⌘ *Author's Choice*

Identification and Structure Determination of Novel Anti-inflammatory Mediator Resolvin E3, 17,18-Dihydroxyeicosapentaenoic Acid*⑤

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Background: Endogenous mediators that control aberrant inflammation are of interest as potential targets of new therapeutics.
Results: Here, we identified a novel omega-3 fatty acid-derived anti-inflammatory mediator 17,18-diHEPE, denoted as resolvin E3.
Conclusion: Resolvin E3 has a potent inhibitory action on neutrophil chemotaxis both *in vitro* and *in vivo*.
Significance: The significance of this study is the identification of a novel endogenous lipid mediator with a potent anti-inflammatory property.

Bioactive mediators derived from omega-3 eicosapentaenoic acid (EPA) elicit potent anti-inflammatory actions. Here, we identified novel EPA metabolites, including 8,18-dihydroxyeicosapentaenoic acid (8,18-diHEPE), 11,18-diHEPE, 12,18-diHEPE, and 17,18-diHEPE from 18-HEPE. Unlike resolvins E1 and E2, both of which are biosynthesized by neutrophils via the 5-lipoxygenase pathway, these metabolites are biosynthesized by eosinophils via the 12/15-lipoxygenase pathway. Among them, two stereoisomers of 17,18-diHEPE, collectively termed resolvin E3 (RvE3), displayed a potent anti-inflammatory action by limiting neutrophil infiltration in zymosan-induced peritonitis. The planar structure of RvE3 was unambiguously determined to be 17,18-dihydroxy-5Z,8Z,11Z,13E,15E-EPE by high resolution NMR, and the two stereoisomers were assigned to have 17,18R- and 17,18S-dihydroxy groups, respectively, using chemically synthesized 18R- and 18S-HEPE as precursors. Both 18R- and 18S-RvE3 inhibited neutrophil chemotaxis *in vitro* at low nanomolar concentrations. These findings suggest that RvE3 contributes to the beneficial actions of EPA in controlling inflammation and related diseases.

In many human diseases, uncontrolled inflammation is suspected as a key component of pathogenesis (1). Acute inflam-mation is an indispensable host response to foreign challenges or tissue injury that, if unopposed, could lead to loss of tissue structure and function. In healthy conditions, inflammatory processes are self-limiting and self-resolving, suggesting the existence of endogenous control mechanisms in the course of acute inflammation and resolution (for reviews see Ref. 2, 3). Therefore, the identification of such endogenous anti-inflammatory and/or pro-resolution mechanisms is of wide interest.

Omega-3 polyunsaturated fatty acids, such as eicosapentaenoic acid (EPA)[2] and docosahexaenoic acid (DHA), both of which are enriched in fish oils, have beneficial effects in many inflammatory disorders, including cardiovascular disease, arthritis, colitis, and asthma (4, 5). Omega-3 polyunsaturated fatty acids are widely held to act via several possible mechanisms, serving as an alternative substrate producing less potent products (6) or being converted to potent anti-inflammatory and protective mediators, namely resolvins, protectins, and maresins (7–11). Resolvin (Rv) E1 (5S,12R,18R-trihydroxy-6Z,8E,10E,14Z,16E-EPE) and RvE2 (5S,18R-dihydroxy-6E,8Z,11Z,14Z,16E-EPE) are biosynthesized by human polymorphonuclear leukocytes (PMNs) via the 5-lipoxygenase (5-LOX) pathway from a common precursor 18-hydroxyeicosapentaenoic acid (18-HEPE) (8–10). 18-HEPE formation *in vivo* is related to dietary intake of EPA (9), and a recent study demonstrated two parallel stereospecific pathways, 18R- and 18S-, in the biosynthesis of E series resolvins both in human sera and murine exudates (12). Resolvins were first isolated from the self-limited inflammation and were found to serve as stop signals for PMN infiltrations in murine peritonitis (13). These lipid mediators are not only anti-inflammatory but also

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⑤ This article contains supplemental Figs. S1–S4, Tables S1 and S2, and "Methods".
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[2] The abbreviations used are: EPA, eicosapentaenoic acid; HEPE, hydroxyeicosapentaenoic acid; EPE, eicosapentaenoic acid; LOX, lipoxygenase; MRM, multiple reaction monitoring; PMN, polymorphonuclear leukocyte; PG, prostaglandin; LTB₄, leukotriene B₄.

promote resolution back to the noninflamed state to maintain tissue homeostasis (14).

Here, we report the identification of a novel EPA-derived anti-inflammatory mediator formed via the leukocyte 12/15-LOX pathway. The basic structure of this bioactive EPA metabolite was identified as 17,18*R/S*-dihydroxy-5*Z*,8*Z*,11*Z*,13*E*,15*E*-EPE, denoted 18*R*-RvE3 and 18*S*-RvE3, respectively.

EXPERIMENTAL PROCEDURES

Materials—Racemic 18-HEPE and 15-HETE-d8, LTB$_4$-d4, and PGE$_2$-d4 were purchased from Cayman Chemical. Zymosan A was purchased from Wako Chemicals. Synthetic RvE2 was prepared as in Ref. 15. Dexamethasone, A23187, and soybean 15-LOX (type I-B) were purchased from Sigma. Male C57BL/6 mice (7–8 weeks) were purchased from CLEA Japan, Inc. 12/15-LOX-deficient mice on C57BL/6 background were purchased from The Jackson Laboratory. Animal studies were approved by the University of Tokyo Animal Committee.

Isolation of Peripheral Blood Leukocytes—Peripheral blood neutrophils and eosinophils were isolated from nonatopic healthy subjects as described previously (16). Briefly, red blood cells (RBC) were removed from 40 ml of heparinized peripheral blood using Dextran T-500 (GE Healthcare) and mononuclear cells by centrifugation over 1.083 g/ml Histopaque (Sigma). After hypotonic cell lysis to remove any remaining RBC, neutrophils and eosinophils were separated by a CD16-negative selection using CD16-labeled magnetic microbeads and autoMACS (Miltenyi Biotec). Ethical approval was obtained from the University of Tokyo Research Ethics Committee.

Neutrophil and Eosinophil Incubations—Neutrophils or eosinophils were divided into 5×10^5 cells in 0.5 ml of Hanks' balanced salt solution and stimulated with calcium ionophore (A23187, 2 μM) at 37 °C in the presence of 18-HEPE (1.6 μg). After 30 min, 1 ml of ice-cold methanol was added to stop the reaction.

Mediator Lipidomics, Product Isolation and Extractions—Samples were extracted by solid-phase extraction using Sep-Pak C18 cartridges (Waters) with a deuterium-labeled internal standard (LTB$_4$-d4). LC-MS/MS-based lipidomic analyses were performed using an HPLC system (Waters UPLC) with a linear ion trap quadrupole mass spectrometer (4000 QTRAP; Applied Biosystems) equipped with Acquity UPLC BEH C$_{18}$ column (1.0 mm × 150 mm × 1.7 μm; Waters). Samples were eluted with mobile phase composed of water/acetate (100:0.1, v/v) and acetonitrile/methanol (4:1, v/v) (73:27) for 5 min and ramped to 30:70 after 15 min to 20:80 after 25 min and held for 8 min, ramped to 0:100 after 35 min, and held for 10 min with flow rates of 70 μl/min (0–30 min), 80 μl/min (30–33 min), and 100 μl/min (33–45 min). MS/MS analyses were conducted in negative ion mode, and fatty acid metabolites were identified and quantified by multiple reaction monitoring (MRM). Quantitation was performed using calibration curves constructed for each compound, and recoveries were monitored using added deuterated internal standards (15-HETE-d8, LTB$_4$-d4, PGE$_2$-d4). Compounds were monitored with MRM transitions of 18-HEPE (317 > 259 *m/z*), RvE3 (333 > 213 *m/z*), and PGE$_2$ (351 > 271 *m/z*).

Transfection Studies—HEK293 cells (3.0×10^5 cells) were transiently transfected with 1.0 μg of pCAGGS (17) or pCAGGS containing mouse 12/15-LOX or human 15-LOX cDNA using Lipofectamine 2000 transfection reagent (Invitrogen). After 24 h, cells were incubated with 18-HEPE (10 μM) or arachidonic acid (10 μM) and calcium ionophore (A23187, 10 μM). After 30 min, ice-cold methanol was added to stop the reaction.

Enzymatic Conversion of 18-HEPE with Soybean 15-LOX—Soybean 15-LOX (type I-B) was incubated with racemic 18-HEPE, 18*R*-HEPE, or 18*S*-HEPE in borate buffer (10 ml, pH 9.0) at 4 °C. The reaction was terminated at 90 min, and hydroperoxide intermediates were reduced with excess NaBH$_4$. Incubations were extracted by using Sep-Pak C18 cartridges (Waters). To separate and isolate conversion products, reverse-phase HPLC was carried out by using a Waters XBridge C$_{18}$ column (100 mm × 4.6 mm × 5 μm) with mobile phase methanol/water/acetate (65:35:0.0035, v/v/v) at 0.7 ml/min of flow rate.

Murine Zymosan-induced Peritonitis—Peritonitis was performed as in Ref. 13. Compounds were administered intravenously through a tail vein and followed by 1 ml of zymosan A (1 mg/ml) into peritoneum. Peritoneal lavages were collected at 2 h, and cells were enumerated. Differential leukocyte counts were performed by Wright-Giemsa stain and light microscopy. For LC-MS/MS analysis, mice were sacrificed at 48 h after zymosan challenge, and peritoneal exudates were collected.

Neutrophil Chemotaxis Assay—Chemotaxis experiments were conducted using EZ-TAXIScan chamber (Effector Cell Institute, Tokyo, Japan). The EZ-TAXIScan is a visually accessible chemotactic chamber, in which one compartment containing chemoattractant and another compartment containing cells are connected by a microchannel (18, 19). PMNs were isolated from mouse bone marrow. Bone marrow cells were obtained by flushing femurs of C57BL/6 male mice (8–10 weeks) with Hanks' balanced salt solution supplemented with 20 mM HEPES, pH 7.4, and 0.5% FCS. RBC were lysed with 0.2% NaCl, and the PMNs were isolated over a 62% Percoll gradient by centrifugation for 30 min at $1,000 \times g$.

The EZ-TAXIScan chamber was assembled with a 260 μm wide × 4 μm thick silicon chip on an untreated slide glass and filled with RPMI 1640 medium, 0.1% BSA. One microliter of test compounds was then added directly to the lower and upper reservoir. PMNs (6×10^3 cells) were preincubated with test compounds for 5 min and were added to the lower reservoir of each of the six channels and allowed to line up by removing 10 μl of buffer from the upper reservoir. One microliter of chemoattractant (LTB$_4$, 10 nM) was then added to the upper reservoir, and PMN migration at room temperature was recorded every 30 s for 40 min and was analyzed with TAXIScan Analyzer 2 software.

NMR Experiments of RvE3—^{1}H and two-dimensional NMR spectra of RvE3 (10 μg, 30 nmol of compound V, and 20 μg, 60 nmol of compound VI, respectively) in CD$_3$OD (0.25 ml) were recorded at 298.1 K on a Varian Unity Inova 800 instruments (800 MHz for ^{1}H NMR) equipped with a cold probe. Chemical shifts are denoted in δ (ppm) relative to residual solvent peaks as internal standard (CD$_3$OD, ^{1}H δ 3.31).
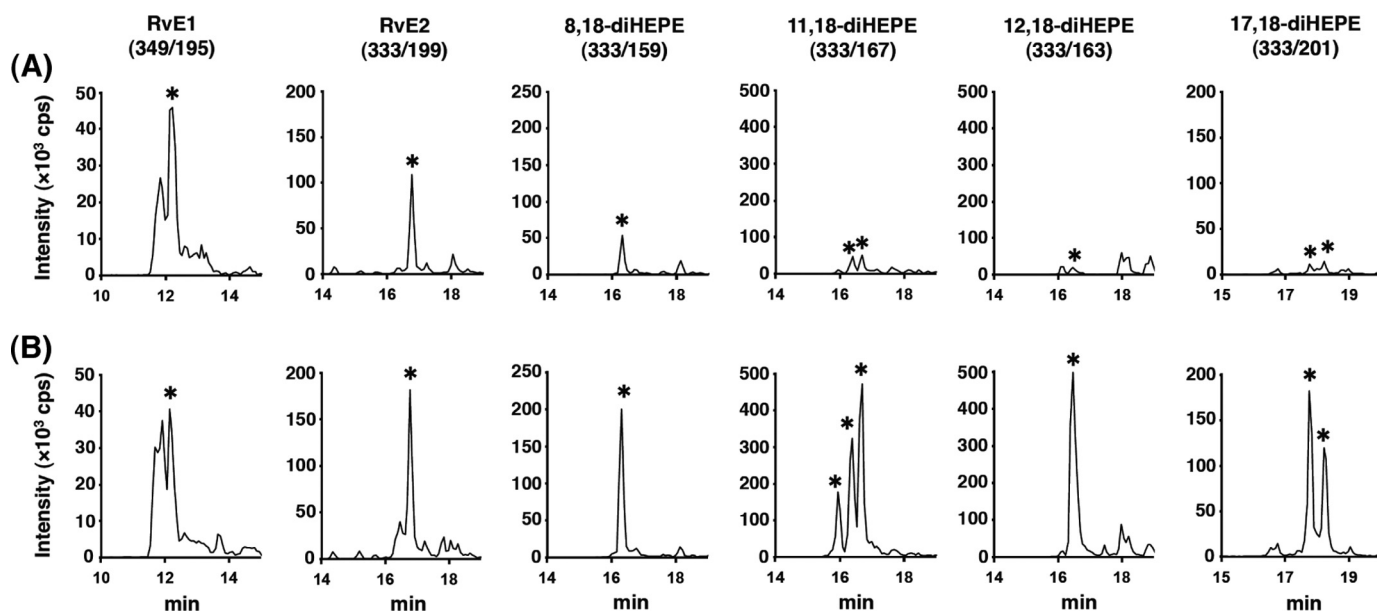
FIGURE 1. **Formation of 18-HEPE metabolites from human leukocyte incubations.** MRM chromatograms of the 18-HEPE incubation products with human PMN (*A*) and eosinophils (*B*) were separated by reverse-phase HPLC. 18-HEPE metabolites were monitored by MRM mode using established transitions for RvE1 (349/195 *m/z*) and RvE2 (333/199 *m/z*) as well as predicted transitions for 8,18-diHEPE (333/159 *m/z*), 11,18-diHEPE (333/167 *m/z*), 12,18-diHEPE (333/163 *m/z*), and 17,18-diHEPE (333/201 *m/z*). Peaks of each metabolite are marked by *asterisks*.

Statistical Analysis—Results are expressed as mean ± S.E. Statistical significance was determined by Student's *t* test; $p < 0.05$ was considered significant.

RESULTS

Formation of Novel Metabolites by Eosinophils—Because human PMNs incubated with 18-HEPE in the presence of a calcium ionophore produced E series resolvins (*i.e.* RvE1 and RvE2) via 5-LOX pathway (7), we questioned whether other EPA metabolite(s) with potent anti-inflammatory property could be formed by other cell types. Because our previous study demonstrated that eosinophils are recruited to the inflamed loci during the resolution phase of acute peritonitis and promote resolution by producing pro-resolving mediators (20), we focused on eosinophils. Human eosinophils were isolated from peripheral blood and were incubated with racemic 18-HEPE in the presence of a calcium ionophore. Unbiased target lipidomics using liquid chromatography-tandem mass spectrometry (LC-MS/MS)-based analyses were performed, and several hydroxylated products were identified using MRM with established or predicted precursor-product ion pairs. Side-by-side MRM chromatograms of products from human PMN and eosinophil incubations with 18-HEPE (Fig. 1, *A* and *B*, respectively) clearly demonstrate that human PMN converted 18-HEPE into RvE1 and RvE2, as reported previously (10, 12). However, isolated human eosinophils converted 18-HEPE into novel 8,18-dihydroxy-EPE (8,18-diHEPE), 11,18-diHEPE, 12,18-diHEPE, and 17,18-diHEPE in addition to RvE1 and RvE2. Because human eosinophils express higher levels of leukocyte-type 15-LOX than PMNs (21), we reasoned that 8,18-diHEPE, 11,18-diHEPE, 12,18-diHEPE, and 17,18-diHEPE might be formed via the 15-LOX pathway. To this end, we isolated eosinophils from wild type or mice deficient in leukocyte-type 12/15-LOX, a murine orthologue of human 15-LOX (22),

and incubated them with 18-HEPE. As expected, much less of the novel products was detected from the incubation of 12/15-LOX-deficient murine eosinophils (Fig. 2).

To further confirm the 12/15-LOX-dependent formation of novel products, HEK293 cells were transiently transfected with expression plasmid encoding 12/15-LOX and were incubated with 18-HEPE in the presence of calcium ionophore. Cells expressing mouse leukocyte-type 12/15-LOX or human 15-LOX efficiently converted 18-HEPE into 8,18-diHEPE, 11,18-diHEPE, 12,18-diHEPE, and 17,18-diHEPE (Fig. 3*A*). As reported previously (22), when arachidonic acid was used as a substrate, murine 12/15-LOX exhibited a major arachidonic acid 12-LOX activity (C10-hydrogen abstraction), whereas the human orthologue acts predominantly as 15-LOX (C13-hydrogen abstraction) (Fig. 3*B*). Similarly, murine 12/15-LOX formed predominantly 8,18-diHEPE and 12,18-diHEPE from 18-HEPE possibly through C10-hydrogen abstraction, whereas human 15-LOX preferentially converted 18-HEPE into 11,18-diHEPE and 17,18-diHEPE, possibly through C13-hydrogen abstraction (Fig. 3*A*). Side-by-side comparison of arachidonic acid- or 18-HEPE-derived products from eosinophil incubations also demonstrated the species difference between mouse and human (Fig. 3, *C* and *D*). These results indicate the different enzyme species convert 18-HEPE to a same series of oxidized products with different ratios both *in vitro* and in cells.

To synthesize 18-HEPE conversion products *in vitro*, racemic 18-HEPE was incubated with soybean 15-LOX. A total of six products (compounds I–VI) carrying chromophore of conjugated triene were generated (Fig. 4*A*). Based on the MS/MS spectra, compounds I–IV were assigned as 11,18-diHEPE, and compounds V and VI were assigned as 17,18-diHEPE (Fig. 4*B*). 11,18-diHEPEs and 17,18-diHEPEs had UV absorbance peaks at 268 and 273 nm, respectively, demonstrating the presence of
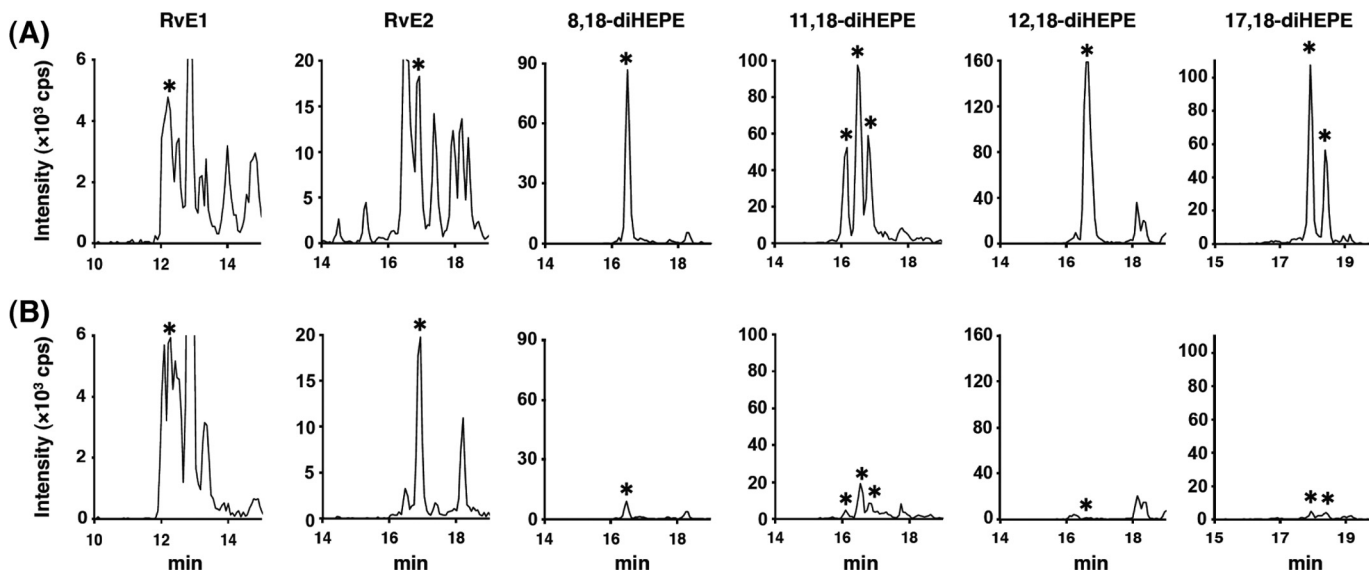
FIGURE 2. **12/15-LOX-dependent formation of 18-HEPE metabolites from mouse eosinophils.** Lipidomic profiles of 18-HEPE incubation products of mouse eosinophils (*A*) and 12/15-LOX-deficient mouse eosinophils (*B*) were compared.
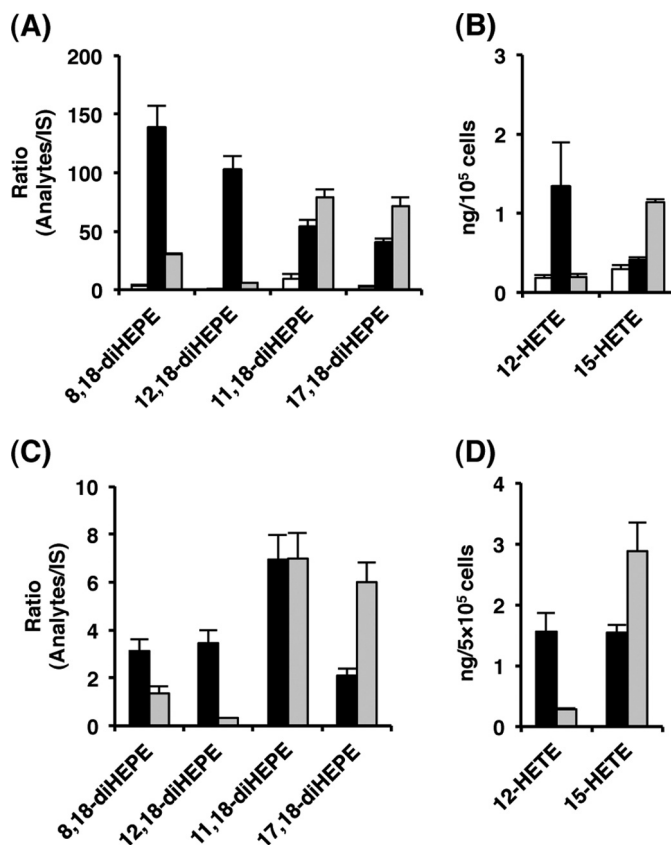

FIGURE 3. **Formation of 18-HEPE metabolites by cells expressing mouse 12/15-LOX or human 15-LOX.** Lipidomic profiles of 18-HEPE (*A*) or arachidonic acid (*B*) incubation products of HEK293 cells transiently transfected with mock (*white bars*), mouse 12/15-LOX (*black bars*), or human 15-LOX (*gray bars*) cDNA plasmids are shown. Lipidomic profiles of 18-HEPE (*C*) or arachidonic acid (*D*) incubation products of mouse (*black bars*) or human (*gray bars*) eosinophils are shown. Relative production was determined by calculating peak area ratio of each analyte to deuterium-labeled internal standard (LTB$_4$-d4). Values represent mean ± S.E., n = 3–5.

conjugated triene structures (Fig. 4*B*). These enzymatically generated compounds co-eluted with eosinophil-derived products at 16.2 min (compound I), 16.6 min (compounds II and III), 16.9 min (compound IV), 18.1 min (compound V), and 18.5 min (compound VI), respectively (Fig. 5). Therefore, we used these

enzymatically generated compounds to further assess their biological activities *in vivo*.

Inhibition of PMN Infiltration in Murine Peritonitis—Earlier studies demonstrated that nanogram doses of E series resolvins (RvE1 and RvE2) significantly reduced PMN infiltration *in vivo*
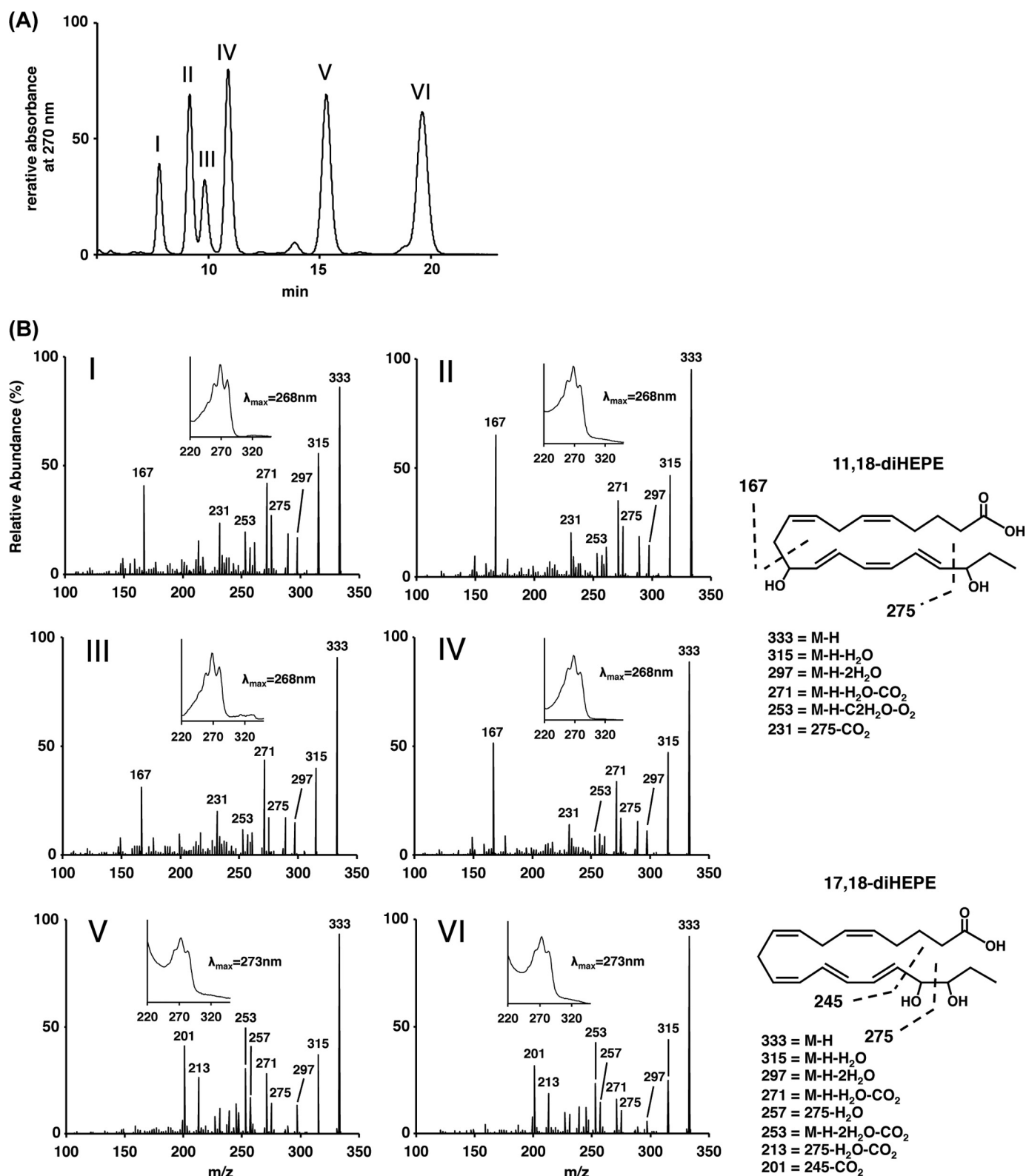
FIGURE 4. **Enzymatic formation of 18-HEPE metabolites and their anti-inflammatory properties *in vivo*.** *A,* reverse-phase HPLC chromatogram of the 18-HEPE incubation products with soybean 15-LOX monitored with UV absorbance at 270 nm. *B,* UV and tandem mass spectra of major products isolated from soybean 15-LOX incubation. Based on the MS/MS spectra, compounds I to IV were assigned as 11,18-diHEPE with corresponding fragments at *m/z* 333(M-H), 315(M-H-H_2O), 297(M-H-2H_2O), 271(M-H-H_2O-CO_2), 253(M-H-2H_2O-CO_2), and diagnostic fragments at *m/z* 275, 231(275-CO_2), and 167. Compounds V and VI were assigned as 17,18-diHEPE with corresponding fragments at *m/z* 333(M-H), 315(M-H-H_2O), 297(M-H-2H_2O), 271(M-H-H_2O-CO_2), 253(M-H-2H_2O-CO_2), and diagnostic ions at *m/z* 275, 257(275-H_2O), 245, 213(275-H_2O-CO_2), and 201(245-CO_2).
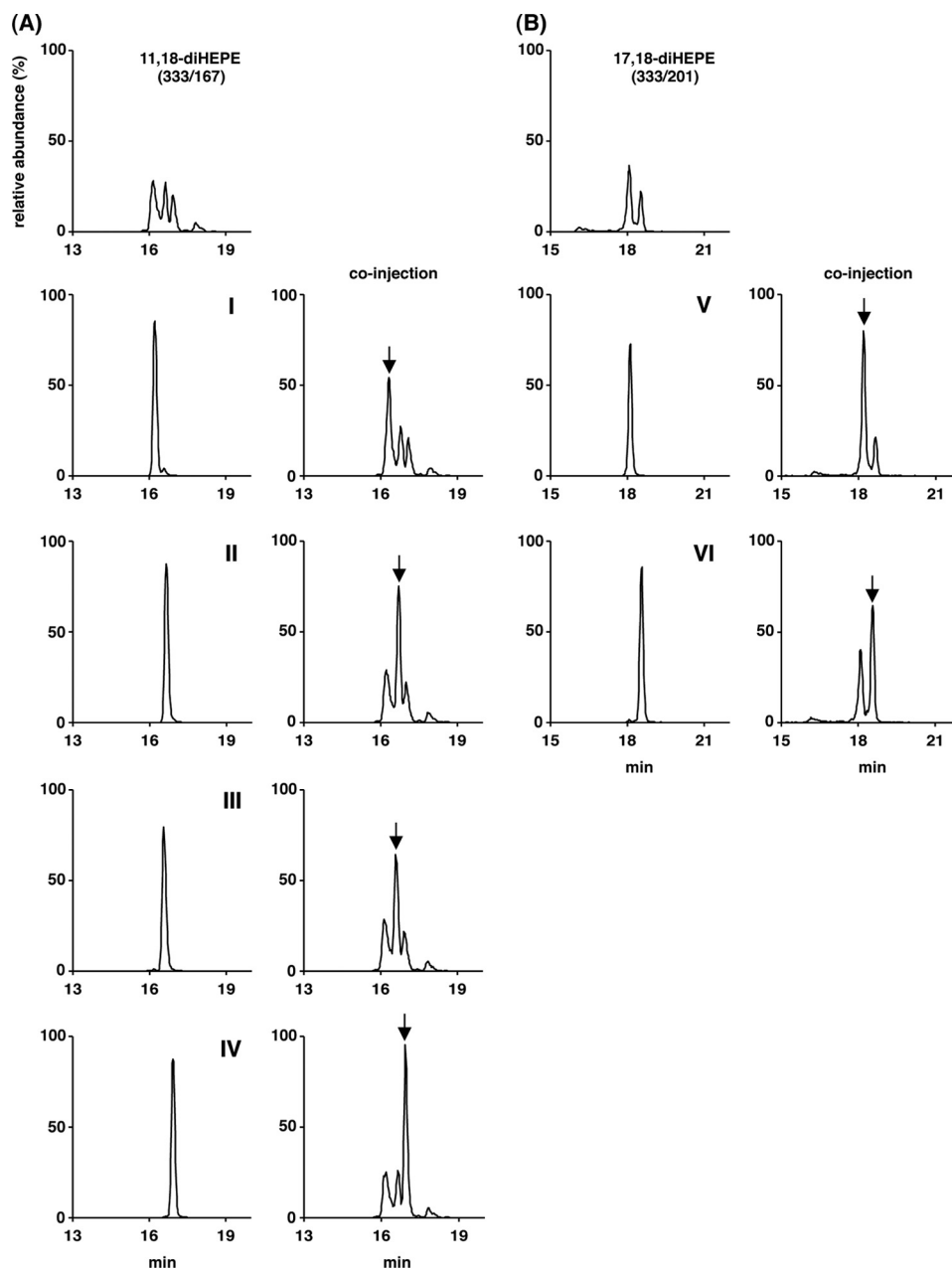
FIGURE 5. **Comparison of eosinophil-derived 18-HEPE metabolites with enzymatically generated products.** *Top panel*, MRM chromatograms of 11,18-diHEPE (*A*) and 17,18-diHEPE (*B*) obtained from eosinophil incubation with 18-HEPE. *Lower panels*, MRM chromatograms of compounds I–VI obtained from soybean 15-LOX-catalyzed synthesis and co-injection of these enzymatically generated products with eosinophil derived products. Note that compounds II and III co-eluted in this liquid chromatographic condition.

(8–10). We determined whether enzymatically generated compounds displayed anti-inflammatory actions *in vivo*. Zymosan A, a glucan from a yeast cell wall, was used to induce sterile peritonitis characterized by acute PMN infiltration. Administration of 10 ng/mouse of the 17,18-diHEPE isomers (compounds V and VI) significantly blocked PMN infiltration by 27.9 ± 8.2 and $43.7\% \pm 1.3\%$, respectively (Fig. 6*A*). In contrast, little effect was observed by the administration of 11,18-diHEPEs (compounds I–IV), which show the structure-specific activity of 17,18-diHEPEs. Compound V at 100 ng/mouse and compound VI at 10 ng/mouse dramatically inhibited PMN infiltration and were almost as potent as higher doses of RvE2 (1 μg/mouse) or dexamethasone (10 μg/mouse) (Fig. 6*B*). For

comparison in this model, EPA administration at 100 ng/mouse gave no effect on PMN numbers ((3.36 ± 0.7) $\times 10^6$ *versus* (3.31 ± 0.3) $\times 10^6$ cells, $n = 4$), suggesting that EPA requires metabolic conversion to exert its actions on leukocyte infiltrations *in vivo*. Given their biosynthetic route and their potent anti-inflammatory actions, the two isomers of 17,18-diHEPE (compounds V and VI) were collectively denoted RvE3.

Structure Determination of RvE3 Isomers by NMR—Minute amounts of the two enzymatically prepared RvE3 isomers (30–60 nmol) were analyzed by high field NMR. The planar structures of the two RvE3 isomers (compounds V and VI), including the positions of the hydroxy groups and the geometries of the olefins, were unambiguously established using [1]H
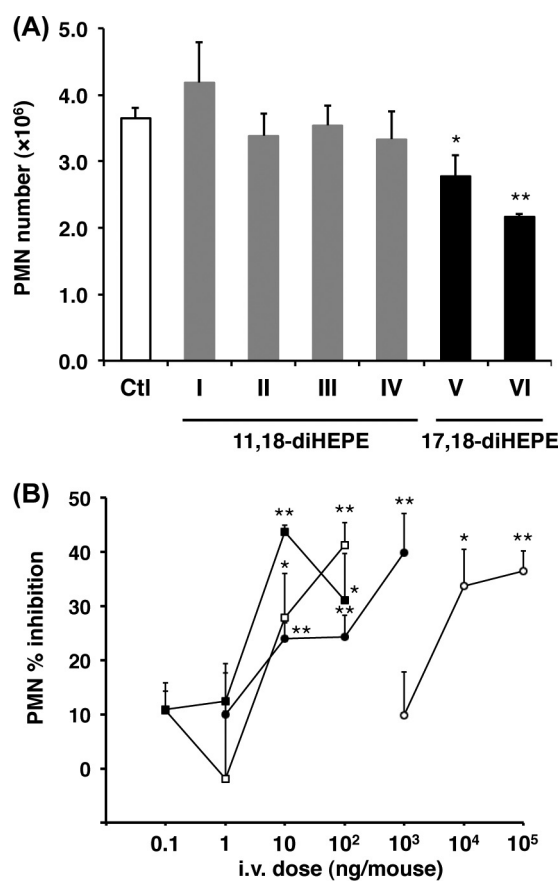
FIGURE 6. **Inhibition of PMN infiltration in zymosan-induced peritonitis.** *A*, compounds I–VI were injected intravenously (10 ng/mouse) via tail vein followed by peritoneal injection of zymosan A (1 mg/ml). After 2 h, peritoneal lavages were collected, and PMN leukocyte numbers were counted. Values represent mean $\pm$ S.E., $n = 3–12$, *, $p < 0.05$; **, $p < 0.01$ as compared with vehicle control. *B*, dose-dependent comparison of the actions of compound V (□), compound VI (■), RvE2 (●) and dexamethasone (○) on PMN infiltration. Values represent mean $\pm$ S.E., $n = 4–12$, *, $p < 0.05$; **, $p < 0.01$ as compared with vehicle control.
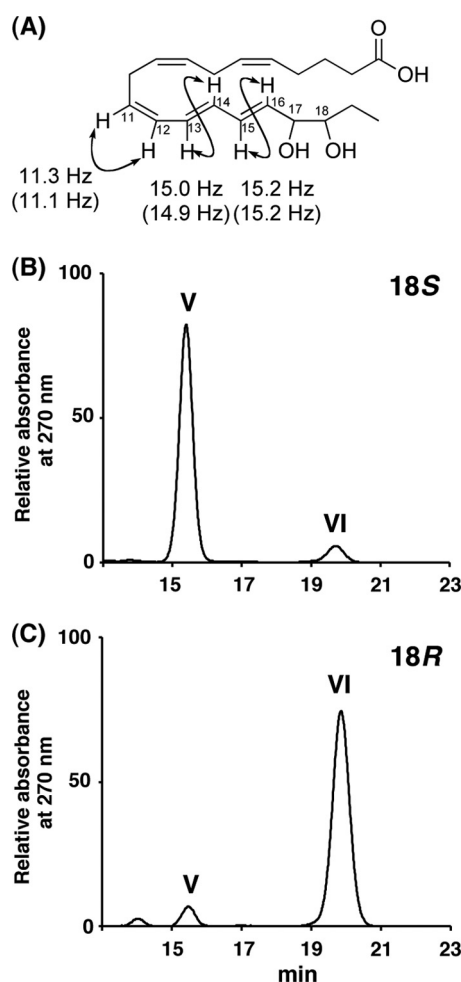

FIGURE 7. **Physical and spectroscopic properties of RvE3.** *A*, ^{1}H-^{1}H coupling constants of conjugated triene and full structure of RvE3 (compound V and VI). *B* and *C*, reverse-phase HPLC chromatogram of synthetic 18*S*-HEPE or 18*R*-HEPE incubation products with soybean 15-LOX monitored with UV absorbance at 270 nm.

NMR and ^{1}H-^{1}H COSY spectra (CD$_3$OD, 800 MHz) (supplemental Figs. S1 and S2 and supplemental Table S1). The coupling constants of the olefinic protons from C11 to C16 clearly indicated that both RvE3 isomer possessed 11*Z*,13*E*,15*E*-conjugated trienes (Fig. 7*A*). Therefore, we concluded that RvE3 is 17,18-dihydroxy-5*Z*,8*Z*,11*Z*,13*E*,15*E*-EPE and that compounds V and VI are stereoisomers of 17,18-hydroxy groups.

Stereoselective Synthesis of 18R- and 18S-RvE3—The 18*R*- and 18*S*-HEPE enantiomers were chemically synthesized (supplemental Fig. S3) and were submitted to the 15-LOX reaction to better understand stereochemical structures of RvE3 isomers. Separate treatment of the synthesized 18*R*- and 18*S*-HEPE by soybean 15-LOX delivered the different diastereomers of 17,18-diHEPEs. The ^{1}H NMR spectra and the HPLC retention time of 17,18*S*-diHEPE and 17,18*R*-diHEPE were found to be identical to those of compounds V and VI, respectively (Fig. 7, *B* and *C*). Importantly, these enzymatically generated products co-eluted with 17,18-diHEPEs formed *in vivo* within inflammatory exudates of murine peritonitis (Fig. 8). By combining these results, the structures of endogenously formed RvE3 isomers were determined to be 17,18*S*-dihydroxy-5*Z*,8*Z*,11*Z*,13*E*,15*E*-EPE (compound V, 18*S*-RvE3) and

17,18*R*-dihydroxy-5*Z*,8*Z*,11*Z*,13*E*,15*E*-EPE (compound VI, 18*R*-RvE3), respectively. Endogenous levels of 18*R*-RvE3, 18*S*-RvE3, and their precursor 18-HEPE in inflammatory exudates of murine peritonitis were ~14, 32, and 222 pg/mouse, respectively. These levels were comparable with that of PGE$_2$ (100 pg/mouse) in this model. Moreover, when EPA was supplemented, we could detect much higher levels of 18*R*-RvE3, 18*S*-RvE3, and 18-HEPE in the peritoneal fluid (supplemental Table S2).

Inhibition of PMN Chemotactic Migration—Next, we examined whether these RvE3 isomers affect PMN chemotaxis *in vitro*. Murine PMNs isolated from bone marrow were applied to an EZ-TAXIScan chemotaxis chamber (18) in which a stable chemoattractant gradient was formed (Fig. 9*A*). Cell movements were time-lapse recorded such that the path and migration speed of an individual PMN could be determined. Both 18*S*- and 18*R*-RvE3 at nanomolar concentrations significantly reduced PMN migration speeds toward LTB$_4$ (Fig. 9, *B* and *C*). Thus, both RvE3 isomers directly act on PMNs to control chemotaxis as did 15-epi-lipoxin A$_4$ tested in parallel for direct comparison. These PMN responses were not observed by EPA,
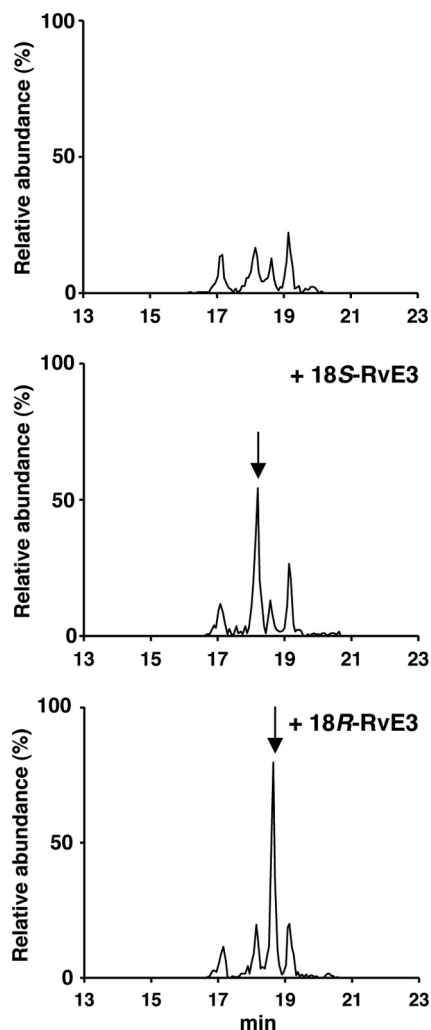
FIGURE 8. **RvE3 formation *in vivo*.** Comparison of endogenously formed 17,18-diHEPEs in mouse inflammatory exudates with enzymatically generated RvE3 isomers. *Top panel*, MRM chromatogram with established transition of 333/201 *m/z* to monitor 17,18-diHEPEs present in murine peritoneal exudates 48 h after zymosan challenge. *Middle* and *lower panel*, MRM chromatogram obtained from co-injection of enzymatically generated 18*S*-RvE3 (*middle panel*) or 18*R*-RvE3 (*lower panel*) with 17,18-diHEPEs present in murine peritoneal exudates 48 h after zymosan challenge.

a metabolic precursor of RvE3, when introduced at equal concentrations in the chambers (Fig. 9*B*).

RvE3 had little effect on cyclic AMP, intracellular calcium level, and morphology of mouse bone marrow PMNs (supplemental Fig. S4). Also, RvE3 treatment had little effect on the LTB_4-induced calcium influx. This result suggests that the inhibitory effect of RvE3 on PMN chemotaxis is not simply due to LTB_4 receptor antagonism or cytotoxic action.

DISCUSSION

The results of this study uncover the structure and anti-inflammatory property of a new EPA-derived mediator RvE3. Human and mouse eosinophils generated RvE3 from 18-HEPE via leukocyte-type 12/15-LOX pathway. Enzymatically generated RvE3 had the same physical properties as endogenously biosynthesized products and displayed a potent anti-inflammatory action by stopping PMN infiltration in zymosan-induced peritonitis. The structures of the two diastereomeric RvE3
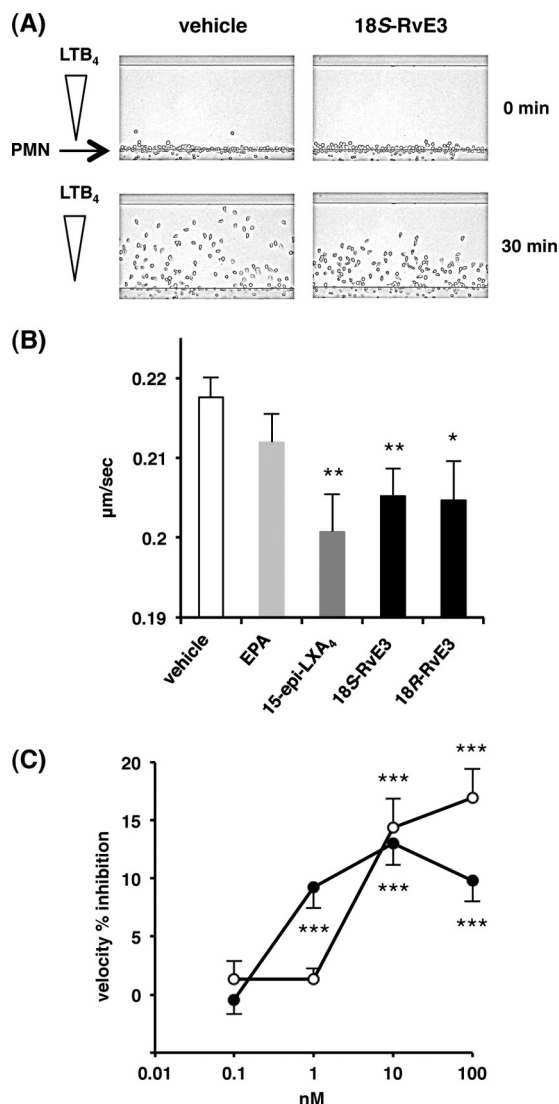

FIGURE 9. **RvE3 reduces mouse PMN chemotaxis efficiency.** Effect of RvE3 on chemotaxis of mouse bone marrow PMNs toward LTB_4. *A*, bone marrow PMNs were incubated with 10 nM 18*S*-RvE3 during the assay. Images of PMNs are shown at 0 and 30 min after addition of LTB_4 (10 nM) as a chemoattractant. *B*, velocity of the motile cells were determined from digital time lapse movies. *C*, concentration dependence of 18*S*-RvE3 (○) and 18*R*-RvE3 (●) on reduced velocity of PMN chemotaxis. Values represent mean ± S.E., $n \geq 20$ cells, *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ as compared with vehicle control.

were determined to be 17,18*S*-dihydroxy-5*Z*,8*Z*,11*Z*,13*E*,15*E*-EPE (compound V, 18*S*-RvE3) and 17,18*R*-dihydroxy-5*Z*,8*Z*,11*Z*,13*E*,15*E*-EPE (compound VI, 18*R*-RvE3), respectively. Both 18*S*- and 18*R*-RvE3 inhibited PMN chemotaxis *in vitro* at low nanomolar concentrations as evidenced by decreased velocity. Reduced chemotaxis of PMN is relevant in many diseases where uncontrolled inflammation is the underlying pathophysiology (1, 2, 7). Hence reduced PMN chemotaxis by RvE3 would be relevant in all conditions where PMN-mediated tissue injury is important.

Although the E series resolvins (*i.e.* RvE1 and RvE2) are formed via the 5-LOX pathway, this study uncovered a novel route of anti-inflammatory cascade via the 12/15-LOX pathway (Fig. 10). EPA is converted to 18-HEPE by aspirin-acetylated COX-2 (8) or cytochrome P450 monooxygenase (23). 18-HEPE is further converted via sequential actions of lipoxygenases,
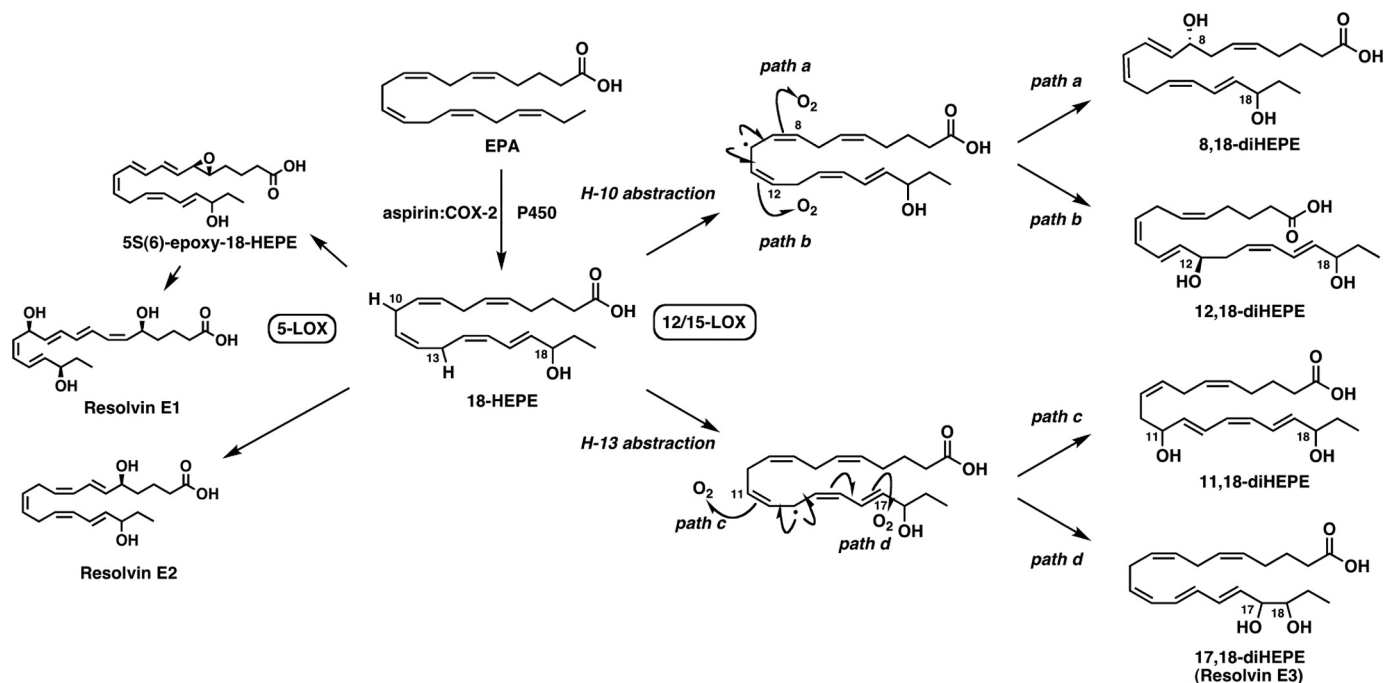
FIGURE 10. **Proposed scheme for biosynthesis of E series resolvins and related products.** E series resolvins are generated from a common precursor 18-HEPE. EPA is converted to 18-HEPE by aspirin-acetylated COX-2 or cytochrome P450 monooxygenase. 18-HEPE is further converted via the sequential actions of lipoxygenases, which leads to formation of E series resolvins. 5-LOX expressed in PMNs converts 18-HEPE into RvE1 and RvE2. The stereochemistry of RvE1 and RvE2 was established (9, 12). In addition, 18-HEPE is converted by 12/15-LOX present in eosinophils or resident macrophages into 8,18-diHEPE, 11,18-diHEPE, 12,18-diHEPE, and 17,18-diHEPE (RvE3). The stereochemistry of the alcohols in 8,18-diHEPE and 12,18-diHEPE is depicted as tentative.

which leads to the formation of E series resolvins. It is likely that hydrogen abstraction from C13 by 12/15-LOX induced a stereospecific oxygen insertion at C11 or C17, leading to the formation of 11,18-diHEPE and RvE3. After 12/15-LOX abstracts pro-*S*-hydrogen from the C13 position of 18-HEPE, molecular oxygen is generally inserted at C17 in an antarafacial fashion (24), resulting in formation of the 17*R*-hydroxylated compounds. Therefore, both RvE3 isomers are likely to have 17*R*-hydroxy groups. Also, hydrogen abstraction from C10 induced the insertion of oxygen at C8 or C12 to form 8,18-diHEPE and 12,18-diHEPE, respectively (Fig. 10). It is important to note that leukocyte-type 12/15-LOX is an enzyme present in murine cells, and in humans there are at least two different enzymes (15-LOX and 15-LOX2) (22). Murine 12/15-LOX exhibits a major arachidonic acid 12-LOX activity (C10-hydrogen abstraction), whereas the human orthologue acts predominantly as 15-LOX (C13-hydrogen abstraction) when arachidonic acid is used as a substrate. We showed the difference in enzymatic property between mouse 12/15-LOX and human 15-LOX when 18-HEPE was used as a substrate. Murine 12/15-LOX formed predominantly 8,18-diHEPE and 12,18-diHEPE possibly through C10-hydrogen abstraction, whereas human 15-LOX preferentially converted 18-HEPE into 11,18-diHEPE and RvE3, possibly through C13-hydrogen abstraction (Fig. 3).

The contribution of eosinophils to the biosynthesis of RvE3 and other 12/15-LOX-derived mediators such as lipoxins and protectins (25–27) is of interest in controlling acute inflammation and resolution. A recent study has demonstrated that eosinophils are recruited to the inflamed loci during the resolution phase of acute peritonitis and promote resolution by producing pro-resolving mediators in mice (20). Besides eosino-

phils, 12/15-LOX is expressed in tissue resident macrophages, dendritic cells, mast cells, and airway epithelial cells (22). Also, the expression level of 12/15-LOX is up-regulated in various cell types by Th2 cytokines, including interleukin(IL)-4 and IL-13 (22). Cells expressing 12/15-LOX might be involved in regulating inflammatory responses by locally producing anti-inflammatory lipid mediators such as RvE3. This is consistent with reports that document exacerbated inflammatory responses in 12/15-LOX-deficient mice in several disease models (25–27).

PMNs constitute the first line of defense against foreign pathogens. In response to stimuli, PMNs migrate into inflamed tissues, where they protect their host by engulfing, killing, and digesting pathogens. Conversely, excessive and sustained PMN activation can cause tissue damage and uncontrolled inflammation. Therefore, PMN function needs to be well controlled, and endogenous mediators that control these PMN responses are of interest. In this study, RvE3 proved to be a potent inhibitor of PMN chemotaxis *in vitro* and also significantly reduced PMN numbers in zymosan-induced peritonitis model *in vivo*. Intravenous administration of 18*R*- and 18*S*-RvE3 in a dose as small as 10 ng/mouse gave 30–45% inhibition of PMN infiltration that was maintained at the 100-ng doses. The characteristics of partial inhibition of PMN infiltration may benefit potential anti-inflammatory roles of these mediators without compromising host defense via immune suppression. Hence, it might be useful to consider RvE3 as a new endogenous anti-inflammatory compound to protect against an aberrant or uncontrolled innate inflammatory response and as a potential new therapeutic.

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