Resolvin E2: Identification and Anti-Inflammatory Actions: Pivotal Role of Human 5-Lipoxygenase in Resolvin E Series Biosynthesis

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Summary

The family of resolvins consists of omega-3 fatty acid-derived mediators, including E series resolvins generated from eicosapentaenoic acid (EPA), and carry potent anti-inflammatory properties. Here, we report the isolation, identification, and bioactions of resolvin E2 (RvE2), which is 5S,18-dihydroxy-eicosapentaenoic acid. RvE2 stopped zymosan-induced polymorphonuclear (PMN) leukocyte infiltration and displayed potent anti-inflammatory properties in murine peritonitis. We also demonstrate that human recombinant 5-lipoxygenase generates RvE2 from a common precursor of E series resolvins, namely, 18-hydroxyeicosapentaenoate (18-HEPE). Furthermore, the initial 5-hydroperoxide intermediate was also converted to a 5(6)-epoxide intermediate in RvE1 formation. These results demonstrate that RvE2, together with RvE1, may contribute to the beneficial actions of omega-3 fatty acids in human diseases. Moreover, they indicate that the 5-lipoxygenase in human leukocytes is a pivotal enzyme that can produce both pro- and anti-inflammatory chemical mediators.

Introduction

The innate inflammatory response is essential in both health and disease. This process helps destroy foreign agents that are potentially harmful to the host and repairs the damaged tissue [1]. Some of the more recent findings have shown that aberrant inflammation is associated with a wide range of diseases, including Alzheimer's disease, cardiovascular disease [2], and cystic fibrosis [3]. These are in addition to the well-appreciated role of inflammation in diseases such as arthritis and periodontitis [4]. Because uncontrolled inflammatory responses can contribute to disease, and the recognition that resolution of inflammation is an active process

[5, 6], a new area for research has emerged focusing on promoting resolution as a possible new therapeutic approach [4, 7–9]. Hence, effective endogenous mechanism(s) that can switch off or dampen, per se, elevated proinflammatory signals as well as cell trafficking that can lead to tissue injury and can promote the clearance of inflammatory cells [7, 8] could be suitable pathways by which to model novel mimetics.

Along these lines, omega-3 polyunsaturated fatty acids (PUFAs), such as eicosapentaenoic acid (EPA) or docosahexaenoic acid (DHA), are widely studied and have been shown to be associated with beneficial actions in many inflammatory disorders, including cardiovascular disease [10, 11], arthritis, inflammatory bowel disease, and asthma [12]. These essential PUFAs were widely believed to potentially act through several possible mechanisms that include the inhibition of proinflammatory eicosanoids and cytokines [13, 14]. Recently, this laboratory [15] uncovered a novel array of bioactive lipid mediators present in inflammatory exudates derived from omega-3 PUFAs [16–19] that carry potent counter-regulatory and anti-inflammatory actions in vivo in murine systems. Together, these findings suggest that essential fatty acids are also important in the resolution of inflammation ([7], and reviewed in [20]). These lipid-derived local chemical mediators are termed resolvins and protectins [20] and serve as stop signals for neutrophil infiltration, a well-appreciated marker cell type in inflamed tissues [1, 9]. The resolvins (resolution-phase interaction products) are derived from omega-3 PUFAs, which, in the presence of aspirin, enable a cyclooxygenase (COX)-2- and lipoxygenase (LO)-mediated conversion of EPA to the 18R series resolvins. The resolvins were first isolated during the spontaneous resolution phase of acute inflammation [18, 19], and the potent bioactive compound 5S,12,18R-trihydroxy-eicosapentaenoic acid (EPE) was denoted as resolvin E1 (RvE1). Recently, we elucidated the complete stereochemistry of RvE1 (5S,12R,18R-trihydroxy-6Z,8E,10E,14Z,16E-EPE), and we found that related double bond isomers are far less potent. Also, a G protein-coupled receptor with nanomolar (nM) potency for RvE1 [16] was demonstrated. This RvE1 receptor-ligand interaction displays potent ability to (1) regulate leukocyte and dendritic cell trafficking, (2) block IL-12 production, (3) counterregulate TNF$_\alpha$-induced NFκB signaling, and (4) protect in murine colitis [17].

Here, we report the identification of a new bioactive member of the E series resolvins generated during resolvin E1 biosynthesis that shares anti-inflammatory actions with RvE1. The basic structure of this novel dihydroxyeicosanoid was determined and was shown to be 5S,18-dihydroxy-EPE, denoted resolvin E2 (RvE2), the reduction product of 5S-hydroperoxy,18-hydroxy-EPE (an intermediate in the biosynthetic pathway of RvE1). We also document the generation of RvE2 by human neutrophils and its anti-inflammatory actions in vivo. Together, these results underscore the pivotal role of the 5-lipoxygenase in generating both proinflammatory and anti-inflammatory mediators.

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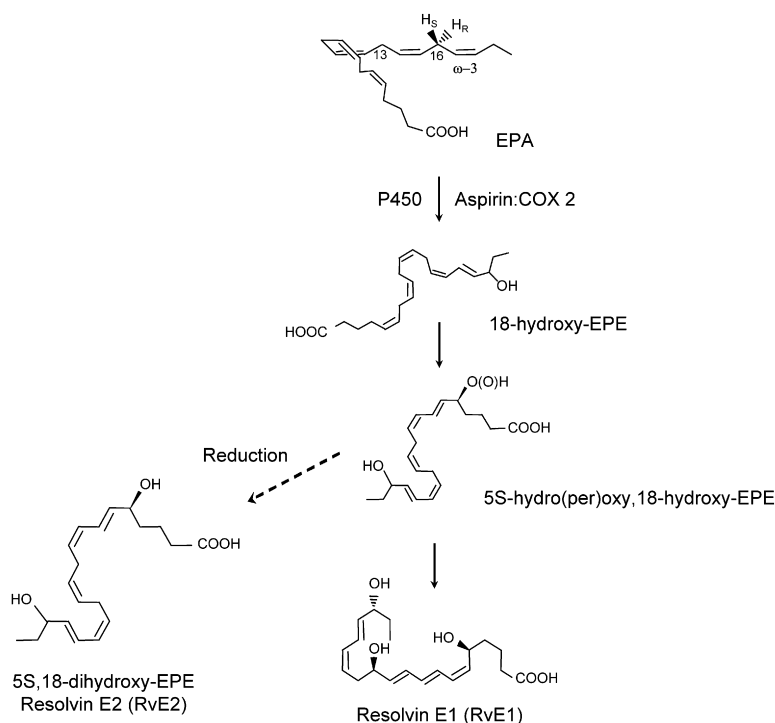[3] These authors contributed equally to this work.

Results and Discussion

Identification of RvE2 in Human Neutrophils

Earlier results from murine exudates obtained during spontaneous resolution [18, 19] gave novel lipid mediators and the proposed biosynthetic pathway of RvE1 (see Figure 1). This led us to hypothesize that 5S,18-dihydroxy-EPE is formed via reduction of the required 5S-hydroperoxy,18R-hydroxy-EPE, an intermediate in the biosynthesis of RvE1 [18]. We questioned whether this compound was formed and carried bioactivity. To test this, human neutrophils (PMNs) were exposed to 18-HEPE, the endothelial-derived product generated from EPA [18], with the presence or absence of the divalent cation ionophore (A23187). Figure 2A shows an HPLC chromatogram of materials isolated from human PMNs. This chromatogram indicates the presence of both RvE1 [16] and a 5S,18-dihydroxy-EPE-derived product that was previously unknown. The chromatographic behavior, i.e., later chromatographic retention time than RvE1, was also consistent with the presence of a dihydroxy-containing product. Incubations with permeabilized PMNs prepared by freeze-thaw displayed slightly less conversion of 18-HEPE to RvE1, as well as formation of the dihydroxy compound (data not shown). We then assessed whether isolated 5-lipoxygenases could convert 18-HEPE to this new product via insertion of molecular oxygen at the carbon 5 position. When analyzed by RP-HPLC, a significant peak at λ_{max} = 237 nm appeared at an earlier retention time than 18-HEPE, from human recombinant 5-lipoxygenase incubation (Figure 2B).

After separation via HPLC, the samples were subjected to analysis on LC-UV-MS-MS to establish the presence of RvE1, as well as to assess the structure of the compound. The MS-MS spectra (Figure 2B, UV spectrum insert and structural fragmentation) are con-sistent with a novel 5S,18-dihydroxy-EPE with corresponding fragments at m/z 333 (M-H), 315 (M-H-H_2O), 297 (M-H-2H_2O), 289 (M-H-CO_2), 271 (M-H-H_2O-CO_2), and 253 (M-H-2H_2O-CO_2), as well as diagnostic ions for carbon 5 and carbon 18 at m/z 303, 275, 257 (275-H_2O), 246, 217, and 115. The UV spectrum of the compound gave a MeOH λ_{max} at 237 nm, consistent with the presence of a conjugated diene structure that is distinct from the dual chromophore of RvE1 [16].

LC-UV-MS-MS analysis of the product from enzyme incubation assigned the major peak as a 5S,18-dihydroxy-EPE and an 8,18-dihydroxy-EPE as a minor product (data not shown). The fragmentation pattern of 5S,18-dihydroxy-EPE was essentially identical to that obtained from human PMN incubations (Figure 2B). Potato 5-LO also generated the same 5,18-diHEPE, which was confirmed with tandem MS fragmentation pattern comparison (data not shown). We next isolated the novel 5,18-diHEPE to assess its potential biological activity compared to RvE1.

RvE2 Reduces Neutrophil Infiltration in Murine Peritonitis

To determine the potential biologic properties of RvE2, we isolated the compound as described in the legend of Figure 2 and compared it to RvE1 in a widely used model of murine peritonitis (Figure 3). Because of the potent bioactions and anti-inflammatory properties of RvE1 [16–18], we intravenously treated mice separately with RvE1 and RvE2 as a comparative side-by-side analysis of the potency of RvE2. When used intravenously, very low doses of RvE1, at only 1 ng per mouse, stopped PMN infiltration by 15.1% ± 5.7% and 19.8% ± 3.2% and by 44.5% ± 3.2% at 10 and 100 ng per mouse, respectively (Figure 3). A direct side-by-side comparison showed that RvE2 reduced PMN infiltration by 11.3% ± 4.8%, 17.7% ± 9.9%, and 33.7% ± 5.0% at 1, 10, and
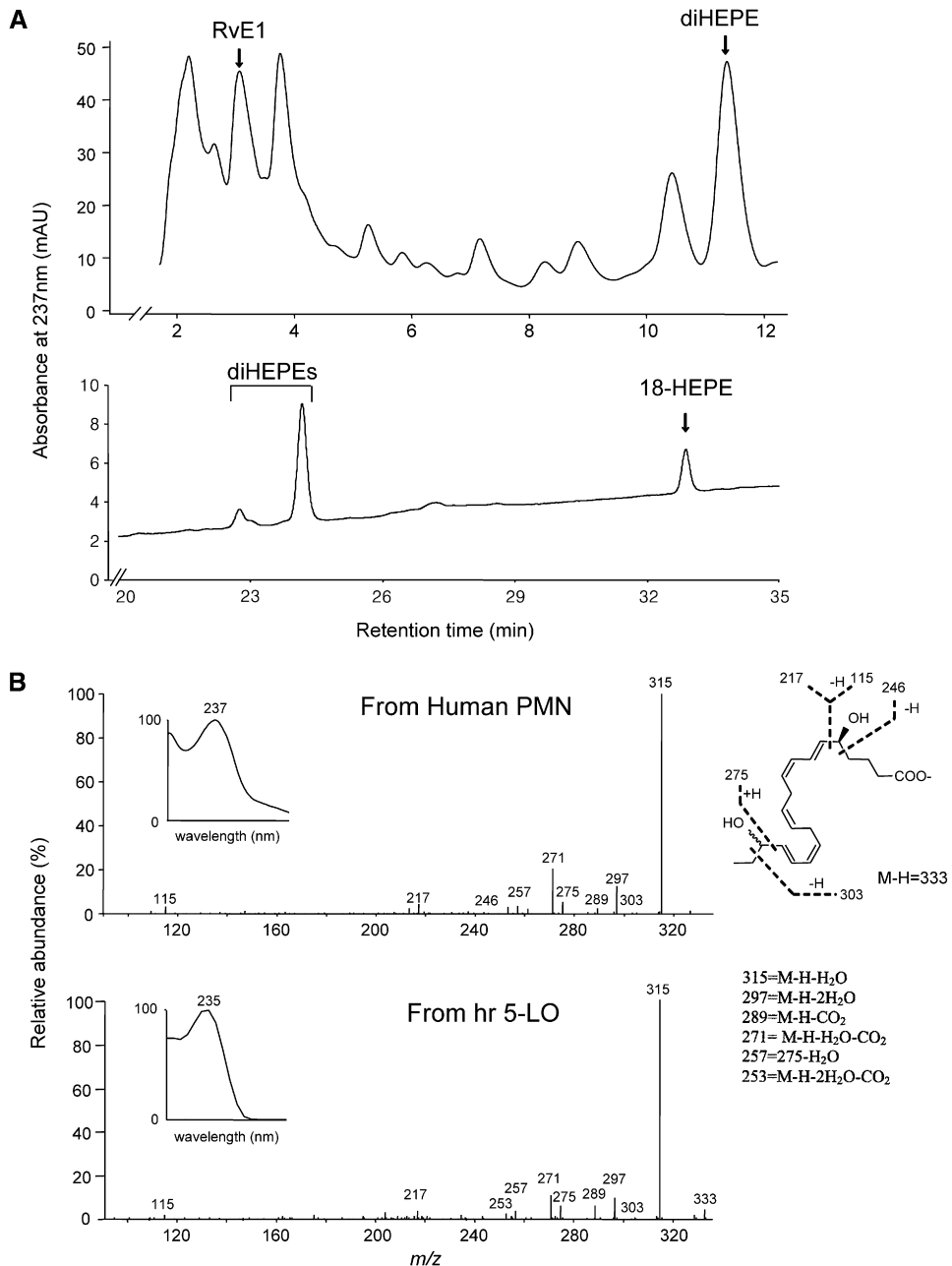
Figure 2. Generation of diHEPEs from Cell and Enzymatic Incubations

(A) RP-HPLC chromatogram of the 18-HEPE incubation product with human PMN (upper plot) and human recombinant 5-LO (lower plot), monitored with UV absorbance at 237 nm.

(B) UV and tandem mass spectra of major products isolated from human PMN (upper) and human recombinant 5-LO (lower) incubation, assigned as 5,18-diHEPE. See Experimental Procedures for conditions of analysis with LC-UV-MS-MS.

100 ng, respectively. RvE2 was not significantly different than RvE1 at any of the doses (p > 0.05). Both RvE1 and RvE2 significantly reduced PMN infiltration in zymosan-treated mice (p < 0.05) and were additive when given together (Figure 3B). In order to assess the mode of action of RvE2, we next compared two different routes of administration. At 10 ng dose per mouse, RvE2 was active via both tail vein (intravenous administration) and via intraperitoneal injection, reducing PMN infiltration by 47.6% ± 6.6% and 34.5% ± 4.5%, respectively (Figure 3C). For the purpose of direct comparison, RvE1 at

these doses did not significantly stop PMN recruitment when administrated intraperitoneally, while it displayed potent actions when administered intravenously. Similar results were obtained with RvE1 when administered in either its free acid or carboxylic methyl ester form (Figure 3D).

Biosynthesis via 5(6)-Epoxide EPE Intermediate and Trihydroxy-EPEs
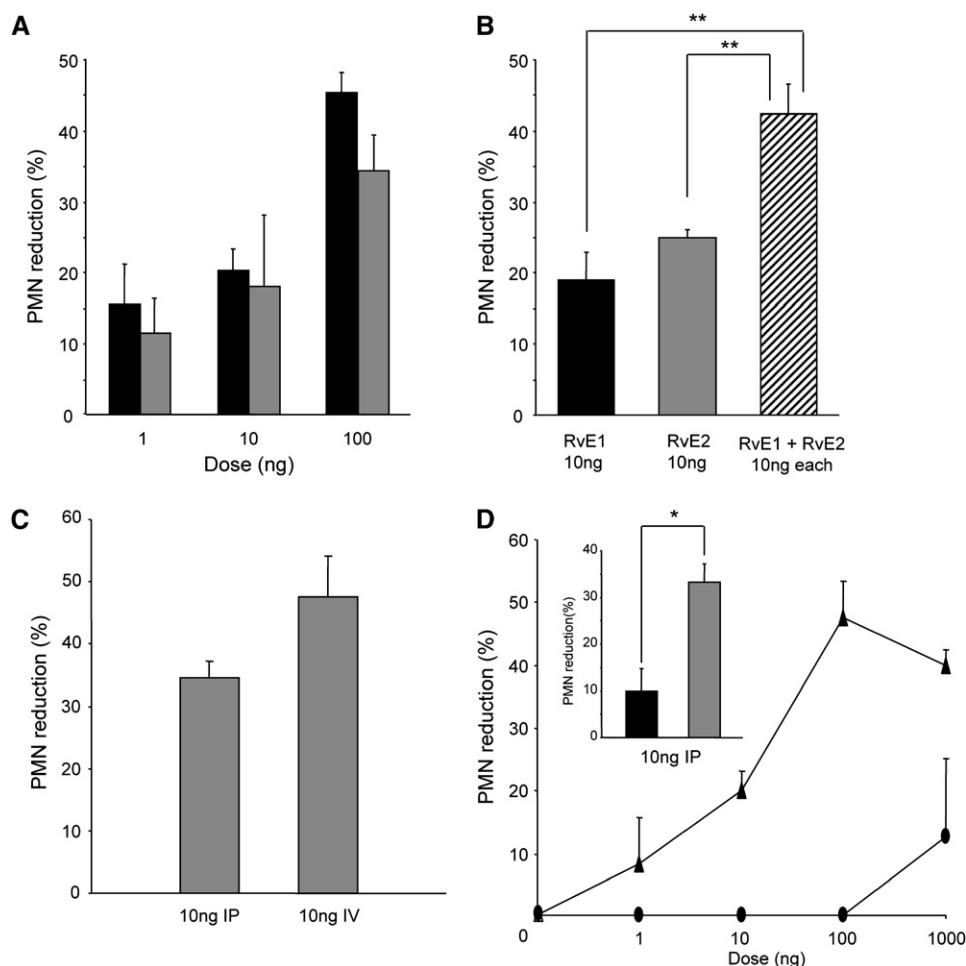When 18-HEPE was incubated with human recombinant 5-LO, several compounds carrying dual chromophores

Figure 3. Anti-Inflammatory Actions of Resolvin E2

At t = 0, 1 mg Zymosan A was injected into mouse peritoneum, immediately followed by intravenous or intraperitoneal injection of resolvin E1 or resolvin E2. After 2 hr, mice were euthanized and peritonea were lavaged with DPBS. The number of PMN infiltrated was enumerated, and percent changes were determined.

(A) Direct comparison between resolvin E1 (black) and resolvin E2 (gray) at 1, 10, 100 ng doses administered intravenously.

(B) Additive bioaction of resolvin E1 and resolvin E2. With the same procedure as that described in (A), 10 ng RvE1, 10 ng RvE2, or 10 ng of each compound were administered intravenously.

(C) Comparison of RvE2 bioaction when administered intravenously and intraperitoneally. A total of 10 ng RvE2 was delivered intravenously or intraperitoneally.

(D) Dose-response curve of RvE1 intraperitoneal administration. 1, 10, 100, and 1000 ng RvE1 methyl ester were delivered into the peritoneum. Inset; 10 ng RvE1 (black) or RvE2 (gray) was delivered intraperitoneally. Results are the mean ± SEM, $n \geq 3$ at each dose of RvE1 or RvE2 for all values. *, $p < 0.02$; **, $p < 0.001$.

of diene-triene structure (cf. [18]) were evident in UV-RP-HPLC analyses. Eluting earlier than confirmed dihydroxy-EPEs, these possible trihydroxy-EPE isomers are categorized as (1) multiple peaks with identical, dual chromophores at 235 nm and 269 nm, eluted at a retention time of 10.5–12.0 min (compound I) and as (2) a single peak with dual chromophores at 235 nm and 275 nm, eluted at 17 min (see Figure 4A) (compound II). With LC-UV-MS-MS analysis, the former is identified as 5,12,18-trihydroxy-EPE, and the latter as 5,6,18-trihydroxy-EPE.

The MS-MS spectrum (Figure 4B, UV spectrum insert and structural fragmentation) of compound I is assigned as 5,12,18-trihydroxy-EPE, with corresponding fragments at m/z 349 (M-H), 331 (M-H-H_2O), 313 (M-H-2H_2O), 305 (M-H-CO_2), 295 (M-H-3H_2O), as well as diag-

nostic ions for fragmentation at carbons 12 and 18 at m/z 195 and 291, respectively. Similar neutral loss fragments were identified from the tandem MS spectrum of compound II (Figure 4C), with additional diagnostic fragments for the vicinal diol at m/z 233 and 203. In addition, the presence of a triene chromophore with a triplet absorption of $\lambda_{max} = \sim 275$ nm assigned compound II's structure as 5,6,18-trihydroxy-EPE. Despite similar tandem mass spectra to RvE1, the 5,12,18-trihydroxy-EPE obtained from enzymatic incubations was considered the likely nonenzymatic hydrolysis products of a 5(6)-epoxide intermediate bearing a 6-*trans* configuration. This is supported by the fact that (1) this product bears a slightly shorter λ_{max} (269 nm), which is characteristic of a synthetic 5,12-all-*trans*-conjugated triene of RvE1 [16], and the fact that (2) none of these products
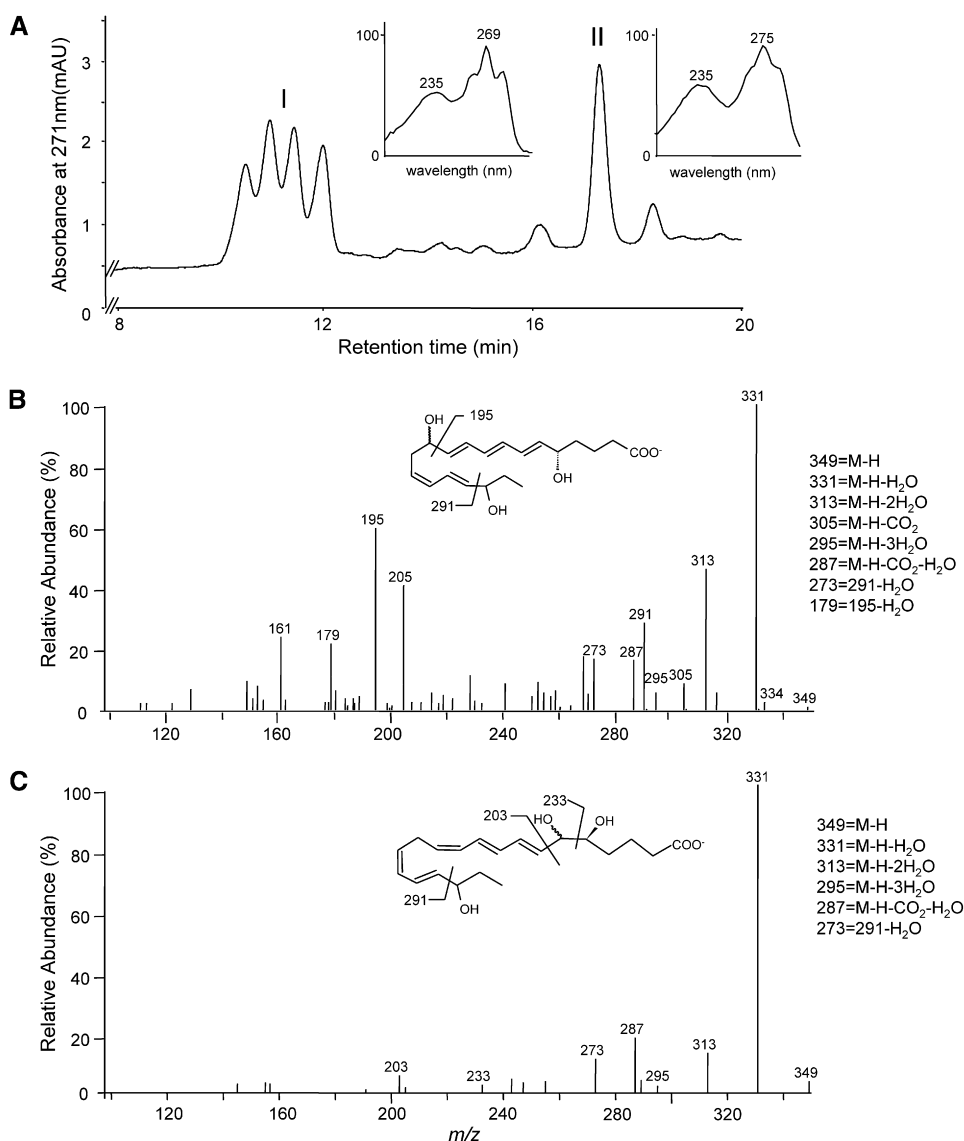
Figure 4. Human Recombinant 5-LO Generates Epoxide Intermediate and Hydrolysis Products

(A) UV chromatogram of dual chromophore-containing compounds. Incubation products were analyzed with the same conditions used for diHEPEs on RP-HPLC (see Figure 2 and Experimental Procedures for detailed conditions). I represents four peaks bearing diene-triene dual chromophores at 235 nm and 269 nm, respectively. II represents a single peak bearing diene at 235 nm and elongated triene chromophore at 275 nm.

(B) LC-UV-MS-MS analysis result of I, assigned as 5,12,18-trihydroxy-EPE.

(C) LC-UV-MS-MS analysis result of II, assigned as 5,6,18-trihydroxy-EPE.

coeluted with synthetic RvE1 [18], which carries the 6-cis double bond critical for its potent bioactivity (data not shown).

Acidic methanol trapping and work-up gave a major peak at 15.9 min (Figure 5B), which showed diagnostic ions consistent with 12-methoxy-5S,18-dihydroxy-EPE. The minor peak at 20.5 min is likely a 6-methoxy-5S, 18-dihydroxy-EPE, although not enough diagnostic fragmentations were present to assign its structure.

RvE2 Production by Human PMNs in Hypoxia
In normal oxygen levels, zymosan treatment did not apparently alter RvE2 generation by PMNs. Of interest, PMNs exposed to the phagocytic stimulus in hypoxic conditions showed a tendency to produce more RvE2

(Table 1) vide infra. However, this increase did not prove to be statistically significant.

The results from this report indicate that RvE2 exhibits potent anti-inflammatory properties similar to those of RvE1. RvE2 stops PMN infiltration in vivo at near the potency level of RvE1, which was in the nanogram range in vivo. The present findings also demonstrate that RvE2 is generated in vivo and thus may act as an endogenous anti-inflammatory mediator generated from EPA. When directly compared to RvE1 generation, greater amounts of the initial 5-hydroperoxide are reduced to RvE2 in incubations with human leukocytes carried out in hypoxic conditions (Figure 2 and Table 1). A hypoxic local environment is encountered at local sites of inflammation, within exudates, and during ischemic reperfusion injury
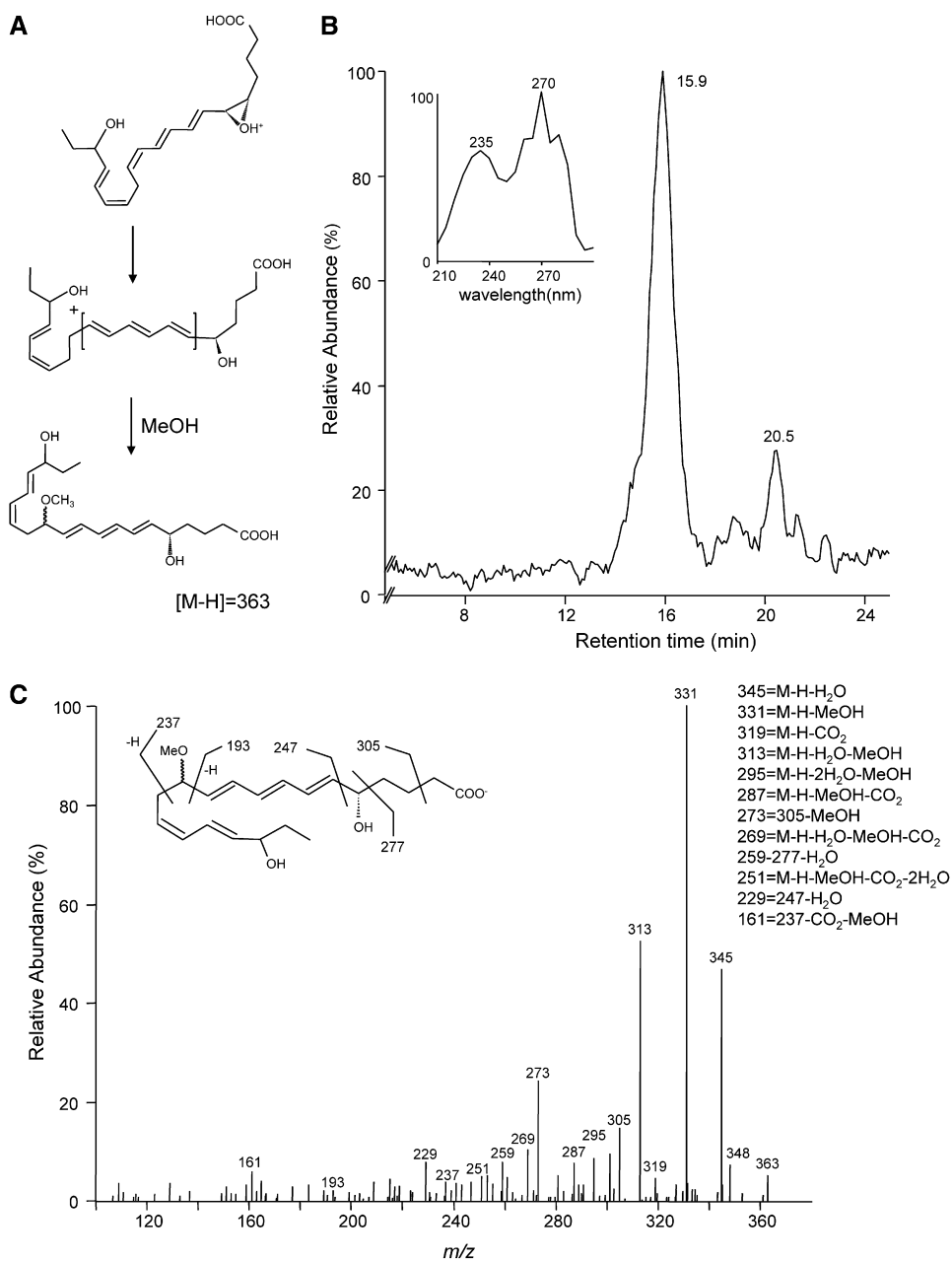
Figure 5. Identification of Methoxy-Trapping Products

(A) Proposed mechanism of acidic O-methoxy product generation. In acidic conditions, 5S(6)-epoxide is protonated and opened to become a carbocation intermediate. Excess MeOH concentration (MeOH:H_2O ~10:1 in this procedure) prefers the addition of a methoxy group to carbocation. Stereochemically favored, the 1,8 addition to produce the 12-methoxy adduct is the major product (MW = 364).

(B) Selective ion chromatogram at m/z 363 with LC-UV-MS-MS and UV spectrum of a peak at a retention time of 15.9 min on this chromatogram.

(C) Tandem mass spectrum of the peak eluted at 15.9 min.

of second organs [1]. In hypoxia chambers, human vascular endothelial cells that were treated with aspirin avidly converted extracellular sources of EPA to 18-HEPE, which is further transformed to RvE1 (Figure 1) by activated human leukocytes [18]. In the present experiments, the hypoxic environment with activated human leukocytes gave a trend toward enhanced biosynthesis of RvE2 (Table 1). Hence, regulated production of RvE1 and RvE2 might still maintain the overall anti-inflammatory activity of this biosynthetic pathway with

EPA as a substrate. Because of its protective anti-inflammatory in vivo action and significant quantities relative to those of RvE1, RvE2 may also share with RvE1 the key regulator role during resolution of this biosynthetic circuit of acute inflammation. Also, as demonstrated here, the anti-inflammatory properties of RvE2 were similar to those of RvE1 when given by intravenous administration. Of interest, when delivered directly to the site of inflammation (intraperitoneally), RvE2 also reduces PMN infiltration and, at equal doses, proved more potent

Table 1. RvE1 and RvE2 Generation by Human PMNs in Normoxic and Hypoxic Environments

Incubation Condition[a]	RvE1, ng	RvE2, ng
Normoxic PMN	0.29	8.05
Normoxic PMN treated with zymosan	0.11	8.15
Hypoxic PMN	0.17	7.61
Hypoxic PMN treated with zymosan	0.12	10.86
Without PMN	0.00	0.00

[a] Results are from human PMN incubations, representative of n = 3 separate donors. PMNs were isolated from human whole blood and were incubated with 5 μg 18-HEPE or activated first with zymosan (100 μg/1 ml) and then incubated with 18-HEPE (5 μg, 37°C, 30 min [pH 7.45]). The same procedures were carried out in parallel in a hypoxia chamber (see Experimental Procedures). After incubations, products were extracted, identified, and quantified by using internal standard and LC-MS-MS-based analysis.

than RvE1, which in this dose range was not as active intraperitoneally. These results suggest that RvE2 has specific target cell types that are distinct from those of RvE1.

Results of the present report demonstrate the identity and structure of RvE2 by using physical methods such as UV spectrometry and MS-MS analyses of the generated products from neutrophil incubations. Earlier results [21] demonstrate that 5-lipoxygenase, with EPA as a substrate, promotes oxygenation at the carbon 5 position of EPA and inserts molecular oxygen in as the S configuration [22]. To establish the structural components of RvE2, we examined in vitro generation with isolated 5-LO and 18-HEPE and documented the insertion of oxygen into the carbon 5 position generating the 5S alcohol group in RvE2. During incubations with isolated human recombinant 5-LO, we also obtained both 5,12,18-triHEPE and 5,6,18-triHEPE, which were formed via aqueous hydrolysis of a 5(6)-epoxide-containing intermediate (see Figure 6). Along with confirming the formation of an epoxide-containing intermediate, we confirmed that human 5-LO also serves as an epoxide synthase in the E series resolvin pathways. It is noteworthy that RvE1 was identified only with human leukocyte incubations and was not obtained in substantial amounts with isolated recombinant 5-LO. These findings strongly indicate that enzymatic hydrolysis of the epoxide intermediate is required for the generation of the potent RvE1 that carries the correct double bond geometry and chirality for the carbon-12-position alcohol. The epoxide-containing intermediate's hydrolysis to RvE1 requires changing from trans to 6-cis regiochemistry, a reaction that likely requires a specific enzyme (see Figure 6B and cf. [16]).

These lipid mediators, namely, resolvins and protectins, actively regulate the resolution of inflammation via novel molecular circuits [7]. As reported here, the anti-inflammatory properties of RvE2 are slightly less potent than those of RvE1, but they are still in the nanomolar range. Thus, it is likely that these lipid mediators, namely, E series resolvins from EPA, may provide a basis for endogenous protection against an aberrant or uncontrolled innate inflammatory response, as well as provide a molecular basis for the beneficial actions of EPA and fish oils noted in human studies. Given the nature of the dihydroxy-EPE products identified here, RvE2 may be partic-

ularly attractive because of the ease of its synthesis as well as its potent bioactions in regulating inflammation. Taken together, the generation of RvE2 in vivo and in vitro provides evidence for a novel lipid mediator derived from EPA and human neutrophils that carry anti-inflammatory properties and the biosynthesis of RvE2 and RvE1 as bioactive mediators generated from the omega-3 fatty acid EPA.

The contribution of 5-LO to biosynthesis of anti-inflammatory mediators such as E series resolvins suggests potential protective roles of this lipoxygenase in addition to its widely appreciated role in leukotriene biosynthesis and production of proinflammatory mediators [8, 9]. This result is consistent with recent reports that document exacerbated inflammatory responses in 5-LO-deficient mice in several disease models. These include more severe disease in EAE (experimental allergic encephalomyelitis) in 5-LO-deficient mice [23] and elevated inflammatory cytokine production in tuberculosis [24], which indicate that 5-LO can produce protective mediators in situ that may include lipoxins and resolvins. Thus, as a therapeutic approach, inhibitors of 5-LO may not come without unwanted side effects; recent concerns have been raised regarding adverse effects with COX-2 inhibitors [25]. The overall pro- or anti-inflammatory role(s) of 5-LO appears to be determined via substrate availability (i.e., arachidonic acid for leukotrienes or EPA converted to E series resolvins) during the time course of an inflammatory response. Hence, it might be useful to consider resolvins such as RvE2 as endogenous agonists of anti-inflammation and as potential new therapeutics.

Significance

Omega-3 PUFAs are considered to be beneficial in human health and disease. However, the molecular basis of EPA's anti-inflammatory actions still remains of interest. Results of the present study demonstrate the identification and potent bioactions of resolvin E2, a newly characterized member of the recently uncovered EPA-derived pathways that generate anti-inflammatory chemical mediators termed resolvins. Human 5-lipoxygenase is demonstrated here to be a pivotal enzyme in the conversion of EPA to these potent, active mediators, by catalyzing the initial 5-hydroperoxide generation of 18-hydroxy-eicosapentaenoic acid and its subsequent 5S(6)-epoxidation, two steps that are essential in resolvin E1 biosynthesis. Resolvin E2 was equipotent to the original member, intravenous resolvin E1, and was additive at low doses, indicating two separate anti-inflammatory cascades. Human neutrophils biosynthesized RvE2 in greater amounts than RvE1, suggesting a significant role for RvE2, which now provides anti-inflammatory approaches that may be useful in treating a wide range of inflammatory disorders.

Experimental Procedures

Human Neutrophil Isolation
Human whole (venous) blood (60 ml) was collected with heparin from healthy volunteers (who declined taking medication for at least 2 weeks before donation; Brigham and Women's Hospital protocol
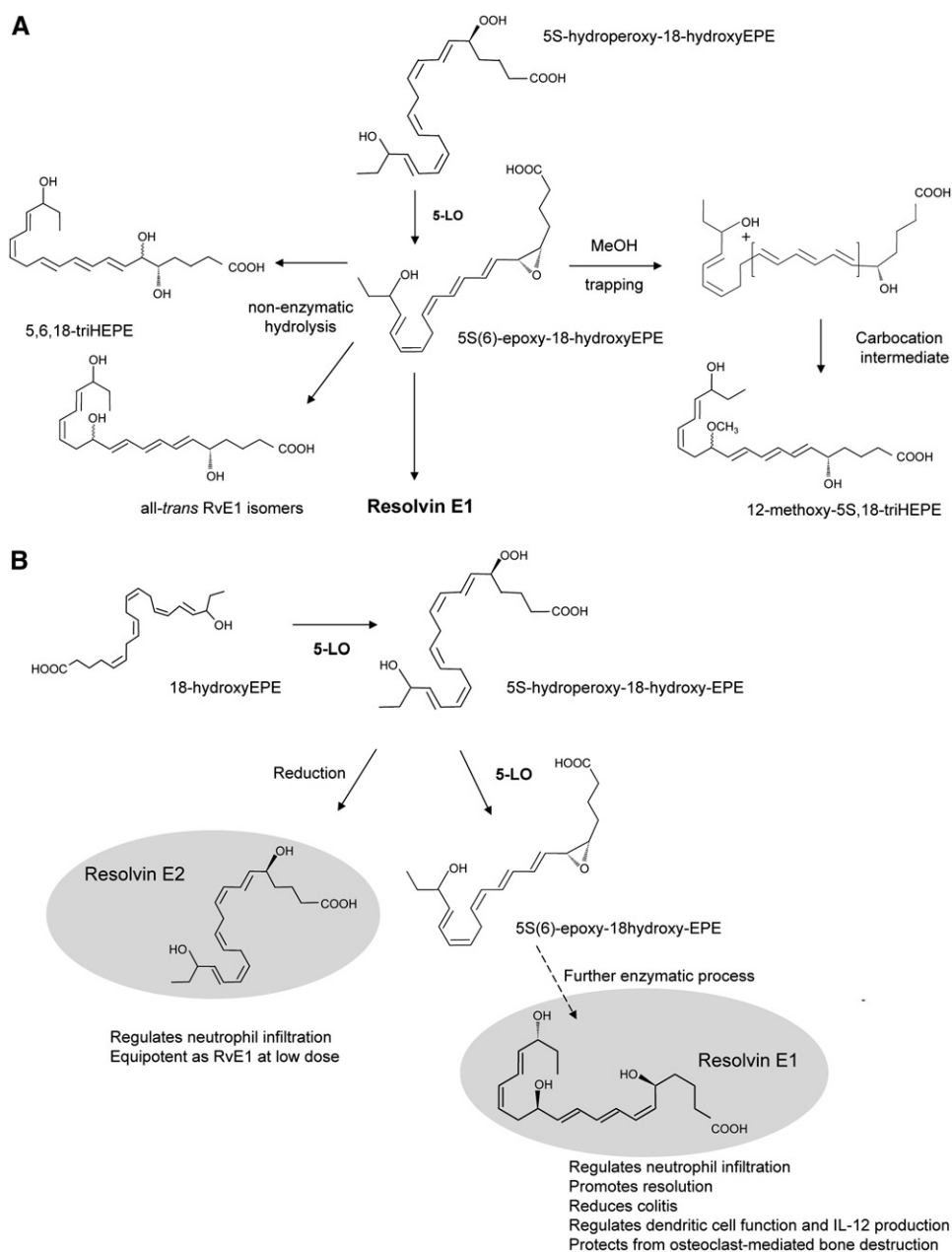
Figure 6. Biosynthesis of E Series Resolvins and the Role of Human 5-Lipoxygenase

(A) The 5-LO biosynthesizes the 5(6)-epoxy intermediate, which can be nonenzymatically hydrolyzed to all-*trans* isomers depicted here. The epoxide intermediate can be identified by acidic methanol trapping (see text).

(B) Human 5-lipoxygenase in the biosynthesis of both resolvin E1 and resolvin E2, potent anti-inflammatory lipid mediators.

88-02642). Human neutrophils (PMNs) were freshly isolated from the whole blood by Ficoll gradient and were enumerated as described in [19]. PMNs were also permeabilized by using rapid freezing in a dry ice-isopropanol bath, were thawed to room temperature, and were taken immediately for incubations, as described in [26]. Both intact and permeabilized PMNs were used within 2 hr of isolation.

Human Neutrophil Incubations
PMNs were divided into 50×10^6 cells in 1 ml DPBS with Ca^{2+} and Mg^{2+}. Intact PMNs were incubated with 18*R/S*-HEPE (16 μg, Cayman Chemical) and with or without calcium ionophore (A23187, 2.5 μM), while permeabilized PMNs were incubated with 18*R/S*-HEPE (16 μg, Cayman Chemical) and with or without ATP (Sigma-Aldrich, 1 mM). All incubations were carried out for 30 min in a 37°C water bath and were then stopped with two volumes of cold methanol.

Human PMN Incubations in a Hypoxic Environment
PMNs for hypoxia experiments were separated into 10×10^6 cells in 0.5 ml DPBS with Ca^{2+} and Mg^{2+}. Media for hypoxia incubations were preconditioned for 24 hr in a hypoxic chamber (COY laboratory products), with 1% oxygen with nitrogen, carbon dioxide (5%), and water vapor. Cells were conditioned (37°C/5% CO_2) for 60 min at pH 7.45 in an incubator at normoxic conditions or in a hypoxic chamber. Cells were then incubated for 30 min with 5 μg EPA (Cayman Chemical) or 18-HEPE or were activated first with 50 μg zymosan (Sigma-Aldrich) and then incubated with substrate. The reactions were stopped with two volumes of ice-cold methanol.

Enzymatic Conversion of 18-HEPE with Human 5-Lipoxygenase
Potato or human recombinant 5-lipoxygenase (Cayman Chemical) was incubated with 18*R/S*-HEPE (Cayman Chemical) at 37°C, in

the presence of CaCl$_2$ and ATP as activators [26, 27]. After 30 min, the reaction was quenched with two volumes of cold MeOH, and hydroperoxide intermediates were reduced with excess NaBH$_4$.

MeOH Trapping

The reaction mixture was poured on 10× volume of acidic ice-cold methanol (pH 3 after mixing) containing d5-PGE$_2$ as an internal standard. A total of 5 min later, this acidic methanol solution was diluted with 5 ml MiliQ water, the pH was adjusted to 3.5, and the sample was directly loaded on a solid-phase extraction column.

High-Pressure Liquid Chromatography and LC-MS-MS Analysis

Incubations were extracted along with an internal standard, by using C18 solid-phase extraction (Alltech Associates). To separate and quantify conversion products of 18-HEPE, RP-HPLC was carried out by using a 1100 series liquid chromatography system (Agilent) with a reverse-phase Luna C18(2) column with dimensions of 150 mm × 2 mm × 5 μm (Phenomenex). A gradient from 55:45:0.01 to 85:15:0.01 (MeOH:H$_2$O:acetic acid) was used as the mobile phase.

Tandem MS-based structure analysis of lipid mediators was performed to determine the production of methanol-trapped products and other 18-HEPE-derived oxygenated products by using a Finnigan LCQ liquid chromatography ion trap tandem mass spectrometer (San Jose, CA) equipped with a Luna C18(2) (100 mm × 2 mm × 5 μm) column, and a photodiode array detector that monitored UV absorbance ∼0.1–0.2 min before samples entered the MS-MS [7].

Murine Peritonitis

Peritonitis was performed with 6- to 8-week-old FVB male mice (Charles River Laboratories) fed laboratory Rodent Diet 5001 (Purina Mills). After anesthetization with isoflurane, compounds or vehicle was administered intravenously via tail vein injections or intraperitoneally. Each test compound or vehicle alone was suspended in 5 μl ethanol, mixed in 120 μl sterile saline, and rapidly injected either intravenously or intraperitoneally. Zymosan A (Sigma-Aldrich) was suspended in 1 ml saline (1 mg/ml) and injected intraperitoneally immediately following compound administration. A total of 2 hr after the zymosan injection, in accordance with Harvard Medical Area Standing Committee on Animals protocol no. 02570, mice were sacrificed with an overdose of isoflurane. Peritoneal lavages were collected and enumerated via light microscopy. Differential leukocyte counts were performed by using a modified Wright-Giemsa stain (Sigma-Aldrich) and were differentiated via light microscopy.

Statistical Analysis

All results in the figures and text are expressed as mean ± SEM of n ≥ 3 mice per group. Statistical significance was determined by Student's t test; $p < 0.05$ was considered significant.

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