Brief Definitive Report

Novel Functional Sets of Lipid-derived Mediators with Antiinflammatory Actions Generated from Omega-3 Fatty Acids via Cyclooxygenase 2–Nonsteroidal Antiinflammatory Drugs and Transcellular Processing

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Abstract

Aspirin therapy inhibits prostaglandin biosynthesis without directly acting on lipoxygenases, yet via acetylation of cyclooxygenase 2 (COX-2) it leads to bioactive lipoxins (LXs) epimeric at carbon 15 (15-epi-LX, also termed aspirin-triggered LX [ATL]). Here, we report that inflammatory exudates from mice treated with ω-3 polyunsaturated fatty acid and aspirin (ASA) generate a novel array of bioactive lipid signals. Human endothelial cells with upregulated COX-2 treated with ASA converted C20:5 ω-3 to 18*R*-hydroxyeicosapentaenoic acid (HEPE) and 15*R*-HEPE. Each was used by polymorphonuclear leukocytes to generate separate classes of novel trihydroxy-containing mediators, including 5-series 15*R*-LX_5 and 5,12,18*R*-triHEPE. These new compounds proved to be potent inhibitors of human polymorphonuclear leukocyte transendothelial migration and infiltration in vivo (ATL analogue > 5,12,18*R*-triHEPE > 18*R*-HEPE). Acetaminophen and indomethacin also permitted 18*R*-HEPE and 15*R*-HEPE generation with recombinant COX-2 as well as ω-5 and ω-9 oxygenations of other fatty acids that act on hematologic cells. These findings establish new transcellular routes for producing arrays of bioactive lipid mediators via COX-2–nonsteroidal antiinflammatory drug–dependent oxygenations and cell–cell interactions that impact microinflammation. The generation of these and related compounds provides a novel mechanism(s) for the therapeutic benefits of ω-3 dietary supplementation, which may be important in inflammation, neoplasia, and vascular diseases.

Key words: dietary PUFA • eicosanoids • leukocytes • cardiovascular disease

Introduction

Numerous reports of the past 25 years suggest that supplementation of dietary omega-3 polyunsaturated fatty acids (ω-3 PUFA) with linseed, canola, or fish oils has beneficial effects in human diseases and laboratory animals (for review see references 1 and 2). These have included lively discussions of potential antithrombotic, immunoregulatory, and antiinflammatory responses relevant in arteriosclerosis, arthritis, and asthma (1) as well as antitumor and antimetastatic effects (3). Their potential for preventative actions in cardiovascular diseases was recently bolstered with the finding that major dietary ω-3 PUFAs, eicosapentaenoic acid (C20:5 ω-3; EPA) and docosahexaenoic acid (C22:6 ω-3; DHA), have a dramatic effect on ischemia-induced ventricular fibrillation and can protect against sudden cardiac death in dogs (4). Emergence of such possible preventative and/or therapeutic actions of ω-3 PUFA supplementation in infant nutrition, cardiovascular diseases, and mental health has led to a call for recommended dietary intakes by an international workshop (5). Also, the Gruppo Italiano per lo Studio della Sopravvivenza nell'Infarto Miocardico-Prevenzione trial evaluated the effects of ω-3 PUFA supplementation with >11,300 patients surviving myocardial infarction taking ~1 g of ω-3 PUFA daily (n = 2,836) along with recommended preventive treatments including aspirin, and reported a significant benefit with a decrease in

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cardiovascular death (6). However, the cellular and molecular mechanism(s) for dietary ω-3 protective actions in all of the studies to date remain largely unexplained.

It is believed that the actions of the major lipid of fish oil, C20:5, are based upon (a) preventing conversion of arachidonic acid (C20:4 ω-6; AA) to proinflammatory eicosanoids (i.e., prostaglandins [PGs] and leukotrienes [LTs]); (b) serving as an alternate substrate producing 5-series LTs that are less potent; and/or (c) conversion by cyclooxygenase (COX) to 3-series prostanoids (i.e., PGI_3) with potencies equivalent to their 4-series PG counterparts to maintain antithrombotic actions (1, 3, 4). These and other explanations offered (1–5) have not been generally accepted because of the lack of molecular evidence in vivo and the high concentrations of ω-3 PUFA required to achieve putative "beneficial actions" in vitro.

Although the proinflammatory roles of LT and PG are well appreciated (7, 8), there is new evidence that other eicosanoids derived from arachidonate, namely lipoxins (LXs) and their endogenous analogues, the aspirin-triggered 15 epimer LXs (ATLs), are potent counterregulators of PMN-mediated injury and acute inflammation (9–11). At least two isoforms for COX, the classic site of action for nonsteroidal antiinflammatory drugs (NSAIDs), have been uncovered (COX-1 and 2) that appear to serve separate physiologic and pathophysiologic roles in humans (12). Each COX isoform carries dual enzymatic activities, a *bis*oxygenase and a peroxidase. Inhibition of COX-2 is the current focus of several pharmaceutical companies, as selective inhibition of COX–2 without blocking COX-1 could reduce unwanted side effects associated with traditional NSAIDs (13). In this regard, acetylation of COX-2 by the classic NSAID, aspirin (ASA), prevents the formation of prostanoids (12), but the acetylated enzyme remains active in situ to generate 15*R*-hydroxyeicosatetraenoic acid (15*R*-HETE) from C20:4 (14, 15) that is released and converted by activated inflammatory cells to the 15-epimeric LXs. Synthetic analogues of these natural local mediators with prolonged biological half-life display potent antiinflammatory properties (11, 16), providing evidence that cell–cell interactions can be responsible for conversion of AA to mediators that possess antiinflammatory properties.

Oxidation of C20:4 via P450 in endothelial cells (ECs) may also lead to 11,12-epoxyeicosatetraenoic acid that appears to block EC activation (17), while nonenzymatic oxidation of EPA can downregulate EC adhesion molecules (18). As PMN–vessel interactions are pivotal to recruitment and PMN-dependent tissue injury, the local signals involved in their "cross-talk dialogue" are of interest. Our finding that aspirin-acetylated COX-2 remains active in vivo (14) to generate specific ATLs that can be effectors of well established antiinflammatory reactions offers a mechanism for beneficial effects of ASA that cannot be attributed to inhibition of prostanoids alone (8, 12). New therapeutic applications for ASA and related NSAIDs continue to emerge. However, they usually require molecular definition to provide a rationale. This includes the reported prophylactic benefit of ASA in colorectal cancer and the lower risk of a second myocardial infarction (for review see reference 19). In view of the qualitatively overlapping beneficial profiles assigned to dietary ω-3 PUFA in human disease (1–6), we sought evidence for novel pathways for lipid-derived signals that could possibly provide a molecular basis and also serve as markers for these beneficial actions.

Materials and Methods

Zymosan, hematin, NADPH, and ASA were from Sigma-Aldrich. EPA (Cayman Chemical) and other synthetic standards, hydroxy fatty acids, and intermediates used for identification were purchased from Cascade Biochem Ltd. *Bacillus megaterium* was from American Type Culture Collection. Materials used in liquid chromatography tandem mass spectrometry (LC/MS/MS) analyses were from vendors given in reference 20.

Human PMNs were freshly isolated from venous blood of healthy volunteers (that declined taking medication for 2 wk before donation; Brigham and Women's Hospital protocol no. 88-02642) by Ficoll gradient and enumerated. Human umbilical vein or microvascular ECs (HUVECs or HMVECs, respectively) were cultured for transendothelial migration (10). HMVEC monolayers (one, two, or three passages) were seeded ($\sim 2 \times 10^5$ cells/cm^2) on polycarbonate permeable supports precoated with 0.1% gelatin for incubations with NSAIDs and PUFA.

Inflammatory exudates were initiated with intrapouch injection of TNF-α (R&D Systems) into 6 d dorsal air pouches (16) with 6–8-wk-old male FVB mice (fed standard rodent diet 5001 containing 0.26% n-3 fatty acids) followed by ASA (500 μg) at 3.5 h and 300 μg C20:5/pouch at 4 h. At 6 h, pouches were lavaged (3 ml saline), and exudate cells were enumerated and activated (4 μM A_{23187}, 37°C, 20 min). Inhibition of TNF-α–stimulated (100 ng/pouch, FVB strain) PMN infiltration with intravenous tail injection of either 18*R*-hydroxyeicosapentaenoic acid (HEPE), 5,12,18*R*-HEPE, or 15-epi-LXA_4 analogue was determined (16) with pouch lavages taken at 4 h.

Specific [^{3}H]LTB_4 (NEN Life Science Products) binding was performed with human embryonic kidney (HEK)293 cells stably transfected with human LTB_4 receptor (11). Human recombinant COX-2 (a gift from Dr. R.A. Copeland, DuPont Merck, Wilmington, DE) was overexpressed in *Sf9* insect cells (American Type Culture Collection) with microsomal fractions ($\sim$8 μl) suspended in Tris (100 mM, pH 8.0) as in reference 21. NSAIDs were incubated (i.e., ASA, $\sim$1 mM) at 37°C for 30 min before addition of PUFA (20 μM), and conversions were also monitored using 1-^{14}C–labeled C20:4 or C20:5 (NEN Life Science Products). *B. megaterium* was grown in Bacto Nutrient Broth (Fisher Scientific) at 30°C with shaking. To prepare standards for 18*R*-HEPE, a biogenic synthesis was used with *B. megaterium* sonicates incubated with NADPH (2 mM) and C20:5 (330 μM) in 2 M Tris buffer, pH 8.1. Similar conditions were employed to convert LTB_5 (15 μM) to novel products; see Results. Incubations were extracted (9) with deuterium-labeled internal standards (15-HETE and C20:4) for LC/MS/MS analysis (14, 16) using a Finnigan LCQ equipped with a LUNA C18-2 (150 × 2 mm; 5 μM) column and a rapid spectra scanning UV/Vis detector. Also, a Chiralcel OB-H column (J.T. Baker) was used to determine *R* and *S* alcohol configurations of monohydroxy-PUFA using isocratic (hexane/isopropanol 96:4 vol/vol). Detailed procedures for isolation, quantitation, and structural determination of lipid-derived mediators were recently reported (20) and used here essentially as described for the elucidation of the novel products.

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Results and Discussion

Because ASA triggers formation of epimeric forms of naturally occurring bioactive eicosanoids (9), we tested the concept that NSAIDs might promote the formation of novel mediators from ω-3 PUFAs. Inflammatory exudates formed in murine air pouches via intrapouch injections of TNF-α with ω-3 and ASA on board (2 h) generated several novel compounds (Fig. 1). These mice were fed a standard rodent diet containing 0.26% ω-3 PUFA. LC/MS/MS analyses of the exudate-derived materials demonstrated monohydroxy acids, depicted in selected ion chromatograms from acquired results recalled at m/z 317 (Fig. 1 A), i.e., 18-hydroxy-EPA (18-HEPE) and 5-HEPE, which coeluted with synthetic 5*S*-HEPE as well as novel trihydroxy-containing compounds derived from C20:5. LC retention times and MS/MS spectra (Fig. 1, B and C) gave product ions consistent with structures shown in the respective insets, namely m/z 317 = $[M-H]^-$, 299 = $[M-H]^-–H_2O$, 273 = $[M-H]^-–CO_2$, 255 = $[M-H]^-–H_2O,–CO_2$. Diagnostic ions consistent with 18-HEPE identification were present at m/z 259 (Fig. 1 B) and 5-HEPE at m/z 115 (Fig. 1 C). These criteria were used throughout for identification. The stereochemistry of the alcohol at carbon 18 was established for exudate-derived 18-HEPE using a chiral column, and a reference 18*R*-HEPE was prepared via biogenic synthesis using *B. megaterium* (see Materials and Methods). This microbe monooxygenates fatty acids and, for example, converts C20:4 to 18*R*-HETE (22, 23). The alcohol configuration at position 18 proved to be >98% *R*. These findings indicated that murine inflammatory exudates exposed in vivo to C20:5, ω-3 and ASA produced 5-lipoxygenase pathway 5-series 5*S*-HEPE, a product also identified with human PMNs (24), as well as a novel 18*R*-HEPE, whose route of formation was determined (vide infra). Air pouch inflammatory exudate cells from these ASA- and EPA-treated mice contained predominantly PMN (as in Fig. 1), which were 25–60% lower in number than in exudates formed with TNF-α alone (n = 3). Also, these exudates, when activated with ionophore A_{23187} (4 μM), generated essentially equivalent amounts of 18*R*-HEPE (10.2 ± 4.3 ng/10^6 cells) and 5*S*-HEPE (10.9 ± 2.9 ng/10^6 cells).

Evidence for novel trihydroxy-containing products was also obtained in these inflammatory exudates (Fig. 1 D).

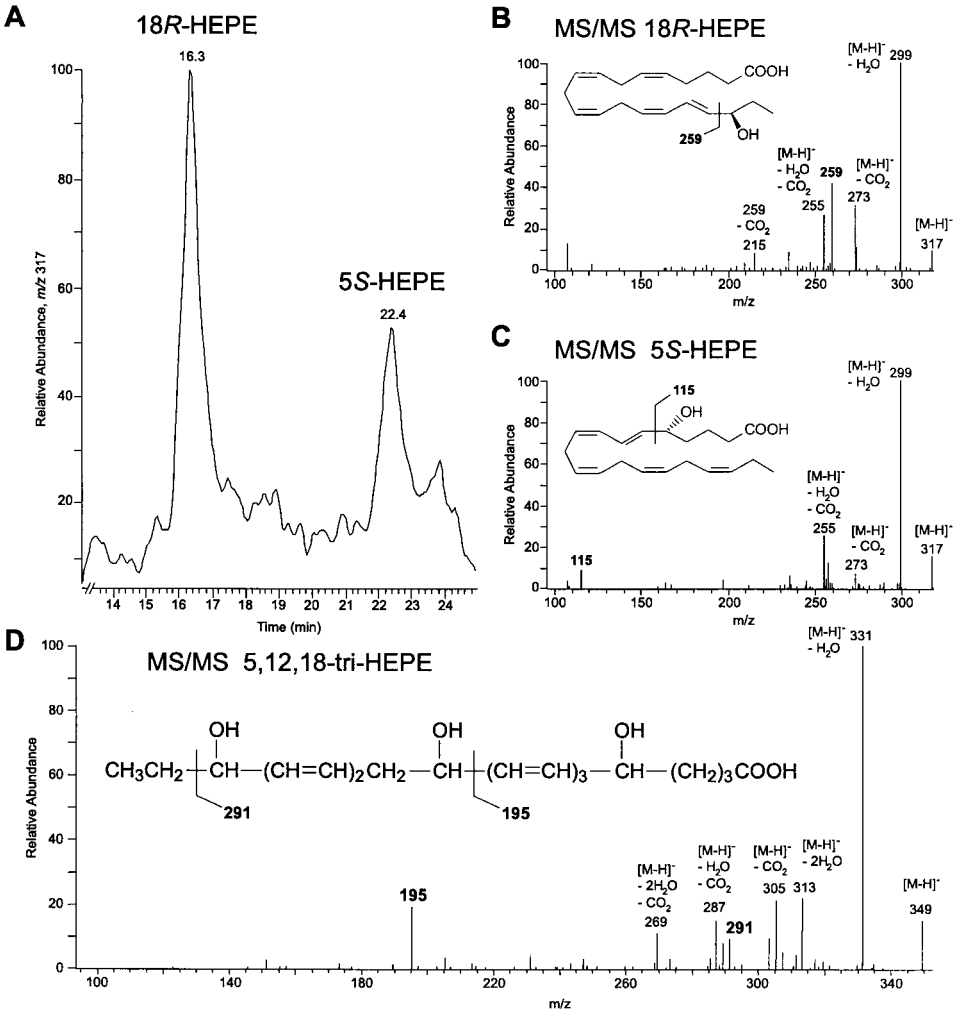

Figure 1. Inflammatory exudates from murine dorsal pouches treated with aspirin generate novel compounds: LC/MS/MS. TNF-α–induced leukocyte exudates collected at 6 h from FVB mice given ASA (3.5 h at 500 μg/air pouch) and EPA (4 h at 300 μg/pouch) contained (2.3 ± 0.5 × 10^6 leukocytes per pouch); see Materials and Methods. (A) Selected ion chromatogram of mono-HEPEs. (B) MS/MS of 18*R*-HEPE. (C) MS/MS of 5*S*-HEPE. (D) MS/MS of 12,15,18*R*-triHEPE (see text for identification of diagnostic ions). Results are representative of n = 4.

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Ions present within MS/MS were consistent with a trihydroxy-containing product from C20:5 with a parent ion at m/z 349 = $[M-H]^-$ and product ions of structural significance present at m/z 291 and 195 that are consistent with fragmentations denoted in the inset (Fig. 1 D). Also, an observed 270 nm UV absorbance maximum, indicative of a conjugated triene, together with the presence of the m/z = 291 (cleavage C17–C18 positions) as well as the 20-carbon structure, implicated that 18*R*-HEPE and the triHEPE were biosynthetically related.

It was of interest to determine whether these new compounds were also generated by human cells and if they possess bioactivities. To this end, human ECs known to induce COX-2 with IL-1β (9) or hypoxia (not shown) were pulsed with EPA and treated with ASA, and extracted materials were subject to LC/MS/MS analysis (Fig. 2 A). Selected ion monitoring at m/z 259 revealed that HUVECs treated with ASA converted EPA to 18*R*-HEPE (Fig. 2 A). Also, HMVECs treated with ASA and EPA generated 18-HEPE (10.6 ng/10^6 cells) and 15-HEPE (6.0 ng/10^6 cells) (n = 2, four determinations; data not shown). These observations implicated the involvement of COX-2 in the generation of these compounds, which proved to be the case with recombinant human COX-2 exposure to ASA and ω-3 PUFA (Table I), findings that may be of clinical significance.

As shown in Table I, linoleic acid (C18:2) was converted to both 13-hydroxy-9Z,11E-octadecadienoic acid (13-HODE; ω-5 oxygenation) and 9-HODE (ω-9), which were greatly diminished by ASA but not completely abolished. AA was converted to 15*R*-HETE (ω-5) as well as 11*R*-HETE (ω-9), consistent with earlier findings (9). ASA triggered the appearance of a lipoxygenase activity that switched to 15*R*-HETE production by acetylated COX-2 (9, 14, 15), which did not appear to influence 11*R*-HETE formation (Table I). 11*R*-HEPE was the major product with EPA and COX-2, with lesser amounts of 15*R*-HEPE (ω-5) and 18*R*-HEPE (ω-2). 1-^{14}C–labeled EPA was used to confirm precursor–product relationships (n = 3). ASA acetylation of COX-2 led to an approximately twofold increase in 18*R*-HEPE (ω-2), with a >85% reduction in 11*R*-HEPE (the ratio of positional oxygenations with C20:5 was 1:1:0.3, with 18*R* ≈ 15*R* > 11*R*). Hence, together they suggested that acetylated COX-2 in ECs (Fig. 2) was a dominant source of 18*R*-HEPE and 15*R*-HEPE.

Interestingly and unlike the isolated COX-2 product profiles, neither 11*R*-HEPE (from C20:5) nor 11*R*-HETE (from C20:4) were major products of the vascular ECs (Table I and Fig. 2). With the selective COX-2 inhibitor NS398 (12, 13), these oxygenations were reduced, and only 18*R*-HEPE formation from EPA appeared to escape inhibition (Table I). These results suggest that ASA treatment at local sites of inflammation along with ω-3 PUFA

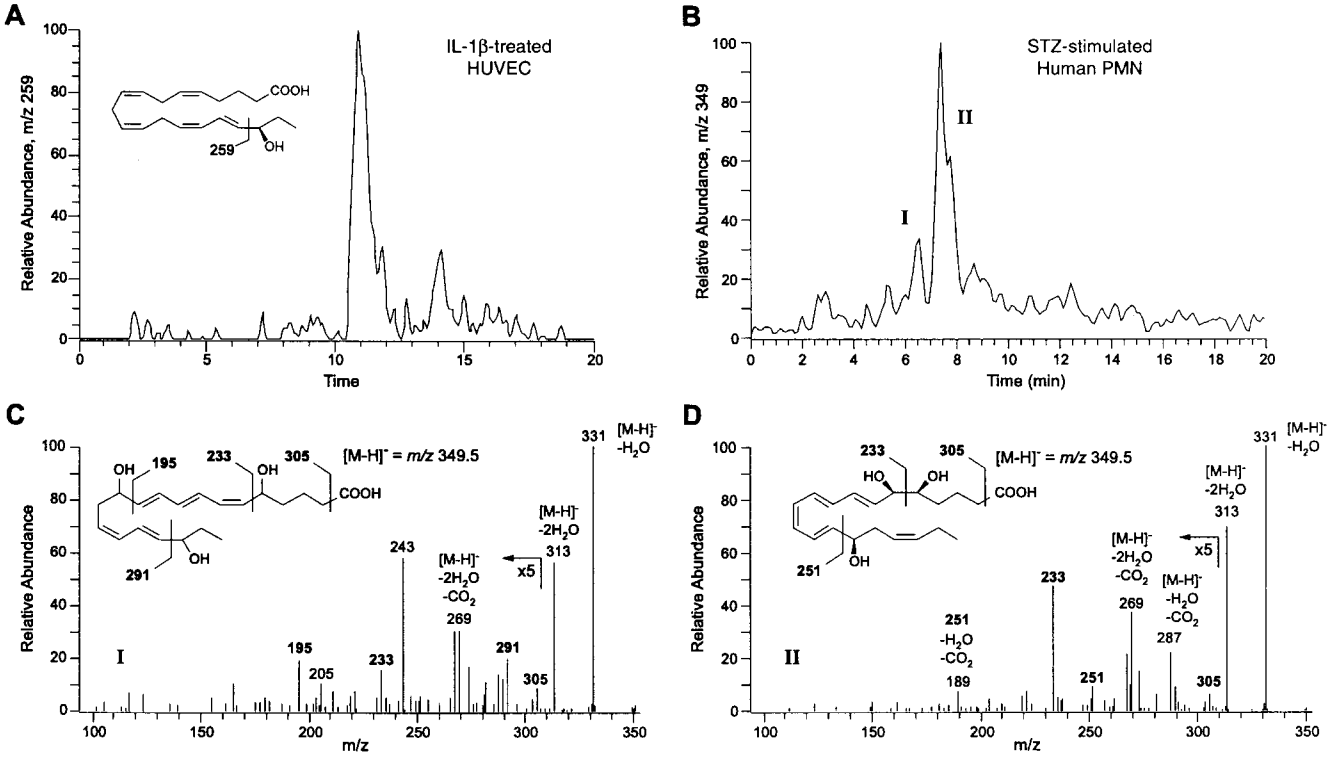

Figure 2. Human ECs and PMNs: novel trihydroxy-containing compounds LC/MS/MS. (A) HUVECs treated with IL-1β (24 h) and ASA. (B) Human PMNs (~ 30 × 10^6/ml) incubated (30 min, 37°C) with serum-treated zymosan (100 ng/ml) and acetylated ASA-recombinant COX-2–derived products from EPA. (C) MS/MS of 5,12,18*R*-triHEPE (see text for ions and fragmentation). (D) MS/MS of 15-epi-LXA_5. Results are representative of n = 3–5 for PMNs and n = 3 for ECs.

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(i.e., EPA; C20:5, ω-3) administration, as exemplified by cytokine-driven acute inflammation (Fig. 1), can convert EPA via COX-2 to 18*R*-HEPE and 15*R*-HEPE.

Because human PMNs convert ASA-triggered, COX-2–derived 15*R*-HETE to 15-epi-LXA_4 (9) and EPA is converted to 5-series LX by human leukocytes (25) as well as trout macrophages (26), we next evaluated whether activated human PMNs engaged in phagocytosis handle acetylated COX-2–derived C20:5, ω-3 products 18*R*-HEPE and 15*R*-HEPE. Serum-treated zymosan, a phagocytic stimulus, initiated the utilization and conversion of acetylated COX-2 C20:5-derived products to two classes of trihydroxy-containing EPE, determined again by selected ion monitoring at m/z 349.5 $[M-H]^-$, the base peak molecular ion for these products (Fig. 2 B). One, shown in Fig. 2 C, gave essentially the same MS/MS observed in Fig. 1 D from murine cells and was consistent with the 5,12,18*R*-triHEPE structure depicted in the inset giving diagnostic ions (Fig. 2 C) at m/z 305, 233, 195, and 291 (Fig. 1 D). This product is an 18*R*-hydroxy–carrying "LTB_5-like" structure (see Fig. 2 D, inset). Indeed, when isolated 18*R*-HEPE was incubated as above with activated PMNs, it was converted to several compounds, including this product. Also, synthetic LTB_5 incubated with *B. megaterium* homogenates and NADPH at pH 8.0 to facilitate hydroxylations (23) was transformed to a trihydroxy product ($n = 3$) with an m/z 291 ion characteristic for the presence of the 18*R* alcohol group as obtained from human PMNs shown in Fig. 2 C. These independent lines of evidence indicated that PMNs take up 18*R*-HEPE, which is converted by their 5-lipoxygenase, to insert molecular oxygen and in subsequent steps to 5-hydro(peroxy)-18*R*-DiH(p)EPE and 5(6)epoxide formation to 5,12,18*R*-triHEPE (an 18*R*-carrying LTB_5-like product) that is likely to possess the stereochemistry of LTB_5 (24), retaining the 18*R* chirality of the precursor.

In an analogous biosynthetic fashion, 15*R*-HEPE was converted by PMN via 5-lipoxygenation to a 5-series LXA_5 analogue (Fig. 2 D) that also retains their C15 configuration. Its MS/MS gave prominent ions, m/z 305, 233, and 251, depicted in the MS/MS spectrum, namely 15-epi-LXA_5, consistent with 15*S*-containing LX_5 structures (5-series) observed from endogenous sources of EPA in trout macrophages (26). In this case, the chirality of the precursor 15*R* is retained by human PMN to give 15-epi-LXA_5 (Fig. 2 D), which is the 5-series ω-3 analogue of 15-epi-LXA_4. As with LX biosynthesis, conversion of both 18*R*- and 15*R*-HEPE by activated PMNs with 5-lipoxygenation was accompanied with a reduction in LTB_4 formation (not shown). Together, these results indicate that isolated human ECs and PMNs (Fig. 2) can generate the novel products observed with inflammatory exudates (Fig. 1).

Transendothelial migration is a pivotal event in PMN recruitment and inflammation and a recognized locus of action for traditional antiinflammatory therapies (27). Endogenous lipid mediators that can control these cell–cell interactions are of interest. Therefore, we isolated and assessed 5,12,18*R*-triHEPE and its precursor 18*R*-HEPE on human PMN transmigration. Both compounds inhibited LTB_4-stimulated PMN transendothelial migration (Fig. 3 A) with an apparent IC_{50} of 5–50 nM for 5,12,18*R*-triHEPE and IC_{50} >1.0 μM for 18*R*-HEPE. Thus, the new 5-series members, namely, 18*R*-carrying trihydroxy-HEPE and 18*R*-HEPE, inhibited PMN migration, as did 15-epi-

Table I. *Hydroxy Compounds Generated from PUFA and Recombinant Human COX-2 with ASA or a Selective COX-2 Inhibitor*

		Hydroxy-containing compounds (ng/incubation)		
PUFA	NSAID	ω-2	ω-5	ω-9
			13-HODE	9-HODE
C18:2	ASA	–	2.9 ± 0.6	25.2 ± 17.8
C18:2	Vehicle alone	–	55.0 ± 18.7	243.0 ± 68.6
			15*R*-HETE	11*R*-HETE
C20:4	ASA	–	234.0 ± 112.5	1.4 ± 1.6
C20:4	Vehicle alone	–	11.5 ± 8.2	1.8 ± 1.0
C20:4	Selective inhibitor	–	5.5 ± 0.8	0.9 ± 0.1
		18*R*-HEPE	**15*R*-HEPE**	11*R*-HEPE
C20:5	ASA	16.2 ± 3.3	16.8 ± 5.8	5.4 ± 3.3
C20:5	Vehicle alone	7.0 ± 3.3	17.9 ± 5.2	40.7 ± 10.3
C20:5	Selective inhibitor	5.8 ± 2.6	0.0 ± 0.6	0.0 ± 0.3

Results are the mean ± SEM, $n = 3$. The selective COX-2 inhibitor NS398 was used at 100 μM. All products were extracted, identified, and quantitated using internal standards and LC/MS/MS. Compounds of interest are shown in bold type.

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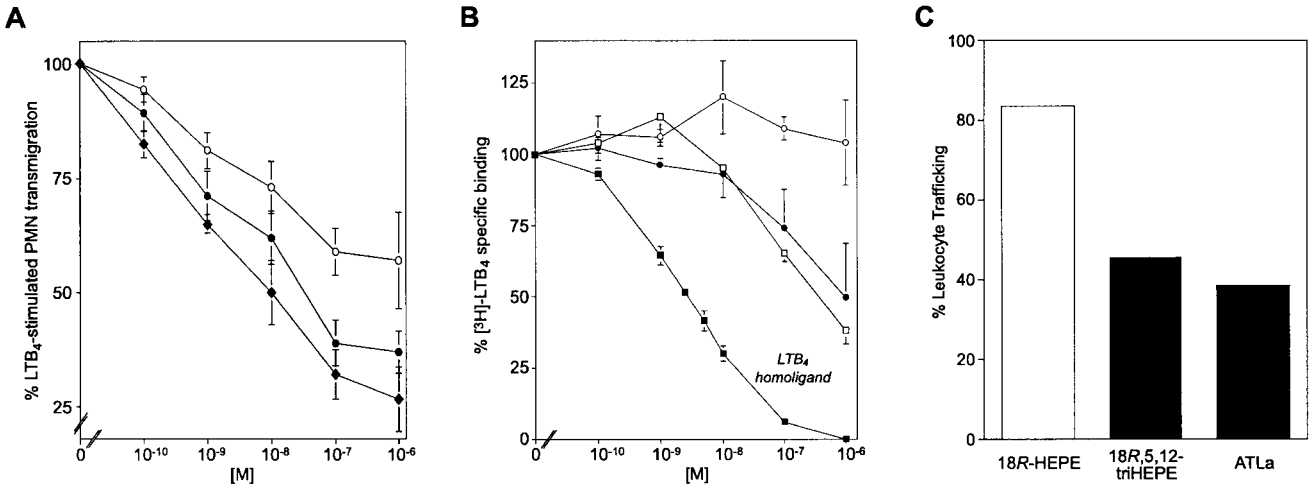

Figure 3. The novel compounds inhibit human PMN transmigration and inflammation in the murine air pouch. (A) Inhibition of PMN transendothelial migration. PMNs were incubated with isolated compounds [10^{-10}–10^{-6} M] 18*R*-HEPE (○), 5,12,18*R*-triHEPE (●), or ATL analogue used for reference (◆), and transmigration was initiated using an optimal amount of LTB_4 [10 nM] (100%) and HUVEC coincubations (90 min, 37°C). Results are the mean ± SEM; $n = 3$. (B) Competition binding with human recombinant LTB_4 receptor. LTB_4 receptor stably expressed in HEK293 cells (~5 × 10^5 cells/incubation) were incubated with [^{3}H]LTB_4 (~1 nM) plus LTB_4 (■) and 18*R*-HEPE (○), 5,12,18*R*-triHEPE (●), or LTB_5 (□). Results are the mean ± SEM from $n = 3$. (C) Inhibition of leukocyte trafficking in 6 d dorsal air pouch. TNF-α (100 ng) was injected intrapouch and 100 ng i.v. tail vein of test compound. ATLa (analogue 15(*S*)-16(parafluoro)-phenoxy-LXA_4; reference 16) was used for comparison. Results represent $n = 4$.

LXA_4 and its omega end analogue (10, 16), tested in parallel for direct comparison (Fig. 3 A). Their rank order of potency was 15-epi-LXA_4 stable analogue > 5,12,18*R*-triHEPE > 18*R*-HEPE.

The G protein–coupled receptor for LTB_4 was identified (28), and to determine whether these 18*R*-containing products interact with the human LTB_4 receptors to block PMNs, this receptor was cloned (11) from reported sequences (28) and stably expressed in HEK293 cells for competition binding experiments (Fig. 3 B). The homoligand LTB_4 effectively competed ($IC_{50} \approx 2.5$ nM). 18*R*-HEPE did not, while both LTB_5 and 5,12,18*R*-triHEPE competed (IC_{50} ~0.5 μM), giving a trend with LTB_5 > 5,12,18*R*-triHEPE. Although the 5,12,18*R*-triHEPE and a related structure (i.e., LTB_5) were substantially less effective than 4-series LTB_4, consistent with the reduced PMN ac-

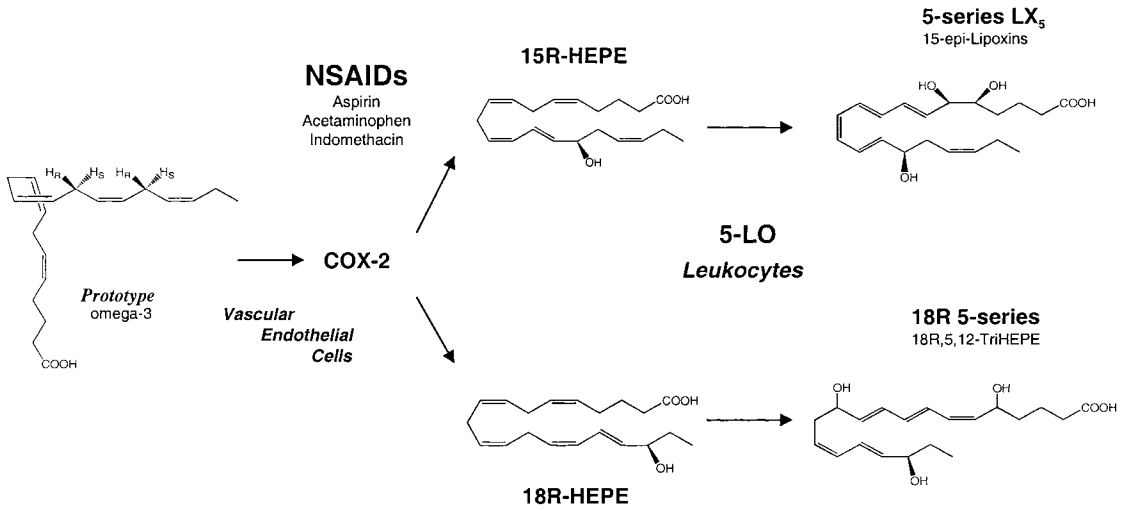

Figure 4. Proposed scheme for generating functional arrays of lipid signals from ω-3 PUFA via transcellular processing: endogenous inhibitors of microinflammation. At sites where COX-2 is upregulated and treated with NSAIDs, PG formation from C20:4 is blocked. Systemic ω-3 PUFAs are converted via a COX-2–NSAID lipoxygenase–type mechanism that abstracts hydrogen in a stereospecific fashion at C16 or C13 in C20:5 to give *R* insertions of molecular O_2 to yield 15*R*-H(p)EPE or 18*R*-H(p)EPE to form epoxides or are reduced to alcohols. The complete stereochemistry for each of the trihydroxy compounds remains to be determined, and the compounds are depicted in their likely configurations. These compounds interact with cells in the local microenvironment, inhibiting PMN recruitment. COX-2–NSAID-dependent hydrogen abstraction and insertion of molecular oxygen occurs with all ω-3 PUFAs containing 1,4-cis pentadiene units tested (see text for details). Thus, sequential oxygenations of ω-3 PUFA during cell–cell interactions generate novel arrays of signals of interest in microinflammation.

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tivity of LTB_5 (24), their potency for displacing [^{3}H]LTB_4 was in the range of currently available synthetic LTB_4 receptor antagonists (not shown). These findings suggest that 5,12,18*R*-triHEPE might serve as a damper for LT-mediated responses in vivo if generated in appropriate quantities within the microenvironment as well as a biotemplate for total synthesis of new classes of receptor antagonists. When administered intravenously into tail at low levels (100 ng), 5,12,18*R*-triHEPE was a potent inhibitor of PMN infiltration into murine dorsal air pouches (Fig. 3 C), as was a 15-epi-LX stable analogue (16) given at equivalent doses for the purpose of direct comparison. 18*R*-HEPE also carried some activity in vivo (<5,12,18*R*-triHEPE), whereas it was far less effective with isolated human PMNs in transendothelial migration and apparently did not interact with recombinant LTB_4 receptors at these concentrations.

Other widely used NSAIDs (i.e., acetaminophen and indomethacin) were also tested with recombinant COX-2 and C20:5 as in Table I to determine whether they altered conversion to HEPE. Each inhibited 11-HEPE by >95%. Interestingly, 18*R*-HEPE and 15*R*-HEPE formation persisted (~1:1 ratio) in the presence of either acetaminophen or indomethacin at concentrations as high as 2 mM, even though the levels of 15*R*- and 18*R*-HEPE were reduced by three to eight times their levels in the absence of inhibitors ($n = 3$). These findings indicate that the oxygenation of ω-3 fatty acids to *R*-containing monohydro(peroxy)-containing products is not restricted to ASA treatment and arachidonate. Hence, these commonly used NSAIDs and selective COX-2 inhibitors (Table I) may still permit PUFA oxygenation by activated ECs exposed to NSAIDs (Fig. 2) and at sites of inflammation where the degree of COX-2 interactions with drugs may be "leaky," permitting novel oxygenation of PUFAs.

Despite the many reports of ω-3 PUFAs (i.e., C20:5) possible beneficial impact in humans (1–6), oxygenation by COX-2 to generate novel bioactive compounds has not been addressed in humans or isolated cells. In fish, both C20:5 and C20:4 are mobilized in macrophages and platelets to produce 5- and 4-series eicosanoids, including PG, LT, and LX, with essentially equal abundance (26). Our results indicate that inflammatory exudates from mice treated with ASA and EPA generate novel compounds (Fig. 1) that are also produced by human ECs, recombinant COX-2, and PMNs (Fig. 2). Given the milligram to gram amounts of ω-3 PUFA taken as dietary supplements (1–6) and the large area of microvasculature that can carry upregulated COX-2, the conversion of EPA by ECs and neighboring cells as observed in our experiments (Figs. 1–4) may represent a significant amount at local microenvironments. These COX-2–NSAID-dependent conversions of ω-3 PUFA are likely to be elevated within inflamed or diseased tissues where COX-2 is upregulated and a determinant that impacts fatty acid metabolism (14) when NSAIDs might be of therapeutic benefit, namely with microinflammation.

Analogous to 15-epi-LX biosynthesis, EPA COX-2–derived 15*R*-HEPE was converted by 5-lipoxygenation with 5(6)-epoxide formation in leukocytes to give the 15-epi-LX_5 series (Fig. 4). The stable analogues of 15-epi-LXA_4, modified at their C15 position through position 20 with bulky groups, resist inactivating enzymes and are more potent in vivo, inhibiting PMN traffic as well as formation and actions of key proinflammatory cytokines (10, 16). Hence, 5-series 15-epi-LXs should act in a similar fashion, as they possess a Δ17–18 double bond and thus could function as an ω-3–derived 15-epi-LX analogue.

Because COX-2–NSAID-dependent oxygenation (e.g., 18*R* and15*R*) led to bioactive compounds in vivo that block PMN transendothelial migration and infiltration, our findings provide a basis for a novel mechanism of action of NSAIDs and dietary ω-3 supplementation, namely the generation of endogenous functional arrays of lipid signals (Table I and Figs. 1 and 2) that could, by dampening key events in microinflammation, mediate some of the beneficial actions noted for ω-3 therapies in human trials (1–6). In this context, 13-HODE, a recognized lipoxygenase product that downregulates platelet–EC interactions (29), is also generated by COX-2 (Table I) and joins this pathway array and class of mediators. As inappropriate inflammatory responses are now recognized as contributors to cardiovascular disease (30) as well as in clinical syndromes where PMN-mediated tissue injury is important (11), our identification of these novel ω-3 PUFA processing pathways evoked by cell–cell interactions and the unexpected impact of NSAIDs opens new avenues for considering the potential clinical protection provided by ω-3 PUFA–based supplementation (as from reference 6) and mechanisms to generate potent local endogenous mediators that control microinflammation (Figs. 3 and 4). Moreover, our findings offer a basis to circumvent unwanted effects of current antiinflammatory therapies as well as potential biochemical indices and/or markers of effective dietary ω-3 supplementation.

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References

1. De Caterina, R., S. Endres, S.D. Kristensen, and E.B. Schmidt, editors. 1993. n-3 Fatty Acids and Vascular Disease. Springer-Verlag, London. 166 pp.
2. Lands, W.E.M., editor. 1987. *Proc. AOCS Short Course on Polyunsaturated Fatty Acids and Eicosanoids*. American Oil Chemists' Society, Champaign, IL. 574 pp.
3. Iigo, M., T. Nakagawa, C. Ishikawa, Y. Iwahori, M. Asamoto, K. Yazawa, E. Araki, and H. Tsuda. 1997. Inhibitory effects of docosahexaenoic acid on colon carcinoma 26 metastasis to the lung. *Br. J. Cancer.* 75:650–655.
4. Billman, G.E., J.X. Kang, and A. Leaf. 1999. Prevention of sudden cardiac death by dietary pure ω-3 polyunsaturated

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fatty acids in dogs. *Circulation*. 99:2452–2457.
5. Simopoulos, A.P., A. Leaf, and N. Salem, Jr. 1999. Workshop on the Essentiality of and Recommended Dietary Intakes for Omega-6 and Omega-3 Fatty Acids. *J. Am. Coll. Nutr.* 18:487–489.
6. Marchioli, R. 1999. Dietary supplementation with n-3 polyunsaturated fatty acids and vitamin E after myocardial infarction: results of the GISSI-Prevenzione trial. Gruppo Italiano per lo Studio della Sopravvivenza nell'Infarto miocardico. *Lancet*. 354:447–455.
7. Weissmann, G. 1991. Aspirin. *Sci. Am.* 264:84–90.
8. Marcus, A.J. 1999. Platelets: their role in hemostasis, thrombosis, and inflammation. *In* Inflammation: Basic Principles and Clinical Correlates. J.I. Gallin and R. Snyderman, editors. Lippincott Williams & Wilkins, Philadelphia. 77–95.
9. Clària, J., and C.N. Serhan. 1995. Aspirin triggers previously undescribed bioactive eicosanoids by human endothelial cell-leukocyte interactions. *Proc. Natl. Acad. Sci. USA*. 92:9475–9479.
10. Serhan, C.N., J.F. Maddox, N.A. Petasis, I. Akritopoulou-Zanze, A. Papayianni, H.R. Brady, S.P. Colgan, and J.L. Madara. 1995. Design of lipoxin A_4 stable analogs that block transmigration and adhesion of human neutrophils. *Biochemistry*. 34:14609–14615.
11. Chiang, N., K. Gronert, C.B. Clish, J.A. O'Brien, M.W. Freeman, and C.N. Serhan. 1999. Leukotriene B_4 receptor transgenic mice reveal novel protective roles for lipoxins and aspirin-triggered lipoxins in reperfusion. *J. Clin. Invest.* 104: 309–316.
12. Herschman, H.R. 1998. Recent progress in the cellular and molecular biology of prostaglandin synthesis. *Trends Cardiovasc. Med.* 8:145–150.
13. Needleman, P., and P.C. Isakson. 1997. The discovery and function of COX-2. *J. Rheumatol.* 24 (Suppl. 49):6–8.
14. Chiang, N., T. Takano, C.B. Clish, N.A. Petasis, H.-H. Tai, and C.N. Serhan. 1998. Aspirin-triggered 15-epi-lipoxin A_4 (ATL) generation by human leukocytes and murine peritonitis exudates: development of a specific 15-epi-LXA_4 ELISA. *J. Pharmacol. Exp. Ther.* 287:779–790.
15. Xiao, G., A.-L. Tsai, G. Palmer, W.C. Boyar, P.J. Marshall, and R.J. Kulmacz. 1997. Analysis of hydroperoxide-induced tyrosyl radicals and lipoxygenase activity in aspirin-treated human prostaglandin H synthase-2. *Biochemistry*. 36:1836–1845.
16. Clish, C.B., J.A. O'Brien, K. Gronert, G.L. Stahl, N.A. Petasis, and C.N. Serhan. 1999. Local and systemic delivery of a stable aspirin-triggered lipoxin prevents neutrophil recruitment in vivo. *Proc. Natl. Acad. Sci. USA*. 96:8247–8252.
17. Node, K., Y. Huo, X. Ruan, B. Yang, M. Spiecker, K. Ley, D.C. Zeldin, and J.K. Liao. 1999. Anti-inflammatory properties of cytochrome P450 epoxygenase-derived eicosanoids. *Science*. 285:1276–1279.
18. Sethi, S., A.Y. Eastman, and J.W. Eaton. 1996. Inhibition of phagocyte-endothelium interactions by oxidized fatty acids: a natural anti-inflammatory mechanism? *J. Lab. Clin. Med.* 128:27–38.
19. Levy, G.N. 1997. Prostaglandin H synthases, nonsteroidal anti-inflammatory drugs, and colon cancer. *FASEB J.* 11: 234–247.
20. Gronert, K., C.B. Clish, M. Romano, and C.N. Serhan. 1999. Transcellular regulation of eicosanoid biosynthesis. *In* Eicosanoid Protocols. E.A. Lianos, editor. Humana Press, Totowa, NJ. 119–144.
21. George, H.J., D.E. Van Dyk, R.A. Straney, J.M. Trzaskos, and R.A. Copeland. 1996. Expression purification and characterization of recombinant human inducible prostaglandin G/H synthase from baculovirus-infected insect cells. *Protein Expr. Purif.* 7:19–26.
22. Capdevila, J.H., S. Wei, C. Helvig, J.R. Falck, Y. Belosludtsev, G. Truan, S.E. Graham-Lorence, and J.A. Peterson. 1996. The highly stereoselective oxidation of polyunsaturated fatty acids by cytochrome P450BM-3. *J. Biol. Chem.* 271:22663–22671.
23. Ruettinger, R.T., and A.J. Fulco. 1981. Epoxidation of unsaturated fatty acids by a soluble cytochrome P-450-dependent system from *Bacillus megaterium*. *J. Biol. Chem.* 256: 5728–5734.
24. Lee, T.H., J.M. Menica-Huerta, C. Shih, E.J. Corey, R.A. Lewis, and K.F. Austen. 1984. Characterization and biologic properties of 5,12-dihydroxy derivatives of eicosapentaenoic acid, including leukotriene B5 and the double lipoxygenase product. *J. Biol. Chem.* 259:2383–2389.
25. Serhan, C.N., P.Y. Wong, and B. Samuelsson. 1987. Nomenclature of lipoxins and related compounds derived from arachidonic acid and eicosapentaenoic acid. *Prostaglandins*. 34: 201–204.
26. Hill, D.J., D.H. Griffiths, and A.F. Rowley. 1999. Trout thrombocytes contain 12- but not 5-lipoxygenase activity. *Biochim. Biophys. Acta*. 1437:63–70.
27. Cronstein, B.N., S.C. Kimmel, R.I. Levin, F. Martiniuk, and G. Weissmann. 1992. A mechanism for the antiinflammatory effects of corticosteroids: the glucocorticoid receptor regulates leukocyte adhesion to endothelial cells and expression of endothelial-leukocyte adhesion molecule 1 and intercellular adhesion molecule 1. *Proc. Natl. Acad. Sci. USA*. 89:9991–9995.
28. Yokomizo, T., T. Izumi, K. Chang, T. Takuwa, and T. Shimizu. 1997. A G-protein-coupled receptor for leukotriene B_4 that mediates chemotaxis. *Nature*. 387:620–624.
29. Buchanan, M.R., P. Horsewood, and S.J. Brister. 1998. Regulation of endothelial cell and platelet receptor-ligand binding by the 12- and 15-lipoxygenase monohydroxides, 12-, 15-HETE and 13-HODE. *Prostaglandins Leukot. Essent. Fatty Acids*. 58:339–346.
30. Ridker, P.M., M. Cushman, M.J. Stampfer, R.P. Tracy, and C.H. Hennekens. 1997. Inflammation, aspirin, and the risk of cardiovascular disease in apparently healthy men. *N. Engl. J. Med.* 336:973–979.

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