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Novel Docosatrienes and 17*S*-Resolvins Generated from Docosahexaenoic Acid in Murine Brain, Human Blood, and Glial Cells

AUTACOIDS IN ANTI-INFLAMMATION*

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Docosahexaenoic acid (DHA, C22:6) is highly enriched in brain, synapses, and retina and is a major ω-3 fatty acid. Deficiencies in this essential fatty acid are reportedly associated with neuronal function, cancer, and inflammation. Here, using new lipidomic analyses employing high performance liquid chromatography coupled with a photodiode-array detector and a tandem mass spectrometer, a novel series of endogenous mediators was identified in blood, leukocytes, brain, and glial cells as 17*S*-hydroxy-containing docosanoids denoted as docosatrienes (the main bioactive member of the series was 10,17*S*-docosatriene) and 17*S* series resolvins. These novel mediators were biosynthesized via epoxide-containing intermediates and proved potent (pico- to nanomolar range) regulators of both leukocytes reducing infiltration *in vivo* and glial cells blocking their cytokine production. These results indicate that DHA is the precursor to potent protective mediators generated via enzymatic oxygenations to novel docosatrienes and 17*S* series resolvins that each regulate events of interest in inflammation and resolution.

Chemical mediators and autacoids such as local acting lipid mediators derived from arachidonic acid are well established regulators of key events of interest in host defense, coagulation, inflammation, and cancer (1). The tight control of the enzymes that regulate conversion of unesterified arachidonic acid to key classes of mediators, including prostaglandins, thromboxane, leukotrienes, and lipoxins highlights the importance of arachidonic acid as an essential fatty acid and precursor of these potent bioactive eicosanoids (2). The potential for dysregulation of each of the individual classes of eicosanoids has been suspect as important molecular events associated with several human diseases, including, inflammatory diseases, atherosclerosis, cardiovascular disorders, Alzheimer's disease, and cancer. The control in physiologic systems of these lipid mediators is an ongoing area of intense investigation, because early results indicated that deficiency disease can be initiated by exclusion of fat from the diet (3), and arachidonic acid was also uncovered as the precursor to prostanoids that play key roles in the regulation of parturition and renal function (4, 5).

Results from recent studies indicate that arachidonic acid is not the only fatty acid precursor that is transformed to potent bioactive mediators in inflammation and resolution (6, 7). Both DHA[1] and eicosapentaenoic acid (EPA), the well-known ω-3 fatty acids present in fish oils, appear to be effective as dietary supplements in the treatment of a wide range of human disorders (8–11). For example, ω-3 fatty acid supplementation is reported to have a beneficial impact in treating asthma, atherosclerosis, cancer, and cardiovascular disorders (for a recent review, see Ref. 12). Of interest, the lack of ω-3 fatty acid consumption has also been shown to correlate with mental depression (13). Several clinical trials aimed at testing the therapeutic value of ω-3 supplementation have convincingly established that ω-3 fatty acids can display beneficial actions reducing the incidence and the severity of disease (14). In many of these, aspirin therapy was also used in conjunction, although apparently unintentionally, along with the ω-3 supplementation (*i.e.* see Refs. 14 and 15). Recent results evaluating the impact of aspirin in the transformation of ω-3 in inflammatory exudates *in vivo*, namely acute inflammation and spontaneous resolution, demonstrate that DHA and EPA are each converted via independent pathways to potent bioactive local mediators. These new di- and tri-hydroxy-containing compounds derived from ω-3 fatty acids were termed "resolvins," because they are (*a*) formed within the resolution phase of acute inflammatory response, at least in part, as cell-cell interactions products, (*b*) "stop" neutrophil entry to sites of inflammation, and (*c*) reduce exudates (7). These findings, together with earlier results (16–21), suggest that ω-3 fatty acids, in addition to arachidonic acid, an n-6 fatty acid (2, 4), can serve as precursors for potent

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[1] The abbreviations used are: DHA, C22:6, docosahexaenoic acid; 4*S*-HDHA, 4*S*-hydroxydocosa-5*E*,7*Z*,10*Z*,13*Z*,16*Z*,19*Z*-hexaenoic acid; 4*S*,17*R*/*S*-diHDHA, 4*S*,17*R*/*S*-dihydroxydocosa-5*E*,7*Z*,10*Z*,13*Z*,15*E*,19*Z*-hexaenoic acid; 7*S*,17*R*/*S*-diHDHA, 7*S*,17*R*/*S*-dihydroxydocosa-4*Z*,8*E*,10*Z*,13*Z*,15*E*,19*Z*-hexaenoic acid; 10,17*R*-docosatriene, 10,17*R*-dihydroxydocosahexaenoic acid; 10,17*S*-docosatriene, 10,17*S*-dihydroxydocosahexaenoic acid; 10*R*/*S*-OCH_3-17*S*-HDHA, 10*R*/*S*-methoxy-17*S*-hydroxydocosahexaenoic acid; 17*R*/*S*-H(p)DHA, 17*R*/*S*-hydro(peroxy)docosa-4*Z*,7*Z*,10*Z*,13*Z*,15*E*,19*Z*-hexaenoic acid; COX-2, cyclooxygenase 2; LC-PDA-MS-MS, liquid chromatography-photodiode array detector-tandem mass spectrometry; LO, lipoxygenase; PMN, polymorphonuclear leukocytes; HPLC, high performance liquid chromatography; EPA, eicosapentaenoic acid; PBS, phosphate-buffered saline; TNF, tumor necrosis factor; IL, interleukin.

bioactive molecules with distinct functions (22). Hence, it is likely that the resolvins and related compounds identified might represent the active products responsible, at least in part, for the many reported beneficial responses obtained in clinical studies with patients given high doses of ω-3 supplementation (see Refs. 6 and 7 and references within).

Another aspect for the many years of sustained interest in DHA lies in the fact that the brain is lipid-rich and that DHA is highly enriched in the membranes of brain synapses and in the retina (16). DHA declines in brain neurons with age and may result in loss in mental function (20). Also, it is now clear that DHA is required for fetal brain development and is held to be critical in the newborn for appropriate development and intelligence (23). Hence, from these and many other recent studies (24), it is now apparent that, in humans, DHA serves a critical role in both physiologic and pathophysiologic responses. Our recent finding that aspirin therapy can lead to the biosynthesis of unique series of 17*R* resolvins generated from DHA led us to question whether the significant roles reported for DHA in the many biological systems noted above were related to DHA conversion to potent local bioactive mediators. The reason for this line of thinking is that in most experimental systems, where the actions of either DHA or EPA were assessed either clinically or in animal models, the concentrations required to evoke beneficial actions are usually in the high microgram to high milligram range. In this context, it is difficult to envision direct and specific molecular actions responsible for the many beneficial outcomes from *in vivo* studies. Thus, with the coordinates (physical and biologic properties) in hand for the new aspirin-triggered 17*R* series resolvins obtained using lipidomics and bioassays (see Ref. 7), we determined in the present experiments whether DHA is converted to bioactive mediators *de novo*. The present results indicate that (*a*) DHA is a precursor to a potent family of bioactive docosanoids that include novel docosatrienes as well as the 17*S* epimer resolvin series generated in human blood cells, mouse brain, and by human glial cells; (*b*) these compounds display potent actions on leukocyte trafficking as well as on glial cell functions down-regulating cytokines expression. Hence, our present results indicate that DHA is a precursor in novel biosynthetic pathways to previously unrecognized potent molecules.

EXPERIMENTAL PROCEDURES

Materials—Zymosan A and soybean lipoxygenase (fraction IV) were purchased from Sigma Co. (St. Louis, MO). Docosahexaenoic acid (C22:6, DHA) was from Cayman Chemical Co. (Ann Arbor, MI); other synthetic standards, hydroxy fatty acids, and intermediates used for MS identification and fragment ion references were purchased from Cascade Biochem Ltd. (Reading, UK). Authentic docosanoids of known hydroxy-containing products from DHA used to characterize physical properties, *i.e.* LC-MS-MS diagnostic ions and MS-MS spectra, were 4*S*-hydroxy-5*E*,7*Z*,10*Z*,13*Z*,16*Z*,19*Z*-docosahexaenoic acid (4*S*-HDHA), and the racemate 17*R*/*S*-hydroxy-4*Z*,7*Z*,10*Z*,13*Z*,15*E*,19*Z*-docosahexaenoic acid (denoted 17*R*/*S*-HDHA) purchased from Penn Bio-Organics (Bellefonte, PA). For each, NMR analyses were used to establish stereochemistry (*i.e.* double-bond configurations). Using recombinant enzymes (*i.e.* COX-2, 5-LO, and others) and additional reactions, we prepared and isolated 10,17*S*-docosatriene, 10,17*R*-docosatriene, 7*S*,17*S*-diHDHA, 4*S*,17*S*-diHDHA, 4*S*,5,17*S*-triHDHA, 4*S*,11,17*S*-triHDHA, 7*S*,8,17*S*-triHDHA, and 7*S*,16,17*S*-triHDHA, similar to (6, 25). Additional materials used in LC-MS-MS analyses were from vendors reported in (6, 25).

Biogenic Synthesis of 17S Series Compounds—The reference materials were prepared by incubation of DHA and soybean lipoxygenase (Type IV) and/or 5-LO from potato using different ratios of substrate to enzyme that gave specific product profiles (see below). Each reaction condition used was optimized according to UV spectrometric monitoring and high performance liquid chromatography coupled with a photodiode-array detector and a tandem mass spectrometer (LC-PDA-MS-MS, ThermoFinnigan, San Jose, CA). In brief, soybean lipoxygenase (260 kilounits, 14 mg of protein/ml, 360 kilounits/mg of protein, Sigma, St. Louis, MO) was incubated with DHA (100 μg) in 10 ml of Dulbecco's PBS (phosphate-buffered saline with Ca^{+2}/Mg^{+2}, pH 8.0) at 4 °C. To obtain 17*S*-hydro(peroxy)-DHA (H(p)DHA), aliquots (2 ml) were removed at the initial 15-min interval. To obtain both di- and tri-HDHA, lipoxygenase (260 kilounits), and/or potato 5-LO was added to the remaining 17*S*-H(p)DHA incubations in one round-bottomed flask held at 2–4 °C. At 15-min intervals, product profiles were assessed using LC-PDA-MS-MS following $NaBH_4$ reduction. The 17*R* series resolvins were prepared as in a previous study (7).

Hemoglobin-catalyzed Conversion of 17-H(p)DHA—17*S*-H(p)DHA was prepared and incubated with hemoglobin (Sigma H7379A) to identify non-enzymatic transformation products. Incubations were carried out according to those used for hemoglobin-catalyzed transformation of 15*S*-hydro(peroxy)eicosatetraenoic acid (H(p)ETE) (26). In brief, 17*S*-H(p)DHA (30 μg/ml) was incubated with human hemoglobin (300 μg/ml) in PBS (pH 7.4, 37 °C) for 15 min. Incubations were terminated by acidification to pH 3.2 and taken for immediate solid-phase extraction (SepPak). Mono-, di-, and trihydroxy docosanoid reaction products were each separated by combined column chromatography (CC7) followed by solid-phase extraction. Products were identified and quantitated by LC-PDA-MS-MS lipidomic analysis.

Human Whole Blood, PMN, and Murine Brain Incubations—Human whole (venous) blood (10 ml) was collected with or without heparin from healthy volunteers (that declined taking medication for ~2 weeks before donation; Brigham and Women's Hospital protocol 88-02642). Following collection, whole blood was immediately incubated with DHA (10μg) in a covered water bath for 40 min (37 °C). Human PMN were freshly isolated from the whole blood by Ficoll gradient and enumerated as in a previous study (7). PMN ($30–50 \times 10^6$ cells/ml) were exposed to zymosan A (100μg/ml, Sigma) followed by addition of either 17*S*-hydroxy-DHA (3 μg/ml) or DHA (3 μg/ml). Cell suspensions were incubated for (37 °C, 40 min) in a covered water bath. For identification of epoxide-containing intermediates, isolated human PMN ($30–50 \times 10^6$ cells/incubation) were incubated with 17*S*-hydro(peroxy)-DHA and zymosan A (100 μg/ml) in Hanks' buffer containing 1.8 mM Ca^{2+} and 10 mM HEPES (pH 7.4, at 37 °C for 15 min). The incubations were immediately poured into 10 volumes of cold acidified (apparent pH 3.1) methanol and kept (30 min, 4 °C) before analysis. Lipidomic and lipid mediator profile analyses were performed as in previous studies (7, 27, 28).

DHA Uptake and Agonist-induced Release of Novel Docosanoids—Human glial cells (DBTRG-05MG, ATCC) were cultured as in a previous study (7). Media were removed from semiconfluent monolayers (~75%) and replaced with Hanks' buffer (37 °C, 10 ml) containing HEPES, 10 mM, pH 7.4, and [1-^{14}C]DHA (55 mCi/mmol, American Radiolabeled Chemicals, Inc., 10 μM), or unlabeled C22:6 (~10 μM, Oxford Biomedical Research) was added in EtOH (5–10 μl). Cells were placed in an incubator with an atmosphere of 5% CO_2 at 37 °C for 90 min, and aliquots (100 μl) were removed at 30-min intervals and analyzed by scintillation counting to determine cellular [1-^{14}C]DHA. At 90 min, buffer was removed and cells were washed with Hanks' buffer (10 ml, 37 °C). Hanks' buffer (10 ml, 37 °C) containing HEPES (10 mM, pH 7.4) and $CaCl_2$ (1.6 mM) was added to the flasks, and cells were exposed to either zymosan A (100 ng/ml), calcium ionophore (A23187, 5 μM), or buffer (with vehicle, 0.01% EtOH) alone. Cells were placed in an incubator with an atmosphere of 5% CO_2 (37 °C for 30 min), and aliquots were removed at initiation and 30 min post agonist addition to assess the cellular release of [^{14}C]DHA and ^{14}C-labeled oxygenated products. After 30 min buffer was removed and immediately added to 2 volumes of MeOH (4 °C) and kept at −80 °C for >30 min. Cells were detached, lysed with 5 N NaOH and neutralized with 1 M Tris (pH 8.0) to quantitate cell-associated 1-^{14}C-labeled DHA and novel docosanoids carrying label. For the experiments aimed at determining the sites for DHA incorporation into major classes of phospholipids, triglycerides, and neutral lipids, incubations (37 °C, 30 min) were stopped with $CHCl_3$/MeOH (2:5, v/v) and cells were detached. Lipids were extracted using the method of Bligh and Dyer (29), materials from the organic phase were suspended in chloroform, and the identity of lipid classes was established with authentic standards and standard procedures (29). Phosphorimaging analysis was used for quantitation of the ^{14}C content and demonstrated that 80–86% of the total cell associated DHA was in phospholipid and ~11–18% in neutral lipid pools (range from two separate experiments). DHA was predominantly acylated into phosphatidylethanolamine (39–41%) and phosphatidylcholine (29–36%), findings that are consistent with studies in human platelets (30), neutrophils (31), and a rat glioma cell line (32). Selected samples were taken for additional analysis and extracted, and methyl formate-eluted products were analyzed by PDA-MS-MS for identification as in Ref. 7.

Reversed-phase HPLC radioprofiles were carried out using a 1100 series liquid chromatography system (Agilent Technologies) as done previously (7, 27). Radioactivity in 60-s fractions was quantified with an LS6500 scintillation counter (Beckman).

Substrate Competition with Human rCOX-2—Human recombinant COX-2 was overexpressed in *Sf*9 insect cells (ATCC). The microsomal fractions (~8 μl) were suspended in Tris (100 mM, pH 8.0) as done previously (33). [1-^{14}C]Docosahexaenoic acid (55 mCi/mmol, American Radiolabeled Chemicals, Inc., 10 μM) was added to rCOX-2 microsomal membrane suspensions followed immediately by arachidonic acid (C20:4, Cayman Chemical) at increasing concentrations or vehicle alone (0.5% EtOH). Suspensions were incubated at 37 °C for 30 min and stopped with a solution (4 °C) containing ether:MeOH:citrate (30:4:1, v/v). The organic phase was collected and analyzed by thin layer chromatography at 4 °C using as a mobile phase the organic fraction of ethyl acetate:2,2,4-trimethylpentane:acetic acid:water (110:50:20:100, v/v) as in a previous study (34). Total and product-specific radioactivity were each quantitated using a PhosphorImager. Human rCOX-2 conversion of DHA (10 μM) to specific products was 95 ± 0.5% (n = 6) and was used as the 100% value to determine the impact of arachidonic acid on conversion of DHA.

Acute Inflammatory Exudates: Murine Dorsal Air Pouch and Peritonitis—In the 6- to 8-week-old male FVB mice (fed laboratory rodent diet 5001 (Lab Diet, Purina Mills) containing 1.49% eicosapentaenoic acid, 1.86% DHA, and <0.25% arachidonic acid, inflammatory exudates were initiated by intra-pouch injection of recombinant mouse TNFα (100 ng/pouch, R&D Systems). Four hours later mice were sacrificed, in accordance with the Harvard Medical Area Standing Committee on Animals protocol 02570, air pouch lavages were collected, and cells were enumerated. Inhibition of TNFα (100 ng/pouch) stimulated PMN infiltration, with intravenous tail injection of *S* series resolvins and docosatrienes (as prepared with biogenic synthesis; see below) was determined with pouch lavages taken at 4 h. To assess the impact of novel compounds in peritonitis, mice (FVB) were anesthetized with isoflurane and docosatrienes, and *S* series and *R* series of resolvins (suspended in 120 μl of saline) were administered intravenously and followed (~1–1.5 min) by an intraperitoneal injection of zymosan A (1 mg) in 1 ml of sterile saline. Two hours after the intraperitoneal injections, mice were euthanized and peritoneal lavages were rapidly collected for enumeration. Cytosensor® analyses were evaluated using a Cytosensor® microphysiometer (Molecular Devices) and computer workstation as in previous work (35, 36). Also, reverse transcription-PCR analyses of TNFα-induced IL1β was carried out according to a previous study (7).

RESULTS

Novel Docosanoids via Lipidomics: Formation of 17S Series Resolvins and Docosatrienes by Human Whole Blood and Murine Brain—Because brain and blood share many biochemical and signaling pathways in common (37, 38), we compared the profiles of bioactive docosanoids produced by murine brain, human blood, and leukocytes to those generated by glial cells. To this end, we first determined whether human blood generates bioactive docosanoids such as the recently described resolvins (7) by examining the transformation of DHA in human whole blood and endogenous products produced by murine brain on addition of agonists and utilizing LC-PDA-MS-MS-based lipidomic analysis. Fig. 1*A* shows representative LC-MS selective ion monitoring for [M-1] at *m/z* 375 for trihydroxy-containing docosanoids and *m/z* 359 for dihydroxy- and *m/z* 343 for monohydroxy-containing docosanoids, respectively. These results demonstrate for the first time that, in addition to docosanoids with physical properties similar to those of the aspirin-triggered 17*R* series resolvins generated in resolving murine inflammatory exudates (7), shown in Fig. 1, human blood converts DHA to 17*S* series resolvins as well as novel dihydroxy-containing docosanoids. LC-MS plots of selective ion chromatograms and mass spectra also showed the presence of novel dihydroxy-containing docosanoids. For example, the MS-MS spectrum of a dihydroxy-containing DHA is reported in Fig. 1*B* with prominent fragment ions consistent with the structure shown in the insert, namely 10,17*S*-dihydroxydocosahexaenoate and ions at *m/z* 359 (M-H), 341 (M-H-H_2O), 323 (M-H-2H_2O), 315 (M-H-CO_2), 297 (M-H-H_2O-CO_2), and 277 (M-H-H_2O-CO_2-2H). Additional diagnostic ions consistent with the carbon 10 and carbon 17 alcohol-containing positions were observed at *m/z* 153, 163, 181, 189, 205, 217 (216-CO_2+H), 243 (261-H_2O+H), 261, and 289. The UV spectrum of this docosanoid gave a maximum absorbance wavelength (λ_{max}) of 269 nm with shoulders at 260 and 279 nm indicating the presence of a conjugated triene chromophore (*i.e.* three conjugated dou-

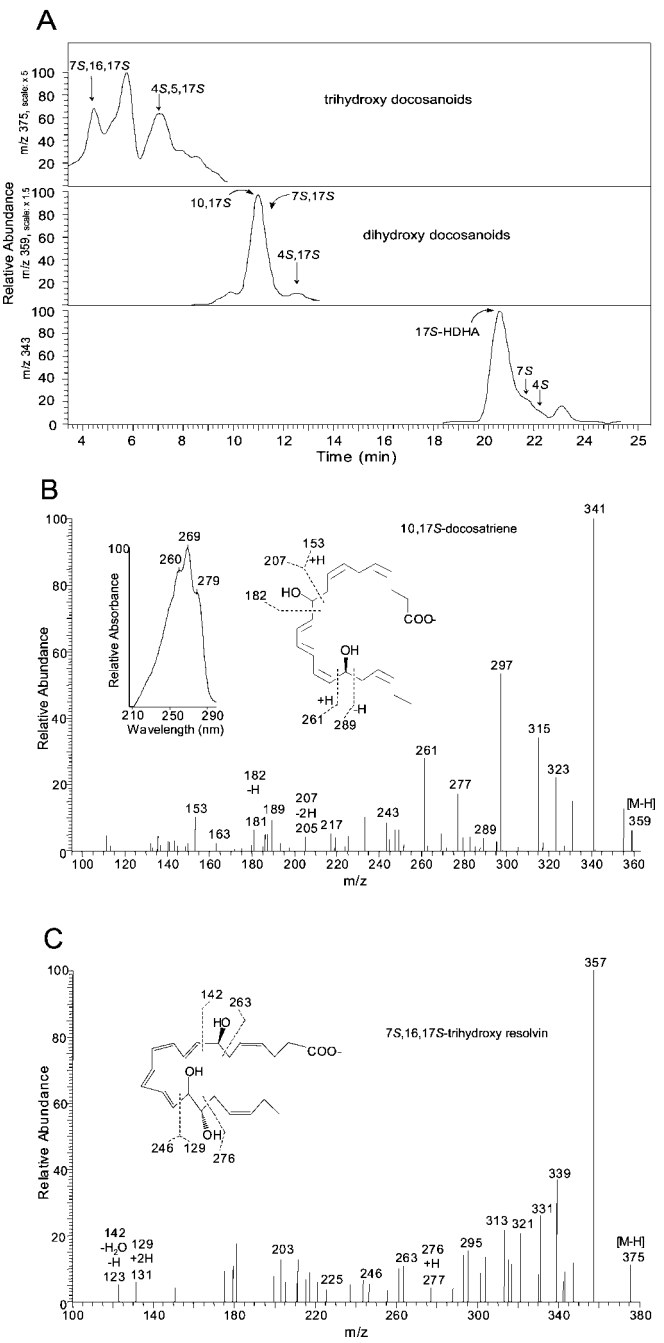

FIG. 1. **Formation of novel docosatrienes and 17*S* series resolvins.** Lipidomic analysis was carried out with heparinized blood incubated (10 ml, 37 °C, 30 min) with DHA and products identified by LC-PDA-MS-MS (see "Experimental Procedures" and text, n = 3). *A*, selected ion chromatograms of trihydroxy-carrying (*m/z* 375, *upper panel*), dihydroxy-carrying (*m/z* 359, *middle panel*), and monohydroxy-carrying (*m/z* 343, *lower panel*) products from DHA. Identification of the ions diagnostic for specific hydroxy group positions present in the trihydroxy, dihydroxy, and monohydroxy products generated are given in the *insets* (see text). *B*, MS-MS and UV (*inset*) spectra of 10,17*S*-docosatriene (depicted with tentative stereochemistry assignments). *C*, MS-MS spectrum of the 17*S* series trihydroxy resolvin: 7*S*,16,17*S*-triHDHA.

ble bonds) in the new structure. The configuration of the alcohol at position 17 was confirmed as being in predominately the *S* configuration using materials prepared with isolated lipoxygenases and DHA as substrate with co-elution experiments (see "Experimental Procedures"). The predominant products, also observed from endogenous sources of DHA in isolated murine brain incubated with ionophore, were 10,17*S*-docosatriene and 4*S*,17*S*-diHDHA ($n = 3$, not shown). Taken together with the chromatographic behavior, these physical properties were consistent with the formation of 10,17*S*-diHDHA (docosatriene), a novel carbon 17 position-oxygenated product formed from DHA.

We questioned whether these 17-hydroxy-carrying docosanoids from human whole blood and murine brain could be formed via 15-lipoxygenation, which is an enzymatic activity in human leukocytes and is induced during the resolution phase of acute inflammation (39) and whether these new compounds were biosynthetically interrelated. Along these lines, earlier results with isolated soybean 15-lipoxygenase showed that this enzyme can rapidly transform DHA to 17*S*-HDHA *in vitro* (34) that were confirmed during the course of the present experiments with isolated enzyme and LC-PDA-MS-MS analyses ($n > 12$, not shown). Results shown in Fig. 1 demonstrate that human whole blood generated 17*S*-hydroxydocosahexaenoic acid (λ_{max} of 237 nm and diagnostic MS-MS ion *m/z* 245) from DHA. This human product of whole blood was matched in physical properties to those of authentic 17*S*-hydroxydocosahexenoate produced via biogenic synthesis (see "Experimental Procedures"). In addition, a novel product 7*S*,16,17*S*-trihydroxydocosahexaenoic acid was uncovered that exhibited an MS-MS spectrum (Fig. 1*C*) with diagnostic ions at *m/z* 123 (142-H_2O-H), 131(129 + 2H), 203 (246-CO_2-H), 225 (263–2H_2O-2H), 246, 263, 277 (276+H), 295 (M-H-2H_2O-CO_2), 313 (M-H-H_2O-CO_2), 321 (M-H-^3H_2O), 331 (M-H-CO_2), 339 (M-H-2H_2O), 357 (M-H-H_2O-CO_2), and 375 (M-H). The presence of this 7*S*,16,17*S*-trihydroxy-containing docosanoid suggested a pathway for its formation (see below) similar to the generation of the anti-inflammatory eicosanoid lipoxin B_4, a 5-lipoxygenase interaction product generated from arachidonic acid (*e.g.* 5*S*,14,15*S*-trihydroxyeicosatetraenoic acid), and the newly described 17*R* epimer termed 7*S*,16,17*R*-trihydroxydocosahexaenoic acid-resolvin (as in 7). Of interest, the 17*R* series and 17*S* series of di- and tri-hydroxy products appear to give different chromatographic retention times (see "Experimental Procedures").

Lipidomic-based analysis employing LC-PDA-MS-MS also identified the presence of distinct docosanoids that were consistent with the presence of and conversion of DHA by both lipoxygenase and cyclooxygenase-like enzymatic activities that act on DHA present in whole blood (Fig. 1*A*). In these analyses, we also identified 13-HDHA (UV λ_{max} 237 nm and diagnostic ion *m/z* 221), a potential cyclooxygenase-2-derived product from DHA (Ref. 7 and data not shown). Along with these compounds shown in Fig. 1*A*, we also observed LC-MS and MS-MS spectra consistent with the production of both 11-HDHA and 14-HDHA the known 12-lipoxygenase products reported earlier (17, 40), as well as the 4*S*- and 7*S*-hydroxy-containing products of DHA that can be generated by a 5-lipoxygenase-like reaction as exemplified by the potato 5-lipoxygenase enzyme (41, 42). It is noteworthy that both clotted whole blood (non-heparinized) as well as heparinized blood generated the identified monohydroxydocosanoid profile (Fig. 1). In contrast, the dihydroxy products from DHA, including the novel docosatriene (10,17*S*-diHDHA), were not observed with clotted whole blood (data not shown). In addition, incubation of 17*S*-hydro(peroxy)-DHA with hemoglobin as used with 15-H(p)ETE conversion (26) produced several new non-enzymatic epoxy alcohols and trihydroxydocosanoids (*i.e.* 16,17*S*-epoxy-15-hydroxydocosapentaenoic acid (16,17*S*-epoxy-15-HDPA), 13,14-epoxy-17*S*-hydroxydocosapentaenoic acid (13,14-epoxy-17*S*-HDPA), 16,17*S*-epoxy-13-hydroxydocosapentaenoic acid (16,17*S*-epoxy-13-HDPA), 15,16-epoxy-17*S*-hydroxydocosapentaenoic acid (15,16-epoxy-17*S*-HDPA), 13,16,17*S*-tri-hydroxydocosapentaenoic acid (13,16,17*S*-HDPA), 15,16,17*S*-tri-hydroxydocosapentaenoic acid (15,16,17*S*-tri-HDPA), 13,14,17*S*-tri-hydroxydocosapentaenoic acid (13,14,17*S*-tri-HDPA); see "Experimental Procedures"). These compounds were not formed in substantial quantities in whole blood (see below) but may be relevant in other pathophysiologic scenarios. It is likely that they were not formed in substantial amounts in these incubations, because the 17*S*-H(p)DHA, once formed, remained intracellular, limiting its ability to interact with heme proteins in a non-selective fashion. The bioactions of these will be reported elsewhere. Together these results indicated that human blood and murine brain converts DHA to several previously unappreciated novel di- and tri-hydroxy-containing docosanoids. Some of these new docosanoids resembled the physical properties of the recently identified 17*R* series resolvins (7), namely those denoted here as their 17*S* carbon position, *e.g.* hydroxy epimers or the 17*S* series resolvins.

A Role for Cell-Cell Interactions in the Formation of 17S Series Resolvins and Docosatrienes—Human PMN were exposed to 17*S*-H(p)DHA to determine if the likely intermediates in the formation of 17*S* series resolvins and docosatrienes were transformed by leukocyte-associated lipoxygenase-like activity (*i.e.* 5-LO, 15-LO) to the novel dihydroxy- and trihydroxy-containing docosanoids identified in blood and murine brain (see Fig. 1). Indeed, PMN freshly isolated from peripheral blood, transformed 17*S*-hydro(peroxy)docosahexaenoic acid to two distinct 5-lipoxygenase products 7*S*,17*S*-diHDHA and the less prominent product 4*S*,17*S*-diHDHA as indicated in the selective ion chromatogram plotted at *m/z* 359 (Fig. 2*A*). The 4*S*,17*S*-diHDHA was identified based on the MS-MS spectrum that displayed diagnostic ions at *m/z* 101, 257, 239 (257-H_2O), 261, 267, 287, 245 (290-CO_2-H), 359 (M-H), 341 (M-H-H_2O), 315 (M-H-CO_2), 297 (M-H-CO_2-H_2O), and 277 (M-H-CO_2-2H_2O-2H) and on its UV spectrum that gave a λ_{max} 234 nm revealing its conjugated diene structure (Fig. 2*B*, *inset*). In addition to these dihydroxydocosanoids, the MS-MS and UV spectral analysis also confirmed the presence and formation of the novel docosatriene 10,17*S*-diHDHA (see Fig. 2*A*), results that are consistent with spectra obtained from murine brain and human whole blood lipidomic analysis (Fig. 1). The apparent ratio for 10,17*S*-docosatriene to the other main dihydroxy product 7,17*S*-diHDHA generated by human PMN was ~1.12 (calculated based on the analysis of UV chromatographic peak areas at their respective λ_{max} 269 and 242 nm using recorded on line PDA spectra). Further MS-MS and UV spectral analysis revealed the formation of ω-22-hydroxy-10,17*S*-docosatriene (Fig. 2*C*), a likely ω-oxidation product of the 10,17*S*-docosatriene in human leukocytes. Human leukocytes and several other tissues possess a prominent ω-oxidation apparatus that can rapidly inactivate local bioactive lipid mediators (25, 43). The structure of ω-22,10,17*S*-docosatriene was determined by analysis of the MS-MS spectrum (Fig. 2*C*). This compound gave ions of diagnostic value at *m/z* 375 (M-H), 357 (M-H-H_2O), 343 (344-H), 339 (M-H-H_2O), 331 (M-H-CO_2), 324 (344-H_2O-2H), 309 (344–2H_2O+H), 300 (344-CO_2), 295 (M-H-2H_2O-CO_2), 273 (290-H_2O+H), 265 (344–2H_2O-CO_2+H), 261 (260+H), 217 (260-CO_2+H), 193, 181, and 163 (181-H_2O). Taken together these findings suggest transformation of 17H(p)DHA via two prominent and distinct metabolic pathways to generate 5-LO-

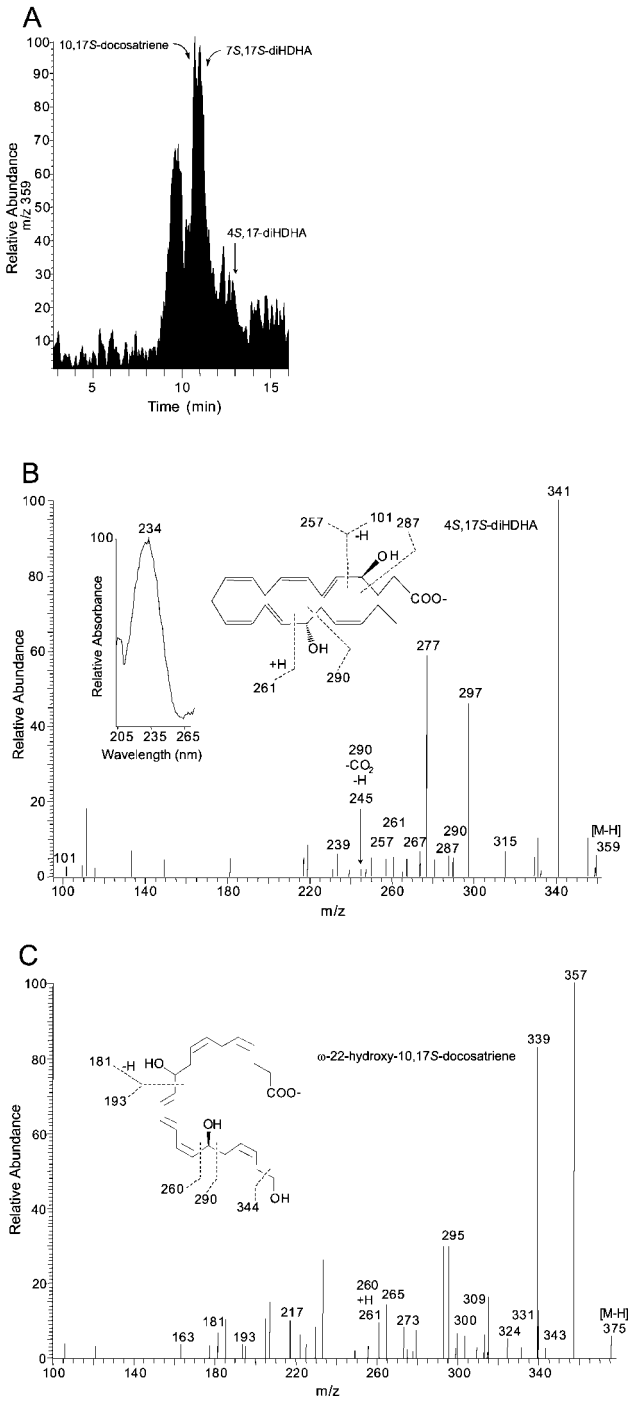

FIG. 2. **Human PMN produce both 17*S* series resolvins and docosatrienes.** Human neutrophils (30–50 × 10^6 cells/incubation) were exposed to zymosan A and 17*S*-H(p)DHA, and products were analyzed using LC-PDA-MS-MS (see "Experimental Procedures," n = 5). *A*, selected ion chromatogram (*m/z* 359) for dihydroxy-DHA products. *B*, MS-MS and UV (*inset*) spectra of the 17*S* series resolvin 4*S*,17*S*-diHDHA. *C*, MS-MS of ω-22-hydroxy-10,17*S*-docosatriene. Identification of 17*S* series diHDHAs and 10,17*S*-docosatriene are indicated with tentative stereochemistry assignments.

like interaction products (*i.e.* the double dioxygenase products 4*S*,17*S*-diHDHA and 7*S*,17*S*-diHDHA) and the novel 10,17*S*-docosatriene.

Human Glial Cells Generate and Release Novel Docosanoids: Docosatrienes—Next, we determined whether the *S* series resolvins and docosatrienes were formed from cellular stores of DHA and if they could also be produced by a single cell type. To this end, we labeled human glial cells with [1-^{14}C]DHA. As anticipated, glial cells rapidly incorporated labeled DHA as indicated by a statistically significant increase in total radiolabel associated with cellular stores ($p > 0.05$, $n = 3$). Radioactivity associated with cellular stores increased from 35 ± 8% at $t = 30$ min to 51 ± 3% at $t = 60$ min and 61 ± 4% at $t = 90$ min post addition of [1-^{14}C]DHA ($n = 3$) indicating that cellular uptake of labeled DHA was time-dependent. Because glial cells are involved in host defense and inflammation in neural tissues, we exposed them to the microbial product, zymosan A, as well as a non-selective stimulus for cellular calcium (calcium ionophore, A23187) to test whether they were agonists for the formation of novel docosanoids from cellular DHA stores. Activation of glial cells induced a statistically significant release ($p > 0.05$, $n = 4$) of cell-associated label as well as formation of the novel docosanoids. Results in Fig. 3*A* indicated release of ~4.1 ± 1.1% of total cellular DHA following exposure to zymosan A and ~5.6 ± 2.4% with calcium ionophore when directly compared with glial cells that were exposed to vehicle alone (1.6 ± 0.6%). Reversed-phase-HPLC radioprofiles demonstrated that zymosan A challenge of glial cells released several distinct 1-^{14}C-labeled compounds from cellular DHA stores (see *inset* of Fig. 3*A*). In addition to distinct docosanoids that carried radioactivity in the profile there was also label associated with material that corresponded to the retention time of native DHA that accounted for 74 ± 6% ($n = 3$) of the total released radioactivity. Exposure of human glial cell to either zymosan A or calcium ionophore gave agonist-dependent and selective loss of [1-^{14}C]DHA from phosphatidylethanolamine that ranged from 13 to 28% and from 3 to 22%, respectively (see Fig. 3*A* and "Experimental Procedures").

To identify the endogenous docosanoids formed from cellular stores of DHA in human glial cells, we analyzed the released DHA-derived products by LC-PDA-MS-MS based lipidomics (as in Fig. 1). Again, activation of glial cells with either calcium ionophore (Fig. 3*B*) or zymosan A stimulated the formation (not shown) of novel 17-lipoxygenation product 10,17*S*-diHDHA as well as its ω-oxidation product 10,17*S*,22-triHDHA (note that both were also produced in blood; see Fig. 1). The structure of the ω-22 product was determined by analysis (at *m/z* 375) of the MS-MS spectrum (Fig. 3*C*). The material beneath the peaks corresponded to 10,17*S*-docosatriene and 17*S*-HDHA (not shown) and gave MS-MS and UV spectra essentially identical to the 17*S*-oxygenation products of DHA that were detected with human whole blood (see 10,17*S*-docosatriene in Fig. 1*B*). Of the theoretically possible multiple isomers, only one major peak eluted 10,17*S*-docosatriene produced by glial cells (see "Experimental Procedures"). The ω-22-hydroxy-4,17-diHDHA was also formed as shown in the LC-MS selective ion chromatogram at *m/z* 375 (Fig. 3*C*). This structure was consistent with the MS-MS (at *m/z* 375) and prominent ions of diagnostic value at *m/z* 216 (260-CO_2-H), 236 (273–2H_2O-H), 247 (303-$^3H_2$O-2H), 272 (290-H_2O), 273, 293 (M-H-2H_2O-CO_2-2H), 300 (344-CO_2), 303, 313 (M-H-H_2O-CO_2), 337 (M-H-2H_2O-2H), 345 (344+H), 357 (M-H-H_2O), and 375 (M-H) together with the UV spectrum (λ_{max} 238 nm, Fig. 3*C*, *inset*). The formation of ω-22-hydroxy-4,17-diHDHA suggests conversion of 17*S*-H(p)DHA by a 5-lipoxygenase-like mechanism that can oxygenate DHA at the carbon 4 position as shown for DHA to give 7*S*- or 4*S*-HDHA (41, 42). Hence, with 17*S*-H(p)DHA as substrate, this LO-like reaction can generate 4*S*,17*S*-diHDHA that is subsequently ω-oxidized at carbon 22.

To assess the potential pathways involved in the formation of these new compounds, human glial cells were exposed to exogenous DHA in the presence of calcium ionophore. Lipidomic-based analysis using LC-PDA-MS-MS as shown in Fig. 4 yielded products consistent with the presence of 15-LO, 12-LO,

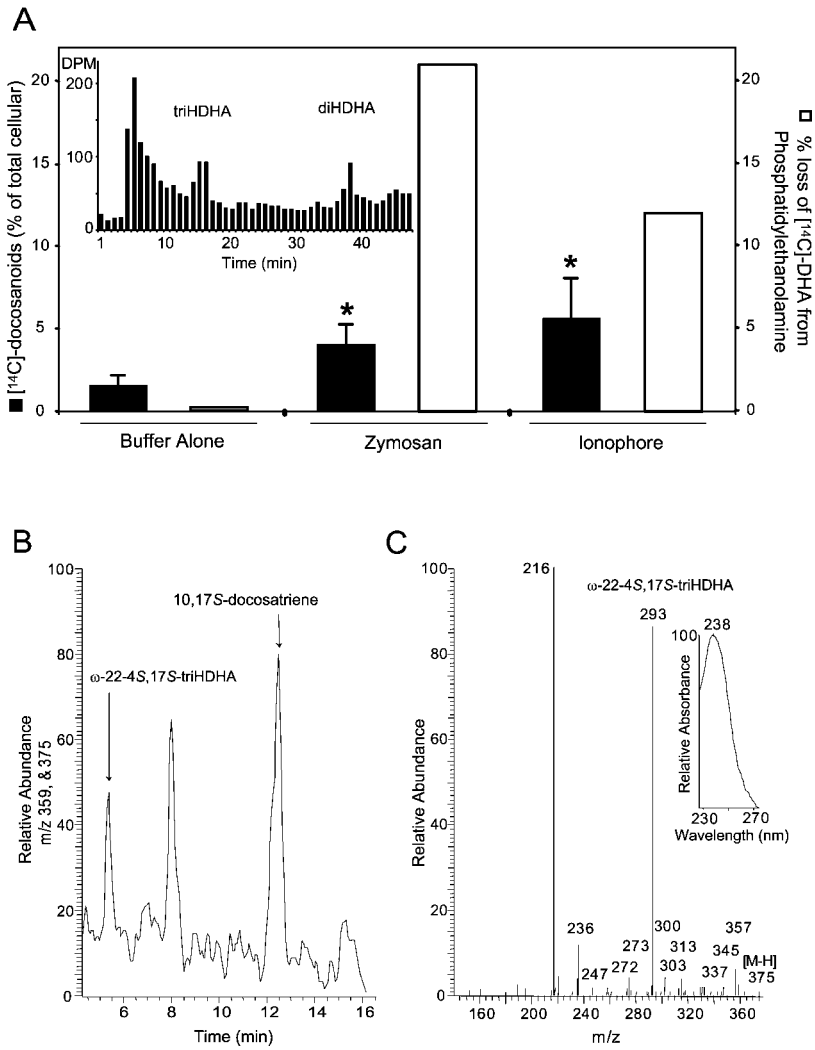

FIG. 3. **Human glial cells release and transform DHA.** *A*, human glial cells were labeled with [1-^{14}C]DHA and exposed to either PBS alone, zymosan A, or calcium ionophore (see "Experimental Procedures"). Released [1-^{14}C]docosanoids were quantified (n = 4). The *asterisk* denotes significant differences from control (PBS alone), $p < 0.05$ analysis of variance, Newman-Keuls test. The *inset* shows a representative HPLC radiochromatogram of released [1-^{14}C]docosanoids (see "Experimental Procedures") from glial cells exposed to zymosan A (n = 3). Phospholipids from glial cells were identified, and the ^{14}C content in phosphatidylethanolamine (*PE*) was determined. The percent loss in PE ^{14}C content from cells exposed to agonists was determined (n = 2). *B*, lipidomic profile of human glial cells exposed to Ca^{2+} ionophore: LC-MS total ion chromatogram of released products. *C*, LC-MS selective ion chromatogram of released DHA-derived products from glial cells exposed to calcium ionophore. MS-MS and UV spectra of ω-22-hydroxy-4*S*,17*S*-diHDHA.

5-LO, and COX-2 activity in glial cells. This was further supported by the formation of stereospecific oxidation products that were also identified in murine brain and blood (see text and Fig. 1). Formation of double oxygenation products 7*S*,17*S*-diHDHA and 4*S*,17*S*-diHDHA was confirmed by MS-MS and UV spectra that proved to be identical to those obtained with human whole blood (Fig. 1). The formation of ω-22,16,17*S*-docosatriene was confirmed by MS/MS (at m/z 375) spectrum (Fig. 4*B*). Its spectrum showed ions at m/z 309 (344–$2H_2O$+H), 345 (344+H), 217 (260-CO_2+H), 243 (260-H_2O+H), 259 (260-H), 271 (290-H_2O-H), 111 (145–$2H_2O$+2H), 143 (145–2H), 231, 277 (M-H-^3H_2O- CO_2), 295 (M-H-$2H_2O$-CO_2), 311 (M-H-H_2O-CO_2-2H), 331 (M-H -CO_2), 339 (M-H-$2H_2O$), 357 (M-H-H_2O), and 375 (M-H). These results suggested formation of a 16,17*S*-epoxide-containing intermediate that opens to form the diol 16,17*S*-diHDHA (see below), which is subsequently transformed by ω-oxidation to give the trihydroxy-containing product.

Substrate Competition with Human rCOX-2—Human COX-2 generates stereospecific oxygenated fatty acids from multiple substrates such as eicosatetraenoic acid, eicosapentaenoic acid (6), and docosahexaenoic acid (7). These lipid mediators as potential COX-2 products are of interest, because COX-2 may regulate homeostasis in the brain, kidney, and digestive tract as well as being potentially linked to inflammatory diseases, fetal development, and carcinogenesis (44). 13-HDHA, a COX-2 product, was identified using LC-MS-MS-based lipidomic analyses in both blood and human glial cell incubations. We investigated the impact of arachidonic acid, the COX-2 substrate for prostaglandins and 15-epi-lipoxin formation (45), on docosahexaenoic conversion by human recombinant enzyme. COX-2 rapidly transformed docosahexaenoic acid to specific docosanoids that were determined using both thin layer chromatography and LC-PDA-MS-MS. The percentage of docosahexaenoic acid transformation at 10 μM substrate by rCOX-2 to specific products mainly 13-hydroxydocosanoid was 94.6 ± 0.5% (n = 3, d = 2 in each). This conversion of DHA was inhibited in a concentration-dependent fashion when exposed to increasing concentrations of arachidonic acid (Fig. 5), which reached 70 ± 15% inhibition at 1 mM arachidonic acid (n = 3). These results indicate that DHA is an effective substrate for oxygenation and can compete for the enzyme's active site *in vitro*. Hence, these findings suggest that within cells or tissues the rate-limiting step for DHA oxygenation is substrate availability. They do not, however, preclude the involvement *per se* of as yet to be defined enzymes or ones that can specifically act on DHA to produce bioactive local mediators (see below).

Anti-inflammatory Properties of 17S Series Resolvins and Docosatriene: Inhibition of PMN Recruitment and Cytokine Gene Expression—To determine whether the 17*S* series compounds in the 17*S* series carry biologic properties, we tested their topical and systemic actions in established *in vivo* models of acute inflammation (Fig. 6*A*). The novel docosanoids proved to be potent inhibitors of TNFα-induced leukocyte trafficking to the air pouch both via topical and systemic application. The 17*S* series resolvins reduced PMN within the exudates by

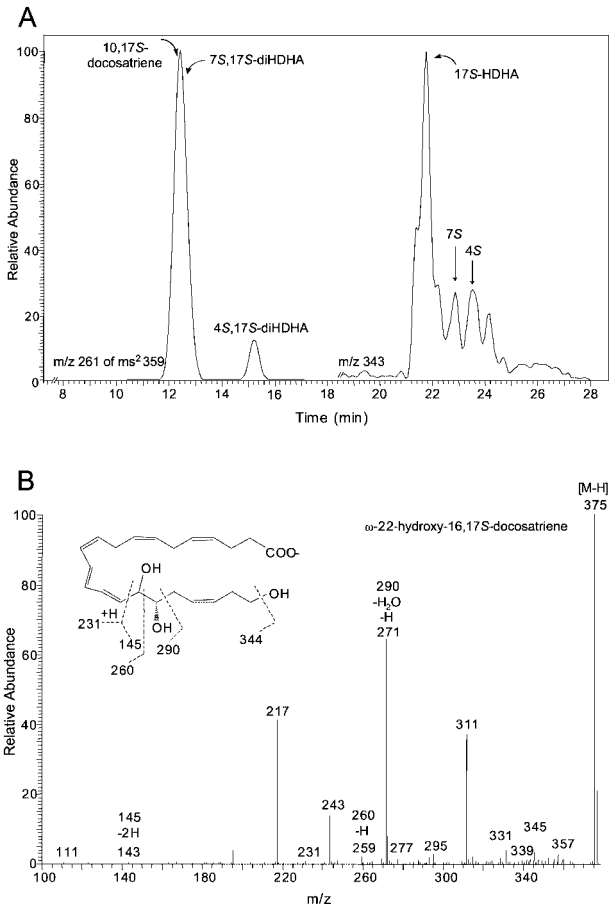

FIG. 4. **Glial cells generate 17*S* series resolvins and 10,17*S*-docosatriene.** Human glial cells were exposed to DHA and calcium ionophore (37 °C, 30 min). The docosanoids were identified by LC-PDA-MS-MS analysis. *A*, LC-MS-selective ion chromatograms showing generation of specific mono-HDHAs (*right*) and di-HDHAs (*left*). *B*, MS-MS of ω-22-hydroxy-16,17*S*-docosatriene.

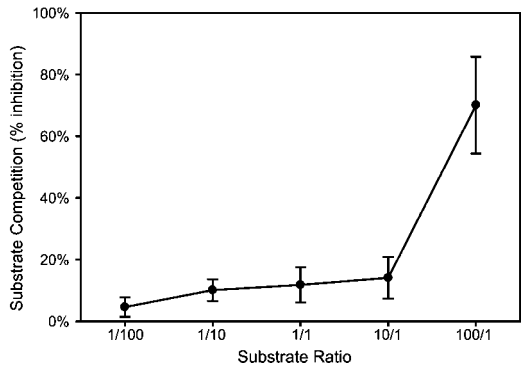

FIG. 5. **Substrate competition at human recombinant COX-2.** Human rCOX-2 microsomal membranes from Sf9 cells were incubated with [1-^{14}C]DHA (C22:6, 10 μM) in the presence of increasing concentrations of unlabeled arachidonic acid (C20:4). COX-2 conversion of [1-^{14}C]DHA and specific product profiles were quantitated (n = 3). rCOX-2 activity is expressed as the inhibition of [^{14}C]C22:6 product formation (95 ± 0.5%; conversion of DHA was set to 100%).

82.2 ± 5.6% given topically and by 49.6 ± 8.2% when given intravenously (n = 4). Systemic treatment with the 17*S* series resolvins also inhibited zymosan A-induced PMN recruitment to the peritoneum by 45.1 ± 0.8% (n = 4), a value that was equipotent to the recently described 17*R* series resolvins (7). The direct side-by-side comparison showed that the 17*R* series resolvins inhibited PMN recruitment by 42.6 ± 8.0% (Fig. 6*A*). Treatment with the isolated novel docosatriene (10,17*S*-diHDHA), a member of the 17*S* series (see Fig. 8), proved to be a potent systemic inhibitor of PMN recruitment as evidenced by its ability to abate PMN in the peritoneal exudates by ~42%. In this regard, it proved to be as potent as indomethacin, a well-characterized non-steroidal anti-inflammatory drug (46), which reduced PMN peritoneal in exudates by ~40% (Fig. 6*A*).

Properties with Glial Cells—The 17*S* series DHA compounds were also evaluated as potential regulators of cytokine-induced gene expression in the human glioma cells (Fig. 6*B*). Semi-qualitative reverse transcription-PCR analyses demonstrated that both the 17*S*-HDHA and 10,17*S*-docosatriene inhibited TNFα-induced IL-1β transcript levels and gave a dose-dependent inhibition with an IC_{50} of ~0.5 nM (Fig. 6*B*). Analysis using microphysiometry can give a rapid real-time indication of receptor ligand-operated cellular events (47). To determine if the 17*S* series docosanoids interact with recognition sites on human glial cells, we evaluated whether these novel lipid mediators could evoke ligand-operated extracellular acidification using a four-channel Cytosensor®. Results shown in Fig. 6*C* demonstrate that the novel 10,17*S*-docosatriene and a mixture of both 17*S* series docosanoids each added to the cells separately evoked rapid ligand-specific extracellular acidification ($p < 0.03$). In sharp contrast, the precursor DHA did not evoke extracellular acidification. These findings are consistent with the ability of both 17*S*-HDHA and 10,17*S*-docosatriene to regulate gene expression in human glial cells (Fig. 6*B*) and suggest the presence of recognition sites for 17*S* series docosanoids in human glial cells.

Trapping of Epoxide Intermediates—To evaluate the formation of potential epoxide-containing intermediates during the biosynthesis of 17*S* series resolvins and docosatrienes, we exposed PMN to excess acidic alcohol and analyzed the extracted materials for the presence of methoxy-trapping products as in previous work (28, 48, 49) that could be formed as direct marker of epoxide-containing biosynthetic intermediates using LC-PDA-MS-MS. Results shown in Fig. 7 demonstrate formation of two prominent 16-methoxy-17*S*-hydroxydocosahexaenoate isomers, denoted *Ia* and *Ib*, as well as two 10-methoxy-17*S*-hydroxydocosahexaenoate isomers, denoted *IIa* and *IIb* in Fig. 7*A*. The 16-methoxy-containing trapping products were identified based on the UV and MS-MS (at *m/z* 373) spectral analysis (Fig. 7*B*) of the corresponding LC peaks in Fig. 7*A*. The presence of a chromophore with a triple band of absorbance at λ_{max} 270 nm with shoulders at 261 and 281 nm (see Fig. 7*B*, *inset*) and diagnostic MS-MS ions of *m/z* 355 (M-H-H_2O) and 329 (M-H-CO_2), 230, 143, 275 (274+H), 241 (274-MeOH-H), 304, 271 (304-MeOH), and 210 (304-H_2O-MeOH-CO_2) were consistent with the racemic alcohol-trapping products at position 16 denoted a and b isomers, namely, 16*R/S*-methoxy-17*S*-hydroxy-docosahexenoic acid. The 10-methoxy-containing trapping products were also identified by the presence of chromophores that gave a λ_{max} 270 nm with shoulders at 260 and 281 nm (see *inset* in Fig. 7), together with diagnostic ions present in their MS/MS at *m/z* 355 (M-H-H_2O), 341 (M-H-MeOH), 329 (M-H-CO_2), 323 (M-H-H_2O-MeOH), 311 (M-H-H_2O-CO_2), 297 (M-H-MeOH-CO_2), 279 (M-H-H_2O-MeOH-CO_2), 152, 177, 203 (221-H_2O), and 119 (196-H-MeOH-CO_2), 304, 275 (274+H), and 227 (304-H-MeOH-CO_2). The results of LC-PDA-MS-MS analysis also uncovered the presence of low levels of 4*S*-OH-, 11-methoxy-, 17*S*-OH-DHA (data not shown). The identification of these major methoxy-trapping products indicated that both a 16,17-epoxide-containing intermediate and 4,5-epoxide intermediate were generated from 17*S*-H(p)DHA by human PMN. The nucleophilic addition of excess MeOH in acidic conditions to form two main groups of methoxy- derivatives at carbon position 10 and/or 16 position is consistent with open 16,17-epoxy and 4,5-epoxy rings and is in

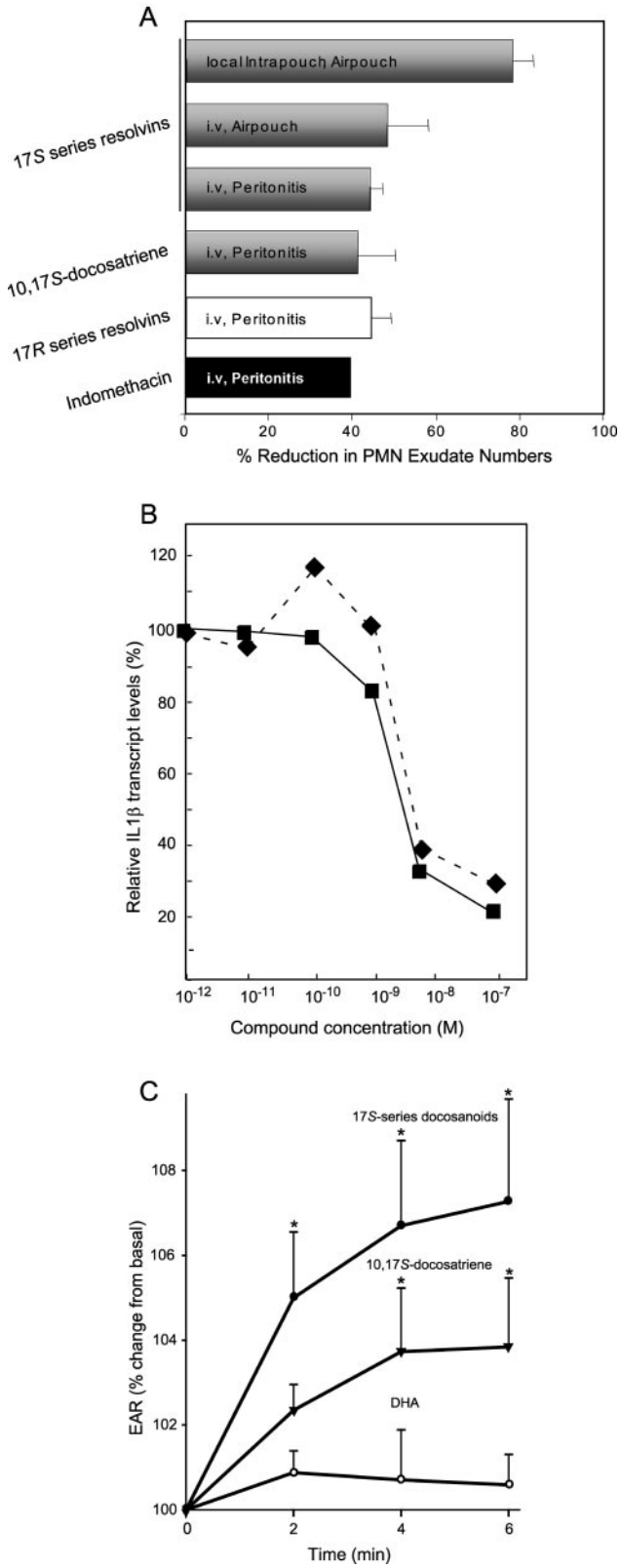

FIG. 6. **Anti-inflammatory properties of 17*S* series resolvins and 10,17*S*-docosatriene.** *A*, inhibition of PMN infiltration in murine peritonitis and dorsal skin pouch following systemic and topical application. Peritonitis: 17*S* series docosanoids (100 ng) or indomethacin (100 ng) was injected intravenously into mouse tails followed by zymosan A into the peritoneum. Mice were sacrificed, and peritoneal lavages were collected (2 h) and cells enumerated ($n = 4$). Air pouch, 17*S* series compounds (100 ng) were injected intrapouch or intravenously followed by intrapouch injection of TNFα. For direct comparison, mice were treated with 17*R* series resolvins via intrapouch injection followed by TNFα. At 4 h, air pouch lavages were collected and enumerated. Values represent mean ± S.E. from three to four different mice; all gave $p < 0.05$ when infiltrated PMN were compared with vehicle control. *B*, inhibition of TNFα-stimulated IL1β transcripts in human glial cells. Human glial cells were stimulated for 16 h with TNFα in the presence of specified concentrations of 17*S*-HDHA (*solid line*) or 10,17*S*-docosatriene (*dotted line*), and expression of IL-1β transcripts was analyzed. Data are representative of $n = 2$. *C*, glial cells: 17*S* series docosanoids evoke ligand-operated extracellular acidification. Changes in extracellular acidification rates (EAR) were analyzed using Cytosensor® microphysiometry. Cells were superfused (100 μl/60 s) with DHA, 17*S* series docosanoids ($n = 3$), or 10,17*S*-docosatriene ($n = 4$). Values are expressed as EAR (μV/s) normalized to baseline (100%), and ligands were added at 2 min.

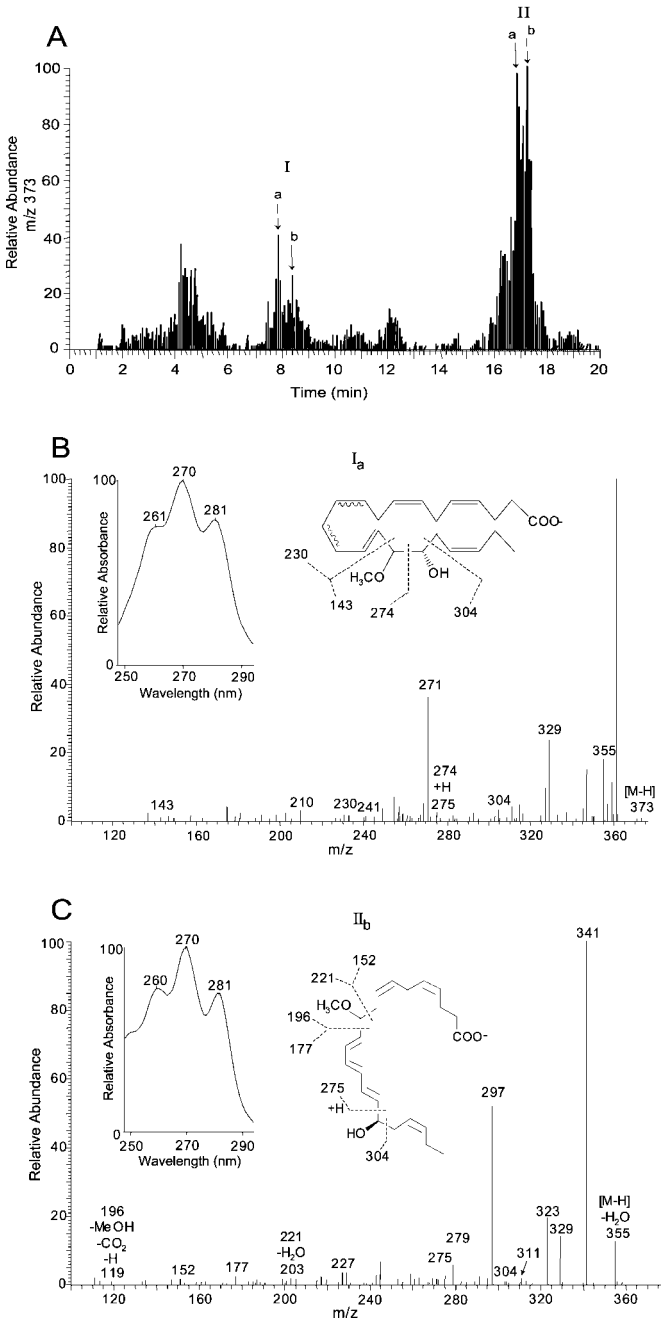

FIG. 7. **Epoxide-containing intermediates in the biosynthesis of docosatrienes and 17*S* series resolvins.** PMN ($30–50 \times 10^6$ cells/incubation) were incubated with 17*S*-hydroperoxy-DHA and zymosan A and stopped, and alcohol-trapping products were extracted. *A*, LC-MS chromatogram obtained from selective ion monitoring for methoxy-trapping products at *m/z* 373 shows two 16-OCH_3 (*Ia* and *Ib*) and two 10-OCH_3 (*IIa* and *IIb*) isomers. *B*, MS-MS and UV (*inset*) spectra of 16-OCH_3 product I; *C*, MS-MS and UV (*inset*) spectra of 10-OCH_3 product II.

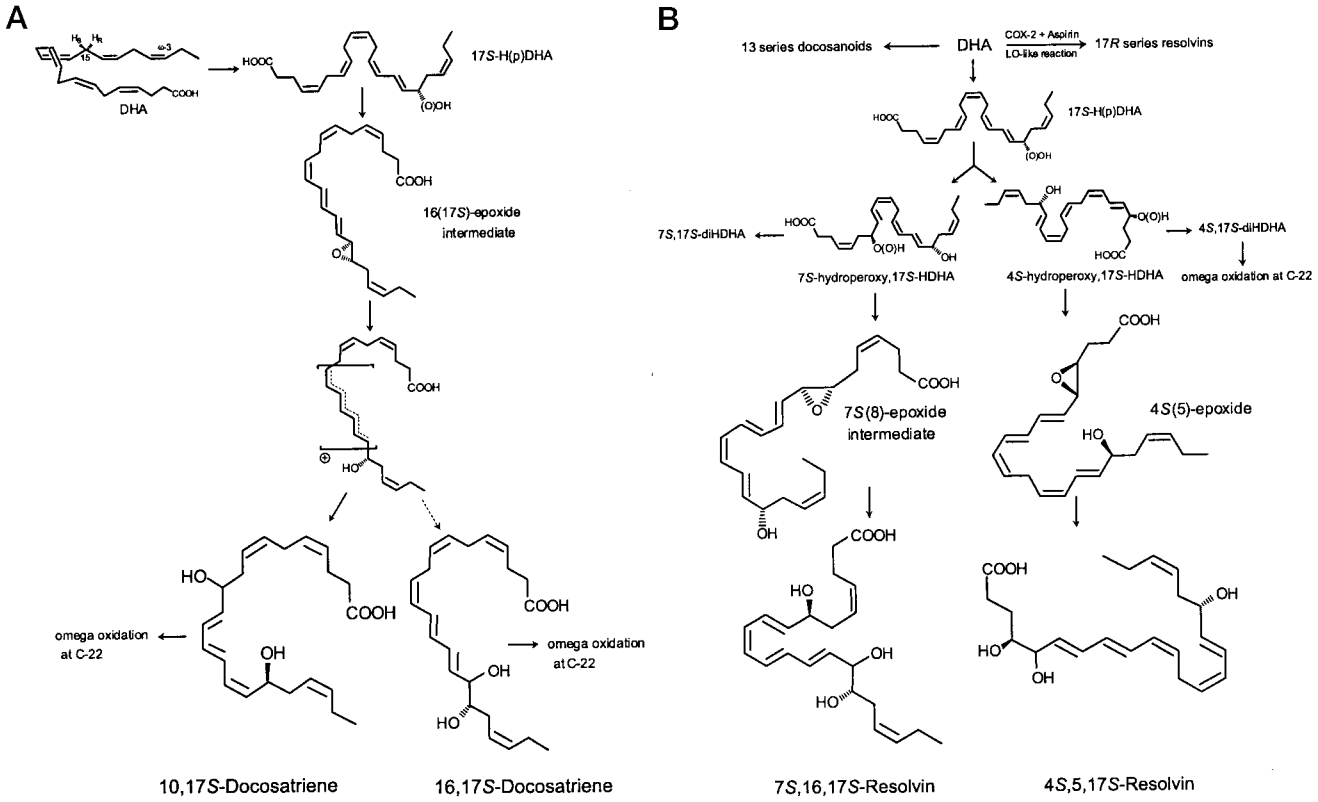

FIG. 8. **Biosynthetic scheme proposed for 10,17*S*-docosatrienes and 17*S* series resolvins.** These transformations may involve novel enzymes in addition to those that serve in the biosynthesis of eicosanoids (see text for further detail). The complete stereochemistry for the docosatrienes (*A*) and 17*S* series resolvins (*B*) remain to be established and are depicted in their tentative configurations based on biogenic total synthesis, lipidomic analyses, and alcohol trapping profiles. The carbonium cation intermediate shown in *A* is also likely to be the enzymatic basis of the epoxide conversion in *B*.

agreement with the involvement of specific epoxy intermediates as those involved in leukotriene and lipoxin generation from arachidonic acid (28, 48, 49).

DISCUSSION

The present results indicate that DHA is a precursor to bioactive products, the basic structures of which were established, and the presence of previously undisclosed pathways in mammalian cells to convert DHA to potent endogenous compounds that display anti-inflammatory properties. Results of alcohol trapping experiments provide evidence for a 16(17)-epoxide intermediate generated from the 17*S*-hydroperoxy precursor in both human glial cells and leukocytes. Taken together with the results in Figs. 1–7, they support the scheme proposed in Fig. 8. In single cell-type systems, as for example, in glial cells, agonists stimulate the release of unesterified DHA predominantly from phosphatidylethanolamine (Fig. 3), which is converted via a lipoxygenase-like reaction to 17*S*-hydroperoxy derivative, which is further transformed to the *trans*-16(17)-epoxide-containing intermediate (Fig. 8*A*).

This *trans* epoxide intermediate can undergo aqueous hydrolysis via a carbonium cation intermediate to produce 10,17*S*-docosatriene carrying a conjugated triene and the 17 position in the *S* configuration retained from 17*S* or the vicinol diol triene (Fig. 8*A*). Once the epoxide intermediate at the 16(17) position is produced via a second hydrogen abstraction mechanism, which appears to be similar to that established for human lipoxygenases and related enzymes such as leukotriene A_4 hydrolase (see Refs. 43, 50–52), it gives a carbonium cation intermediate that can then be converted to the dihydroxy docosatrienes (Fig. 8*A*). Once produced, these dihydroxy-containing docosatrienes exert their actions, in the present studies preventing leukocyte infiltration and down-regulating the production of inflammatory cytokines.

The lipoxygenase product 17*S*-H(p)DHA is converted to 16(17)-epoxide, which, if generated enzymatically, is likely to be a *trans* epoxide by analogy with the formation of leukotriene A_4 (48, 51, 52). If 10,17-docosatriene is generated via non-enzymatic aqueous hydrolysis of the 16(17)-epoxide, the products would be likely to yield the position 10 racemates (10*R*/*S*) with all-*trans* double-bond configurations for the resulting conjugations. We have observed, in human cells and whole blood, production of only one dominant 10,17-dihydroxy product, indicating the biosynthesis and most bioactive product is likely to be enzymatically formed *in situ*. This enzymatic hydrolysis could proceed via a mechanism analogous to the leukotriene A_4 hydrolase. Its double-bond geometry would likely be 10*R*-hydroxy-Δ11*E*,13*E*,15Z as depicted in Fig. 8 (48, 51, 52). Although 10,17*S*-docosatriene is generated endogenously and carries potent bioactivity, the chirality of the 10 position alcohol remains to be established. Nevertheless, once these dihydroxy products are formed and impart their bioactions, they can undergo ω-oxidation at sites of inflammation and within exudates. They can then undergo ω-oxidation at the carbon 22 position (see Fig. 8 and "Results"). This type of ω-oxidation route is usually associated with rapid inactivation of bioactive eicosanoid mediators (for example, see Refs. 43 and 53). These are also novel trihydroxy docosanoids and implicate that the DHAs carry bioinformation. This was further substantiated with the results obtained from *in vivo* experiments indicating that 10,17*S*-docosatriene is a potent regulator of peritonitis-activated leukocyte recruitment.

The major bioactive product generated from endogenous

DHA released by glial cells focused our attention on the formation and actions of 10,17*S*-docosatriene (see Figs. 3, 4, and 6 for bioactions). Our results obtained with glial cells, murine brain, whole blood, and human leukocytes indicate that the biosynthesis of the docosatrienes can occur in a single cell type and appears to involve a 15-lipoxygenase-like activity and/or related enzymes. Whether the newly uncovered isoforms of human 15-lipoxygenase A or B (see Ref. 54 for a recent perspective) or possibly other, yet to be discovered, novel enzymes are involved in this pathway remains to be established. The initial step to form monohydro(peroxy)-containing intermediates is not likely to occur via a classic p450 enzyme as the predominant pathway (55), because we found the production of the 16(17)-epoxide intermediate as direct evidence for formation of alcohol-trapping products, *i.e.* the markers of epoxides that arise from allylic epoxides (Fig. 8). From our present results, it appears that the major enzymatic route to formation in glial cells involves tightly controlled enzymatic transformations. These reactions appear to be akin to those used for eicosanoid biosynthesis and related enzymes but instead handle DHA. These novel pathways may also involve very specialized enzyme systems to produce in brain, neural systems, and cells of hematopoietic origin the docosanoids established in the present study, namely the docosatrienes and 17*S* series resolvins.

The results of the alcohol trapping and labeling experiments also support the scheme in Fig. 8*B* for the formation of 17*S* series resolvins. Once produced, 17-hydroperoxy-DHA can via transcellular biosynthesis be converted by human neutrophils (see Fig. 8*B*) to two main intermediates following the insertion of the hydroperoxy at either the 4 or 7 position in their precursor. This is rapidly transformed to both 7,8-epoxide and 4,5-epoxide intermediates as also observed with the recently elucidated 17*R* series resolvins produced when aspirin treatment is administered. These two pathways in leukocytes give rise to the dihydroxy- and trihydroxy-containing products termed 17*S* series resolvins (Fig. 8*B*). These are the main products derived from human leukocytes as determined here and are observed both in human whole blood and in inflammatory exudates (see Ref. 7). The 17*S* series compounds appear to be equally potent as the 17*R* series compounds *in vivo* as shown in Fig. 6. This is analogous to the flexibility in stereochemistry noted earlier for the carbon 15 position of arachidonic acid as *R* or *S* observed in lipoxin and aspirin-triggered 15-epi-lipoxin formation and action where the chirality of the other two alcohol positions and double bond geometries are highly critical to maintain bioactions (7). Also, there appear to be one or more specific ligand receptor systems responsible for activating glial cell receptors as indicated by the results with microphysiometry analyses (see Fig. 6*C*), a finding that is in line with other lipid mediators derived from arachidonic acid, namely that there are specific receptors that transduce their wide range of actions (see Refs. 43 and 56). The receptor system(s) and cell types identified in the present experiments with DHA and glial cells as well as formation and action of 10,17-docosatriene provide opportunities to uncover novel receptors signaling used by the docosatrienes and resolvins to evoke their actions *in vivo*.

Results from earlier studies demonstrated that DHA is released from membrane-rich sources in retina and synaptic terminals (57, 58) and underscores an important role for this essential fatty acid in neuronal development (20) and function (59). Importantly, the molecular mechanism(s) for these reported DHA actions remained of interest to explain precise actions in many organ systems. Our present results establish that glial cells can release and transform DHA to novel docosatrienes. In addition, during multicellular events and cell-cell interactions, for example, leukocytes can take up 17-HDHA and convert it to resolvins. These results underscore the importance of cell-cell interaction in generating products that neither cell type can generate alone. This theme in multicellular responses appears to be a key mechanism in the generation of a diverse array of bioactive oxygenated lipid mediators. Because fish are rich in DHA and are able to produce essentially equal amounts of 4 and 5 series eicosanoids (49) from arachidonic acid and EPA, it is possible that DHA transformation to both docosatrienes and 17*S* series resolvins is a conserved primordial signaling pathway in certain human tissues. Hence, results from the present experiments establish the enzymatic transformation pathways for the production of docosatrienes and 17*S* series resolvins and document their actions in models *in vivo*. These new pathways and structures could represent the active components responsible for some of the beneficial actions reported with dietary supplementation of DHA. Moreover, these results indicate that DHA is a precursor for two novel families of bioactive mediators, namely docosatrienes and 17*S* series resolvins.

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Novel Docosatrienes and 17*S*-Resolvins Generated from Docosahexaenoic Acid in Murine Brain, Human Blood, and Glial Cells: AUTACOIDS IN ANTI-INFLAMMATION

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