100 ng/ml 15-*S*-HETE-d_8, 100 ng/ml 5-*S*-HETE-d_8, 200 ng/ml 12-*S*-HETE-d_8, 400 ng/ml 13-*S*-HODE-d_4, 5 μg/ml AA-d_8). The resulting solution was extracted three times with 2 ml hexane/ethyl acetate (1:1 vol/vol, containing 0.1% BHT wt/vol and 1 mM EDTA) by vortexing (3 min) and was centrifuged (2000×g, 10 min, 4°C). The combined extracts were evaporated and reconstituted with 100 μl cold methanol/10 mM ammonium acetate buffer, pH (8.5; 60:40 vol/vol), sonicated (3 min in cold water) and were centrifuged (13,000×g, 3 min, 4°C). The extracts were then transferred to deactivated glass high-performance liquid chromatography (HPLC) vials for LC-MS/MS analysis (25 μl injected).

HPLC separations for eicosanoids were performed on a Waters 2695 separations module (Milford, MA, USA), using a Luna Phenyl-Hexyl analytical column (2×150 mm, 3-μm particle size; Phenomenex, Torrance, CA, USA). The column was maintained at 40°C. Gradient elution was performed with a binary solvent system: phase A — 10 mM ammonium acetate (pH 8.5) and phase B — MeOH. The system flow rate was 0.2 ml/min. The linear gradient program was as follows: 40% B (0–0.5 min), 70% B (6–11 min), 80% B (14 min), 100% B (16–18 min) and 40% B (20–27 min). Samples were maintained at 10°C prior to injection. The effluent was introduced into a Finnigan TSQ Quantum Ultratriple quadrupole mass spectrometer (Thermo Sci., Waltham, MA, USA) by negative electrospray ionization (ESI). The ESI voltage was −2.3 kV, and the capillary temperature was 350°C, with N_2 as the nebulizing and sheath gases. Following ionization, deprotonated pseudomolecular ions ($[M-H]^-$) were fragmented by collision-induced dissociation using argon gas, and detection was performed by selected reaction monitoring (SRM) of fragment ions (see Table 2). Quantification was performed by the stable isotope dilution method relative to deuterated internal standards. Eicosanoid levels in colon mucosa were normalized to protein levels. Levels for animals fed the diets for 9–11 weeks were not significantly different and were combined.

2.5. Analysis of epithelial proliferation in colonic crypts

Immunohistochemical (IHC) staining was performed on colon tissue to visualize the Ki67 antigen expressed in proliferating cells. Tissue slices were fixed in formalin and embedded in paraffin. Slices were then deparaffinized, and antigen unmasking was performed by steaming in citrate target retrieval buffer. Ki67 immunostaining using the mib1 Ki67 antibody and the Labelled Streptavidin-Biotin2 System-Horseradish Peroxidase detection protocol (Dako, Carpinteria, CA, USA). Diaminobenzidine staining

Table 2
SRM transitions for LC-MS/MS analysis of eicosanoids

Compound	[M−H]− (−/z)	SRM transition	Collision energy (eV)	Tube lens (V)	Retention time (min)
PGE_3	349	349.20→269.20	18	−57	8.97
PGE_2	351	351.20→271.23	20	−55	10.80
PGE_2-d_4	355	355.20→275.20	16	−45	10.81
LTB_5	333	333.14→195.10	23	−55	13.86
LTB_4	335	335.20→195.00	22	−70	14.82
LTB_4-d_4	339	339.20→197.00	20	−66	14.82
13-HODE	295	295.24→277.18	34	−70	16.47
13-*S*-HODE-d_4	299	299.24→281.18	30	−68	16.47
5-HETEs	319	319.50→115.27	27	−42	19.09
5- *S*-HETE-d_8	327	327.00→115.80	30	−50	18.94
12-HETEs	319	319.24→179.09	26	−55	17.84
12-*S*-HETE-d_8	327	327.24→184.09	22	−55	17.74
15–HETEs	319	319.31→219.30	20	−52	17.37
15-*S*-HETE-d_8	327	327.20→226.05	20	−52	17.37
AA	303	303.10→177.00	31	−75	22.55
AA-d_8	311	311.10→267.48	25	−76	22.54

was performed on a Dako stainer, and counterstaining was with Gill's hematoxylin/eosin (H/E; Fisher Sci., Pittsburg, PA, USA). Quantification of Ki67 labeling was performed using MCID software (Cambridge, UK). Well-oriented, full-length crypts were scored by setting color thresholds with the software. The extension of positive (Ki67-labeled) cells up the crypt was recorded as well as the number of positive cells in the entire crypt and in each longitudinal fifth of the crypt. Ki67 staining was expressed as labeling index (LI; fraction of Ki67-positive cells in the whole crypt) labeling index in each crypt compartment (fifths) as well as the extent of the proliferative zone (EPZ; number of cells from the crypt base to the highest Ki67-positive cell). Ki67 labeling was measured by two analysts whose mean scoring did not differ appreciably (<10% when LI and EPZ were compared for the same images).

2.6. Data analysis

Statistical analyses were performed by two-way multivariate analysis of variance (MANOVA) with diet, genotype and their interaction as the factors (PASW Statistics software version 18; SPSS, Chicago, IL, USA). We ran this analysis for each of the chemical (fatty acids and eicosanoids) as well as biological (IHC) measures separately. Significance was defined as $P<.05$. As the interaction was of primary importance, we present the comparisons of one factor within the levels of the other factor. For colonic fatty acid levels, Box–Cox transformations of raw data were performed prior to MANOVA analysis in order to achieve normality: square root [12:0, 14:0, 16:1, 18:0, 18:1, 20:1, 20:4 and ω3 highly unsaturated fatty acid (HUFA) score], negative square root (16:0) and log (18:2, 18:3, 20:3, 20:5, 22:6).

3. Results

3.1. Fatty acid profiles

Total (membrane bound+free) fatty acid levels in mouse colonic mucosa are shown in Table 3. Fatty acid profiles differed between diets, as expected, as well as between genotypes. The level of AA was roughly twofold to threefold greater in mice fed the oleate diet compared to the fish oil diet for the COX-1 and COX-2 null mice, whereas these levels were similar between the two diets for the wild-type mice. As expected, the level of EPA was much higher (7- to 16-fold) in mice fed fish oil compared to oleate diet. However, there were also differences in EPA levels between genotypes in mice fed fish oil, with COX-1 null mice having roughly half the EPA as COX-2 null and wild-type mice. The AA/EPA ratio observed in the mice fed oleate diet (21:1–28:1) was dramatically decreased (to about 0.8:1–1:1) by administration of fish oil, indicating a significant shift in the relative availability of AA and EPA for COX metabolism. This shift in the balance of substrate available for COX metabolism from ω6 to ω3 fatty acids by the fish oil diet is reflected in the percentage of HUFAs (fatty acids with ≥20 carbons and ≥3 double bonds) that are also ω3 fatty acids (the % ω3 HUFA score) [29]. The ω3 HUFA score was roughly 25%–26% for all mice fed the oleate diet and 68%–76% for mice fed the fish oil diet (Table 3).

The activities of various fatty acid metabolizing enzymes (desaturases, elongases, etc.) were also estimated by calculating the ratios of products/substrates and comparing between treatments (data not shown). While several differences were observed, the most significant findings were that the activity of the fatty acid Δ5-desaturase (as measured by the 20:4/20:3 ratio) was elevated in mice fed oleate diet versus mice fed fish oil (11.1-12:1 vs. 5:1-10:1, respectively). A similar pattern was observed for the activity of stearoyl-CoA desaturase (SCD; as measured by the 18:1/18:0 ratio), with ratios of roughly 8:1–9:1 for mice fed oleate diet versus roughly 3:1–5:1 in mice fed fish oil. Also, within the fish oil diet, COX-1 null mice had increased SCD activity compared to COX-2 null or wild-type mice (5:1 vs. 3:1), which could explain the observation that this group had oleic acid levels similar to the oleate diet animals (see Table 3).

Table 3
Levels of fatty acids in colonic mucosa as a % of total fatty acids

Fatty acid	% of total fatty acids[†,‡,§]					
	COX-1 null		COX-2 null		Wild-type	
	Oleate	Fish oil	Oleate	Fish oil	Oleate	Fish oil
Lauric (12:0)	2.08±0.287	1.71±0.208	2.04±0.431	1.22±0.241	2.03±0.274	1.43±0.202
Myristic (14:0)	2.55±0.278	3.07±0.244[a]	1.91±0.266	2.00±0.297[c]	2.30±0.250	2.32±0.267
Palmitic (16:0)	19.2±1.69	20.9±1.96	16.3±1.68	18.3±2.07	17.9±1.98	18.8±1.83
Palmitoleic (16:1ω7)	5.16±1.08	4.30±0.661	2.91±0.261	3.61±0.679	4.86±0.666	4.96±0.686
Stearic (18:0)	5.96±0.870	8.08±0.790	8.35±1.29	10.9±1.28	6.50±0.818	9.94±0.768[b]
Oleic (18:1ω9)	39.9±2.59	35.2±3.62[a]	37.9±3.98	25.7±3.85[bc]	41.8±3.01	26.5±3.09[b]
Linoleic (18:2ω6)	13.5±0.766	14.9±1.27	15.2±0.782	17.4±1.16	13.9±0.681	15.7±1.12
Linolenic (18:3ω3)	0.434±0.0925	0.425±0.431	0.666±0.174	0.638±0.129	0.450±0.0666	1.15±0.332
Eicosenoic (20:1ω9)	0.743±0.169	1.45±0.246[ab]	0.761±0.121	0.766±0.303[c]	0.917±0.132	0.794±0.0615
Eicosatrienoic (20:3)	0.670±0.106	0.419±0.129[a]	0.856±0.170	0.622±0.111	0.534±0.0932	0.738±0.0998
AA (20:4ω6)	7.28±1.03	2.15±0.808[ab]	9.53±1.62	5.27±0.826[bc]	6.53±1.29	4.77±0.675
EPA (20:5ω3)	0.422±0.104	2.80±0.997[b]	0.487±0.0855[ab]	4.74±0.764[b]	0.319±0.0917	5.21±0.829[b]
DHA (22:6ω3)	2.19±0.282	4.58±1.51[b]	3.13±0.635[ab]	8.75±1.92[b]	1.90±0.380	7.81±1.19[b]
% ω3 HUFA[‖]	24.8±1.79	76.4±2.38[b]	26.1±1.32[b]	68.4±1.53[b]	25.1±2.94	69.5±1.82[b]
AA/EPA	24.6±3.80	0.802±0.176	21.4±2.00	1.13±0.0607	28.1±4.72	1.03±0.129

[†] Total fatty acids were determined by saponification of mucosa, derivatization and analysis by GC-MS.
[‡] Values represent mean± S.E.M.
[§] Means that differ significantly within each row (two-way MANOVA with comparisons of one factor within the levels of the other factor, $P<.05$),are denoted as follows: [a]significantly different than wild-type mice fed the same diet, [b]significantly different than oleate diet for mice with the same genotype and [c]significantly different than COX-1 null mice fed the same diet. Raw data are presented here, but statistical analyses were performed on Box–Cox transformed data as described in the "Materials and Methods" section. MANOVA was not performed for AA/EPA ratios.
[‖] The % of highly unsaturated fatty acids (≥20 carbons and ≥3 double bonds) that are ω3 (the ω3 HUFA score).

3.2. Free AA levels in colonic mucosa

Animals fed the oleate diet generally had higher levels of free AA (intracellular AA freed from the membrane by phospholipase A_2, and measured by LC-MS without saponification) in the colonic mucosa compared to animals fed fish oil, but the difference between diets was only statistically significant for COX-2 null mice (Fig. 3). The differences in free AA between diet groups reflected reduced total levels of AA produced by the fish oil diet (shown in Table 3). For the oleate diet, COX-2 null mice had elevated free AA, which was statistically significant compared to the wild-type mice. Genotype did not significantly affect free AA levels in mice fed fish oil.

3.3. PGE_2 and PGE_3

Mice fed oleate diet had threefold to fourfold higher mucosal PGE_2 concentrations compared to mice fed fish oil in wild-type and COX-2 null mice (Fig. 3). The absence of COX-1 resulted in almost total elimination of PGE_2 in mice fed either diet, unlike the absence of COX-2, which had virtually no effect compared to wild type. Levels of PGE_3 were typically several-fold lower than PGE_2 levels in all groups

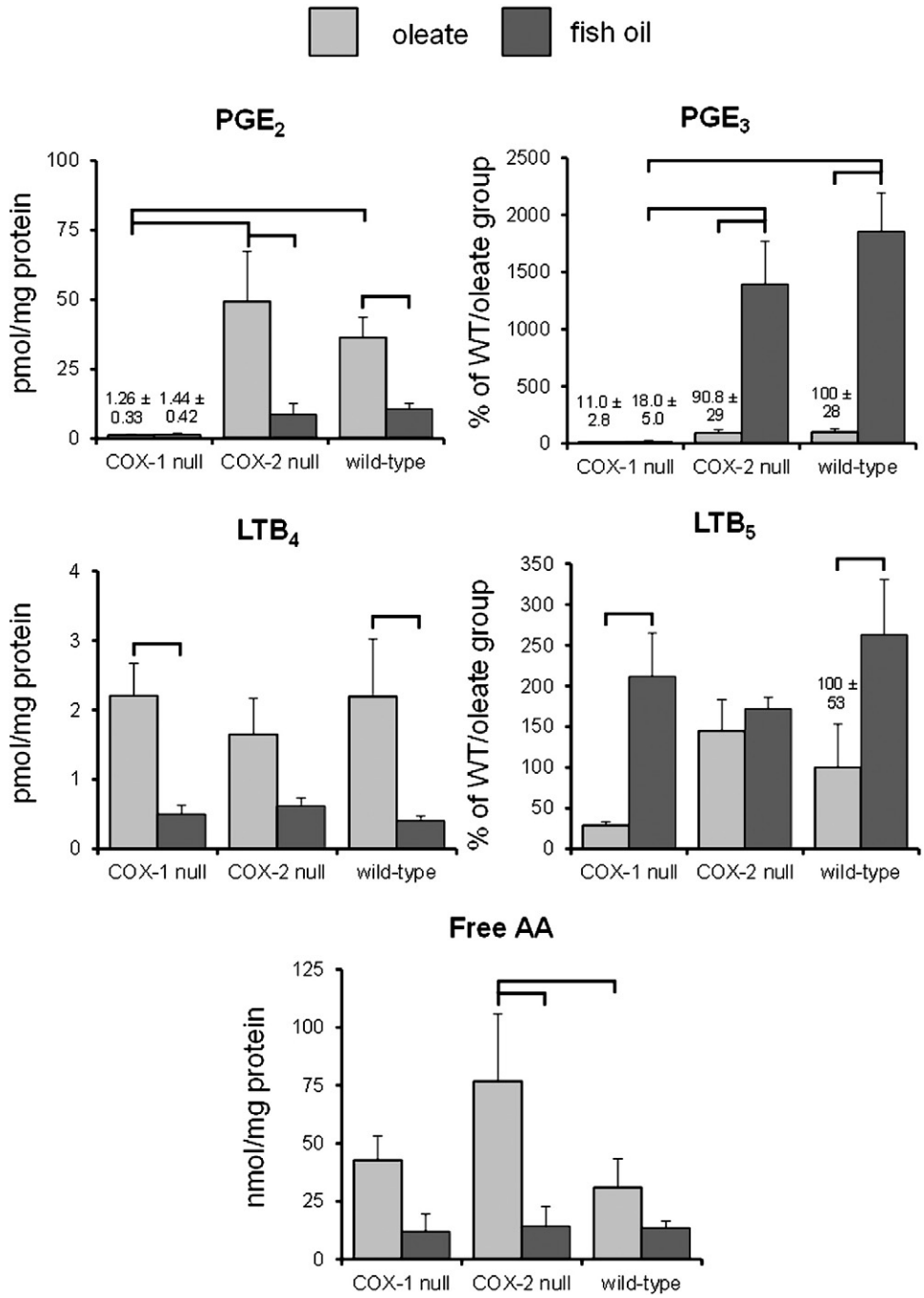

Fig. 3. Levels of free AA and measured PGs and LT in mouse colon mucosa. Eicosanoids were measured LC-MS/MS by the stable isotope dilution method. Error bars represent S.E.M. Brackets indicate significant differences between means (two-way MANOVA with comparisons of one factor within the levels of the other factor, $P<.05$). As no appropriate deuterated internal standard was available for absolute quantification of PGE_3 and LTB_5, these values are expressed as a % relative to wild-type (WT) mice fed the oleate diet, which was set at 100%.

fed the oleate diet, and PGE_3 was increased in wild-type and COX-2 null mice fed fish oil. However, we did not have an appropriate internal standard for quantification, so the absolute levels of PGE_3 were not determined. Similar to PGE_2, the absence of COX-1 resulted in almost total lack of PGE_3 in the mucosa with either diet.

3.4. LTB_4 and LTB_5

Levels of LTB_4 were generally threefold to fivefold higher in mice fed the oleate fat diet than in mice fed fish oil, whereas genotype appeared to have little effect (Fig. 3). Again, we did not have an appropriate internal standard for quantification of LTB_5, but LTB_5 levels increased 2–10-fold in wild-type and COX-1 null mice fed the fish oil diet versus the oleate diet, whereas diet had little impact on LTB_5 levels in COX-2 null mice.

3.5. Hydroxyeicosatetraenoic acids and 13-HODE

LOX products derived from AA [5-, 12- and 15-hydroxyeicosatetraenoic acid (15-HETEs)] and linoleic acid (13-HODE) were quantified due to their utility as biomarkers of transformation and inflammation in colonic mucosa [6,8,30–35]. The levels of HETEs and 13-HODE in mouse colon mucosa are shown in Fig. 4. 12-HETE was by far the most abundant of all the eicosanoids detected, with 15-HETE and 13-HODE also being present at higher levels than PGE_2 or LTB_4. Levels of 12-HETE were roughly 7- to 20-fold greater in mice fed the oleate diet versus mice fed fish oil, with statistically significant differences between diets for every genotype. For mice fed the oleate diet, COX-1 null animals had significantly elevated 12-HETE compared to wild-type and COX-2 null animals. Similar results were observed for 15-HETE. Levels of 15-HETE were roughly fourfold to eightfold greater in mice fed the oleate diet than in mice fed fish oil, with statistically significant differences between diets for every genotype. For mice fed the oleate diet, COX-1 null animals had significantly higher 15-HETE levels compared to wild-type and COX-2 null animals.

Levels of 5-HETE were generally greater in mice fed the oleate diet than in mice fed fish oil, although the only significant difference between diets was observed in COX-1 null animals. No significant differences were observed between genotypes, although COX-1 and COX-2 null mice fed the oleate diet had slightly elevated 5-HETE compared to wild-type mice fed the oleate diet. No statistically significant differences were observed for 13-HODE levels between diets or genotypes, although mice fed the oleate diet had generally greater levels compared to mice fed fish oil. The differences in membrane linoleic acid concentrations (the substrate for 13-HODE synthesis; Table 3) were generally not reflected in 13-HODE levels.

3.6. Colonic proliferation

Proliferation was quantified using Ki67 IHC to assess the biological impact of altering substrate ratios and resultant eicosanoid profiles on normal mucosa. PGE_2 is known to stimulate proliferation in colonic epithelial cells [36,37], while EPA-derived eicosanoids such a PGE_3

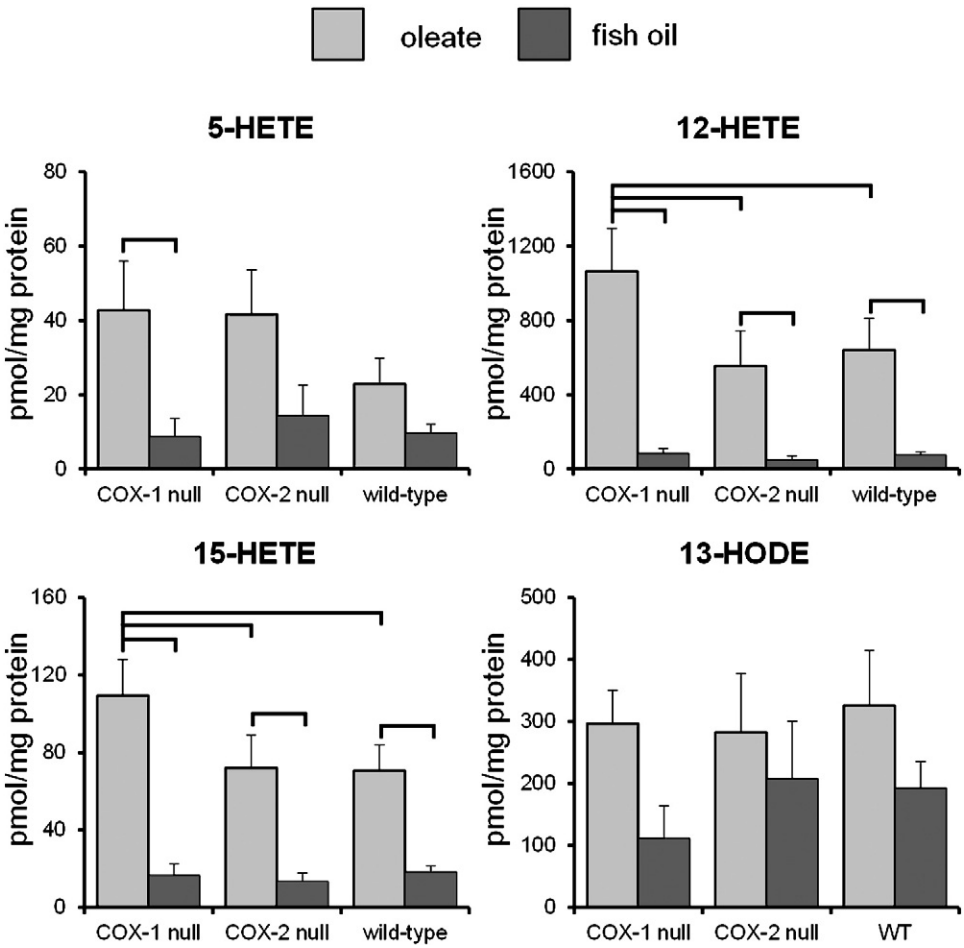

Fig. 4. Levels of HETEs and 13-HODE in mouse colon mucosa. Eicosanoids were measured LC-MS/MS by the stable isotope dilution method. Error bars represent S.E.M. Brackets indicate significant differences between means (two-way MANOVA with comparisons of one factor within the levels of the other factor, $P<.05$).

stimulates proliferation less effectively [13,15]. Apoptosis was not measured due to the extremely low apoptotic rate in normal mucosa (typically <1 apoptotic cell/crypt) [38–40]. Representative Ki67 immunohistochemical staining of colonic tissue is shown in Fig. 5. Overall, diet and genotype had minimal effects on colonic proliferation patterns as quantified by Ki67 IHC (Fig. 6). The EPZ (the number of cells from the crypt base to the highest Ki67-positive cell, expressed as a fraction of crypt height) was similar (0.60–0.79) for all groups. The EPZ was slightly but not significantly, lower for mice fed oleate diet than mice fed fish oil for each genotype. Total Lis (the fraction of Ki67-positive cells in the entire crypt) were between 0.35 and 0.46. Compartment LI values generally decreased toward the top of the crypt, as expected (compartment 1: crypt base, compartment 5: top of crypt). However, the Φ_{40} values (the LI in the upper 40% of the crypt, i.e., compartments 4 and 5) did vary greatly (0.03–0.21) between groups, with COX-2 null mice fed fish oil having a higher Φ_{40} than any other group. It should be noted that the fish oil diet had higher levels of saturated fat than the oleate diet (46.2 vs. 36.2 g/kg, respectively). Saturated fat and fish oil/ω-3 PUFAs have been shown to have opposite anticarcinogenic and anti-inflammatory effects in the colon, and this may be an additional explanation for the lack of observed effects on colonic proliferation between diets [41,42].

The largest and perhaps most important differences were observed in the absolute size of the crypts, shown in Fig. 7. For mice fed the oleate diet, the mean crypt height was significantly higher in COX-2 null mice than in COX-1 null or wild-type mice (34±1.9 cells vs. 19±2.7 and 22±1.2 cells, respectively). This reflects the trend of PGE_2 and AA (membrane and free) levels that were highest in COX-2 null mice (Fig. 3). Fish oil appeared to dampen the effect of genotype on crypt height.

4. Discussion

4.1. Eicosanoids and fatty acids

Administration of fish oil reduced the amount of free AA in colonic mucosa. Free AA levels were surprisingly higher in COX-2 null mice than in wild-type mice fed the oleate diet. This may be due in part to the fact that genetic deletion of COX-1 or COX-2 can induce compensatory up-regulation of cytosolic phospholipase A2 [43–45], resulting in increased release of AA from membrane phospholipids. However, the slight increase in the level of free AA in the mucosa of COX-1 null mice was not statistically significant compared to wild-type mice. Fish oil dramatically altered the AA/EPA ratio relative to the oleate diet. The changes in the AA/EPA ratio are correlated with reduced levels of mucosal PGE_2 and increases in PGE_3. These results are consistent with a previous study in wild-type mice treated with the carcinogen azoxymethane, in which dietary fish oil reduced the PGE_2/PGE_3 ratio from 736:1 to 2.3:1in colonic mucosa [25]. In our study, it appears that these changes were largely mediated through COX-1, as deletion of COX-1 dramatically reduced PGE_2 and PGE_3. Deletion of COX-2 did not affect PGE_2 or PGE_3 synthesis compared to wild-type mice for either diet, suggesting

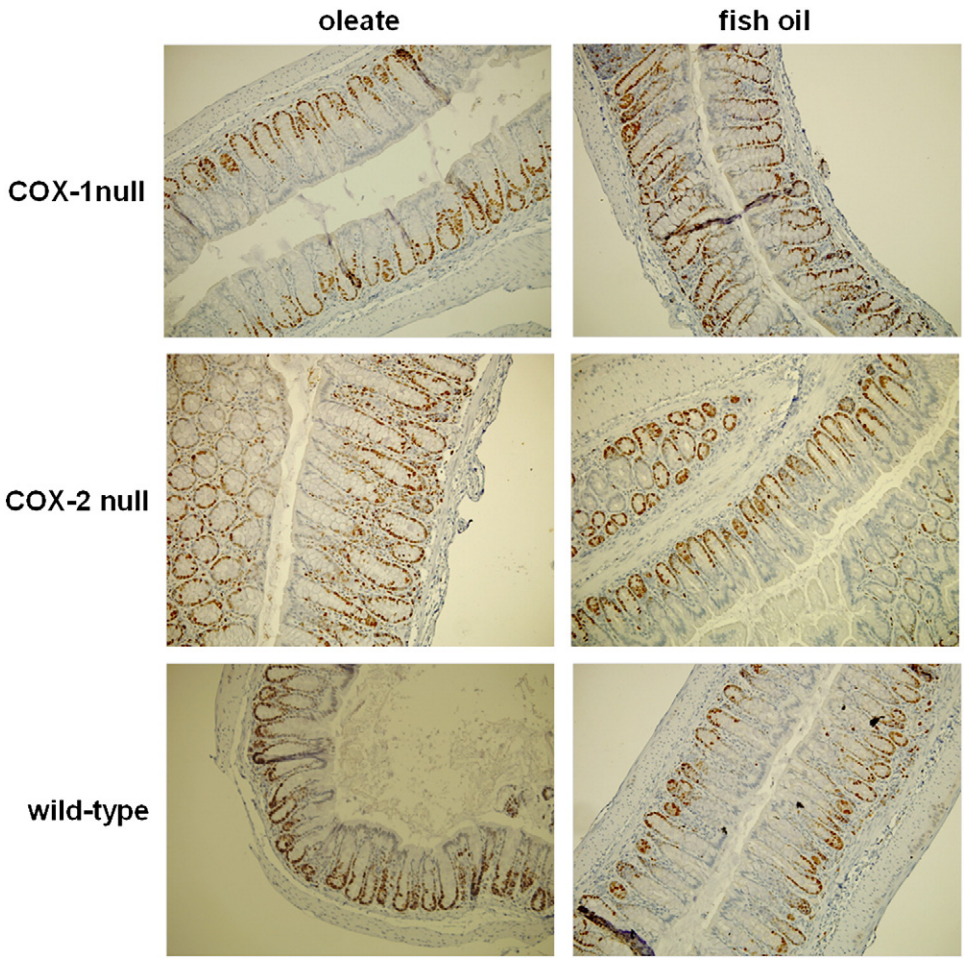

Fig. 5. Representative images of Ki67 IHC for each treatment. Nuclei are stained blue-purple (hematoxylin), while Ki67+ (proliferating) nuclei are stained brown. Magnification is ×40.

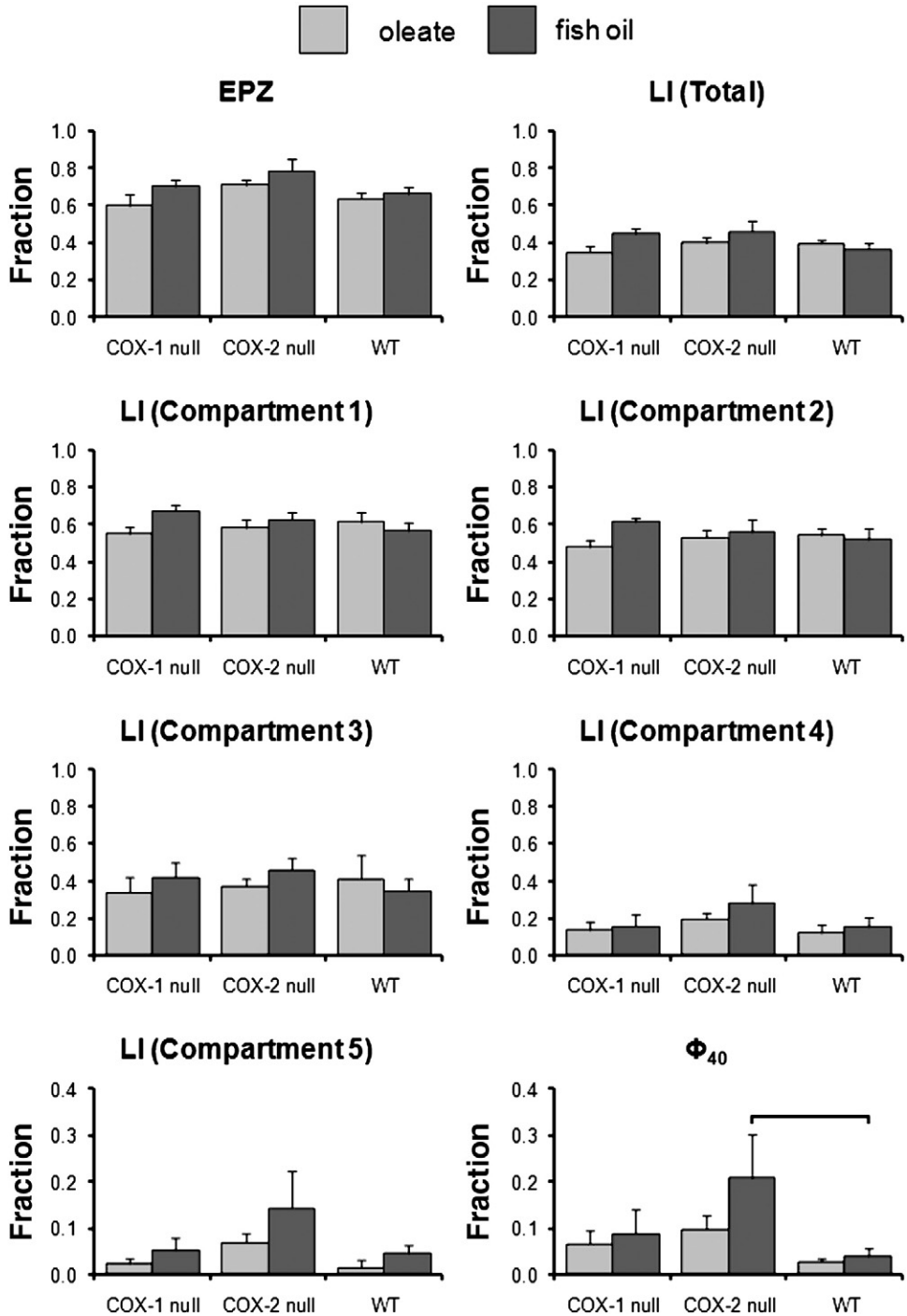

Fig. 6. Proliferation in colon mucosa epithelium as measured by Ki67 labeling of well-oriented, full-length crypts: LI (fraction of Ki67-positive cells) for whole crypts and crypt compartments (fifths) as well as Φ_{40} (the LI in the upper 40% of the crypt, i.e., compartments 4 and 5) and the EPZ (number of cells from the crypt base to the highest Ki67-positive cell). Error bars represent S.E.M. Brackets indicate significant differences between means ($P<.05$). Note the distinct scales for graphs of EPZ, total LI and LIs for compartments 1–4 (0–1) versus graphs of compartment 5 and Φ_{40} (0–0.4). Brackets indicate significant differences between means (two-way MANOVA with comparisons of one factor within the levels of the other factor, $P<.05$). Note that scoring could not be performed for all animals due to poor slicing and arrangement of tissue architecture.

that COX-2 is a minor pathway leading to PGH_2 substrate for E-series PG synthesis in normal mucosa. This is consistent with low expression of COX-2 in normal colonic mucosa of mice (see Fig. 2) [1,26,27]. The small increase in PGE_2 levels in COX-2 null versus wild-type mice fed oleate fat diets may result from the observation that genetic deletion of COX-1 and COX-2 can induce compensatory up-regulation of the other COX isoform, as well as microsomal prostaglandin E synthase-1, to maintain PGE_2 synthesis [43–45]. The PGE_2 data further suggest that COX-1, and not COX-2, would be the major targetable COX isoform for dietary modulation of eicosanoid profiles in normal colonic mucosa.

Similar to the prostanoids, the fish oil diet generally reduced synthesis of AA-derived LTB_4 and increased synthesis of EPA-derived LTB_5 relative to the oleate diet. The fish oil diet also significantly reduced the levels of 12- and 15-HETEs in colonic mucosa, and the same trend was observed for the minor product 5-HETE. The HETEs data for oleate diet animals are consistent with a previous report that the relative levels of HETEs in both normal and inflamed colonic

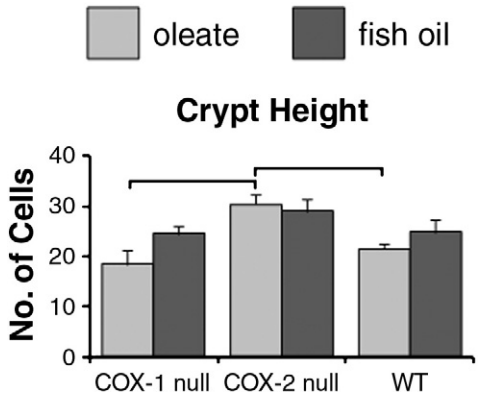

Fig. 7. Crypt height (number of cells in a full-length crypt) in colonic mucosa as measured by H/E staining of well-oriented, full-length crypts. Brackets indicate significant differences between means (two-way MANOVA with comparisons of one factor within the levels of the other factor, $P<.05$). Note that scoring could not be performed for all animals due to poor slicing and arrangement of tissue architecture.

mucosa of rodents are 12-HETE>15-HETE>5-HETE [46]. In human colonic mucosa, reported 15-HETE levels are between 2- and 100-fold higher than 12-HETE in both normal and inflamed mucosa, with 5-HETE being present at much lower concentrations [8,46–49]. These differences between rodents and humans are likely due in part to the fact that 12- and 15-*S*-HETEs are produced by a 12/15-LOX in rodents (which produces predominantly 12-HETE), and by distinct enzymes in humans with 15-HETE predominating [50,51]. Therefore, in humans, the relative levels of 12- and 15-HETEs are likely to be reversed from those seen in mice. The relatively low levels of 5-HETE relative to the other HETEs may be due to the low expression of 5-LOX in normal mucosa [33].

The HETEs data also suggest possible shunting of AA substrate to LOX metabolism when COX-1 is absent since COX-1 null mice fed the oleate diet had virtually no PGE_2 synthesis and significantly elevated synthesis of 12-HETE and 15-HETE relative to wild-type and COX-2 null animals in the context of similar AA levels. A similar but nonsignificant trend in shunting from COX to LOX in was also observed for 5-HETE, but the effect of shunting on eicosanoids derived from 5-LOX was likely minimal as COX-1 null and wild-type animals fed oleate diet had similar LTB_4 levels.

It is interesting to note that the mean net loss of PGE_2 synthesis in COX-1 null mice was roughly 35 pmol/mg (96% of wild-type PGE_2 levels), while the mean increase in total HETEs synthesis was ~482 pmol/mg [1217 pmol/mg total HETEs, a 66% increase from the total HETEs levels in wild-type animals (735 pmol/mg)]. The mean net increases in 5-, 12- and 15-HETEs were ~20 pmol/mg (an 87% increase from wild-type), 423 pmol/mg (66%) and 39 pmol/mg (55%), respectively. Therefore, substrate availability, binding affinity, enzymatic efficiency and compartmentalization of enzymes could play a role in shunting of substrate from COX to LOX pathways in normal mucosa, as the increase in HETEs synthesis was 12-fold greater than the decrease in PGE_2 synthesis.

4.2. Proliferation

The fish oil diet, genotype status and resulting changes in eicosanoid profiles showed little correlation with the proliferation indices in colonic crypts. It is important to note that these animals were not exposed to any carcinogens or inflammatory agents and therefore represent normal colonic mucosa that is not transformed or inflamed. The only statistically significant changes were on absolute crypt size and Φ_{40}. Deletion of COX-2 resulted in increased crypt height in mice fed the oleate diet, with increases in crypt height reflecting the elevated free AA and PGE_2 levels in these animals. The fish oil diet attenuated this effect. In humans, increased crypt height has been associated with reduced colon cancer risk [52,53], while results in animals have been mixed [54–59]. In one study of fish oil feeding, decreased mucosal AA/EPA ratio was associated with shorter crypts, unlike in our study, but increased mucosal PGE_2 levels were associated with larger crypts [60]. Although PGE_2 in colonic pathology is generally associated with inflammation, it should be noted that in other conditions, PGE_2 serves to limit inflammation. This makes it difficult to interpret the effects of PGE_2 in normal colonic mucosa.

The increased LI in compartment 5 (top 1/5th of the crypt) and Φ_{40} in COX-2 null mice fed fish oil was unexpected, given that PGE_2 values were not elevated in this group. It is important to note that these animals were sickly and had a lower survival rate than the other groups (5/10 animals survived for this group, whereas all animals survived in all other groups except COX-2 null mice fed oleate diet, with 9/10 surviving). The increased proliferation may therefore be due to an underlying pathology confounding the experimental treatment in these mice. In addition, COX-2 has been shown to have a protective role in resolution of inflammation and survival of colitis [61–63]. In comparing the effects of genotype on proliferation in animals fed a oleate blend fat diet, COX-2 may have subtle physiological role that is critical for maintaining proliferation at low levels and crypt height at normal levels (EPZ and crypt height were higher in COX-2 nulls). In models of allergic inflammation and colitis, PGE_2 is well known to have a protective role in limiting inflammation [61–65]. This is unlike initiated colon tissue where highly up-regulated COX-2 is known to have tumor-promoting effects [2,3,66]. This calls into question whether prevention in normal risk individuals who do not have any defined pathological changes in the colon would benefit from COX-1 or COX-2 inhibition.

Due to the small sample size of this study, no adjustment was made for multiple comparisons in the statistical analysis. If adjustments for multiple comparisons had been made using the Bonferroni method, the single comparison between the two diets within the same genotype would be the same as the present analysis. The three comparisons between genotypes within each diet would result in the loss of statistical significance for some outcomes. However, the main findings of this study would still be statistically significant.

For fatty acids, the increased % ω3 HUFA, the lower levels of EPA and the lower AA/EPA ratios for the fish oil diet versus the oleate diet would still be significant within each genotype. For the PGs, the near-complete elimination of PGE_2 and PGE_3 synthesis in COX-1 null mice and the reduced PGE_2 and PGE_3 synthesis in mice fed fish oil versus oleate diet in the COX-2 null and wild-type mice would remain significant. The reduction in HETEs synthesis between diets would remain significant for 12- and 15-HETE within each genotype except for 12-HETE in COX-2 null mice. However, the shunting effect from COX to LOX metabolism in COX-1 null mice would no longer be statistically significant. For proliferation, the increased Φ_{40} in COX-2 null mice versus wild-type mice would remain significant, as well as the increased crypt height in COX-2 null mice versus COX-1 null and wild-type mice fed the oleate diet.

In conclusion, the changes in eicosanoid profiles induced by diet and genotype were profound, while corresponding changes in proliferation were slight. The changes induced by fish oil could protect the colon upon carcinogen challenge. Inhibition of COX-2, however, might not have preventive effects in low-risk settings.

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