Resolvins E1 and D1 in Choroid-Retinal Endothelial Cells and Leukocytes: Biosynthesis and Mechanisms of Anti-inflammatory Actions

Haibin Tian,1,2 Yan Lu,1,2 Alexander M. Sherwood,1 Dawuti Hongqian,1 and Song Hong1

PURPOSE. To investigate the biosynthesis of resolvins E1 and D1 (RvE1 and RvD1) in choroid-retinal endothelial cells (CRECs) and leukocytes under inflammatory conditions and to define the mechanisms of anti-inflammatory actions of RvE1 and RvD1 in CRECs and leukocytes, cells crucial to posterior ocular inflammation.

METHODS. RvE1, RvD1, and markers of their biosynthesis were determined by lipidomic analysis. After CRECs or cocultures of CRECs and leukocytes were treated with RvE1 or RvD1 and inflammatory stimuli, inflammatory signaling molecules were quantified by Western blot analysis, ELISA, or protein array. Transmigration of polymorphonuclear leukocytes (PMNs) across CRECs monolayers was quantified.

RESULTS. Inflammatory stimulation increased the biosynthesis of RvE1 and RvD1 from eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), respectively, in coculture of CRECs and leukocytes. CRECs alone did not produce RvE1 and RvD1. RvE1 or RvD1 inhibited the expressions of vascular cell adhesion molecule-1, IL-8, macrophage inflammatory protein-1β, regulated on activation normal T cell expressed and secreted, and tumor necrosis factor-α from CRECs or cocultures of CRECs and leukocytes. RvD1 reduced prostaglandin E2 generation from CRECs. However, neither resolvin affected cyclooxygenase-2 formation. Treating CRECs or PMNs with RvE1 or RvD1 inhibited PMN transmigration across CREC barriers.

CONCLUSION. The interplay of inflammatory stimuli-activated CRECs and leukocytes biosynthesizes RvE1 and RvD1 from EPA and DHA. These resolvins inhibit inflammatory signaling from CRECs and leukocytes and inflammatory activity as PMN transmigration across CRECs barriers. Thus, these resolvins and their biosynthesis pathways are potential targets for novel treatment of inflammatory ocular diseases. (Invest Ophthalmol Vis Sci. 2009;50:3613–3620) DOI:10.1167/iovs.08-3146

Inflammation is a self-defense response of the body against pathogens and injuries. In healthy conditions, the body generates anti-inflammatory substances to halt inflammatory activities, allowing for resolution back to the noninflamed state (homeostasis).1,2 The inflammation process in the posterior of the eye is orchestrated by endothelial cells, leukocytes, retinal pigment epithelium (RPE), retinal neurons, glial cells, and other types of cells present. These cells interact with each other and communicate through signaling molecules, such as lipid mediators and cytokines. Leukocyte infiltration and activation are the key events of inflammation and the consequent pathologic conditions.5 It is well known that leukocytes adhere to, roll along, and infiltrate the endothelial wall of blood vessels during inflammation. Leukocytes release reactive oxygen species to the site attacked by pathogens or injury, causing inflammation. The important roles of inflammation have been observed in posterior eye diseases such as age-related macular degeneration (AMD)5 and uveitis.6 Choroidal neovascularization (CNV) associated with AMD develops after inflammatory events in RPE, Bruch membrane, and choriocapillaris,7 which involves leukocytes.7–9 During CNV progression, an array of inflammation-related factors, including lipid mediators, cytokines, and growth factors, are generated by local inflammation and the accompanying leukocytes.7–9

Recently, a novel family of anti-inflammatory lipid mediators derived from ω-3-eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) was discovered during inflammation and resolution processes. Among these mediators are resolvin E1 (RvE1) or 5,12R,18R-trihydroxy-6Z,8E,10E,14Z,16E-eicosapentaenoic acid)10 from EPA and resolvin D1 (RvD1, 7S,17S-trihydroxy-4Z,9E,11E,13Z,15E,19Z-docosahexaenoic acid)11,12 from DHA. RvE1 and RvD1 have potent anti-inflammatory activities in many models of diseases, such as colitis,13–15 periodontitis,14 acute kidney injury,15 and peritonitis.16 EPA and DHA are the two primary ω-3 polyunsaturated fatty acids in fish oil that have significant beneficial effects in the treatment of many inflammatory diseases. DHA is highly enriched in the retina, brain, and neuronal synaptic membranes and has been critical for neural survival, function, and protection against inflammatory damage.17,18 Clinical epidemiologic data also indicate that diet enriched with ω3 fatty acids, especially DHA and EPA, significantly reduces the risk for AMD.19 This diet also decreases pathologic retinal angiogenesis.20 RvE1 and RvD1 display potent protection against angiogenesis.20 The protective effect of DHA and EPA is mediated partially through the suppression of tumor necrosis factor (TNF-α), colocalized with microglia, macrophages, or both, suggesting that the antiangiogenesis of DHA, EPA, and their bioactive metabolites RvE1 and RvD1 is also mediated through the reduction of local inflammation and inflammatory activities of leukocytes.20 Two decades ago it was found that DHA is oxygenated in retina by lipoxigenases.21

Additionally, increasing the intake of EPA ameliorates CNV and reduces vascular endothelial growth factor (VEGF), intercellular adhesion molecule (ICAM)-1, monocyte chemotactic protein (MCP)-1, and interleukin (IL)-6 in the RPE-choroid complex, inflammatory IL-6, and C reactive-protein in serum.22 It is possible that an EPA-derived product such as endogenous anti-inflammatory RvE1 may be responsible for these actions. These cytokines are important signaling mechanisms in inflammation. Among cytokines, chemokines attract leukocytes to
infiltrate the vascular endothelium. Cytokines and cell adhesion molecules expressed by endothelial cells and leukocytes play critical roles in the recruitment and binding of leukocytes to vascular endothelium.

Given that CRECs and leukocytes are the key elements in posterior ocular inflammation and diseases such as AMD, there are two important questions that must be addressed: Can RvE1 and RvD1 be biosynthesized from EPA and DHA by CRECs or by the interplay of CRECs and leukocytes under inflammatory conditions? Do these two resolvins counterregulate inflammatory signals and activities of CRECs and leukocytes? Here, we set out to find answers to these questions that until now have not been directly approached. The answers may provide fundamental knowledge for developing therapeutic strategies for the treatment of ocular inflammatory diseases.

We used a model of PMN transmigration across the CREC monolayer and in vitro generation of inflammatory factors by CRECs and PMNs. The CREC line was originally derived from a rhesus macaque fetus. Although the origin of the cell line could not be assigned to choroid or retina, results from a published report showed that the expressions of two VEGF receptors, fms-like tyrosine kinase (Flt-1) and kinase insert domain receptor (KDR), were the same as in bovine choroidal endothelial cells but different from those expressed in bovine retinal microvascular endothelial cells, suggesting that CRECs have characteristics more similar to those of choroid endothelial cells than of retinal vascular endothelial cells. CRECs have been confirmed as a useful cell line for studying the mechanisms of AMD pathogenesis. Because CRECs were derived from primate species, which are closer to humans than to rodents, we chose to use this cell line in our study. The assay for PMN transmigration across the endothelial monolayer is commonly used by peers to study inflammation.

PMN transmigration simulates PMN infiltration from blood vessels to inflammatory tissues. Inflammatory stimuli activate endothelial cells and PMNs to produce inflammatory cytokines and chemokines, escalating inflammation in vivo. IL-1β is an important proinflammatory factor in vivo that can induce leukocyte recruitment to the site of inflammation. It can also stimulate human vascular endothelial cells to promote transendothelial neutrophil passage. Therefore, we used IL-1β to stimulate CRECs for simulating PMN infiltration in vivo during the early phase of inflammation.

**Materials and Methods**

**Materials**

DHA, EPA, 5-hydroxy-docosahexaenoic acid (HDHA), 17-HDHA, 5-hydroxy-ecosapentaenoic acid (HEPE), 18-HEPE, resolvin D1, and resolvin E1 were purchased from Cayman Chemical (Ann Arbor, MI). Escherichia coli lipopolysaccharide, TNF-α, and IL-1β were supplied by Sigma (St. Louis, MO). All other reagents were from commercially available sources.

**Mediator Lipidomic Analysis**

Mediator lipidomic analysis was conducted according to the protocol in our previous publications. In brief, it was performed using a liquid chromatography-mass spectrometry-ultraviolet detector-LTQ linear ion trap tandem mass spectrometer (LC-UV-LTQ linear ion trap tandem MS/MS; Thermo, Waltham, MA) equipped with an LC column (150 mm × 2 mm × 5 μm, LUNA C18-2; Phenomenex, Torrance, CA). The mobile phase flows at 0.2 mL/min. The mobile phase eluted as B (methanol/H2O/acetic acid = 60:39:99.01) from 0 to 30 minutes, ramped to methanol from 30.1 to 60 minutes, flowed as methanol for 15 minutes, and finally flowed as B again for 10 minutes. Wideband activation was used for full-scan mode of the MS/MS. Deuterium-labeled internal standard d4-PGD2 (50 ng) was used for each sample. Lipid mediators were extracted and purified according to our established procedures. Briefly, cells and medium of each sample were combined. After the pH of each sample was adjusted to 3.5 with the addition of HCl (1 M) (on ice), 3 vol ice-cold methanol was added into each sample (sample/methanol = 1:3 by volume). Each mixture was vortexed well, then sonicated in a water bath (4°C). After centrifugation (3000 rpm, 15 minutes, 0°C), each pellet was extracted twice with 3 vol methanol (pellet/methanol 1:3). Supernatants from each sample were pooled together and adjusted to the content of 10% methanol with the addition of water, then cleaned up by C18 solid-phase extraction (500 mg; Varian, Palo Alto, CA). Final extracts were reconstituted to methanol for lipidomic analysis. The average recovery for resolvins and internal standard was 85%. The linear range of concentration response for the relevant lipid mediators with the presently used analytical equipment was 50 pg to 100 ng.

**CREC Culture**

The Macaca mulatta CREC line RF/6A (ATCC, Manassas, VA) was cultured in Dulbecco modified Eagle medium (DMEM; Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (Invitrogen), glutamine (Invitrogen), penicillin, and streptomycin (Invitrogen; 37°C, 5% CO2). In these studies, cells from passages 36 to 40 were used.

**Human Leukocyte Isolation**

Human leukocyte-rich buffy coat was purchased from the Blood Center (New Orleans, LA). This buffy coat was freshly isolated from peripheral blood collected from a code-deidentified healthy donor who was not on medication for at least 2 weeks before donation, and the buffy coat was further isolated for monocytes and PMNs in accordance with our published procedures. PMNs and monocytes used for experiments had at least 98% purity by Hoescht 33342 (Sigma) staining and 95% viability by trypan blue (Sigma) exclusion.

**Cellular Biosynthesis of RvE1, RvD1, and Pathway Markers**

To study the role of CRECs in the biosynthesis of RvE1 and RvD1 and its pathways, confluent CRECs (2 × 107 cells/each) were cultured in DMEM containing EPA or DHA (10 μM) for 30 minutes, then stimulated for 1 hour with TNF-α (10 ng/mL), IL-1β (10 ng/mL), and lipopolysaccharide (100 ng/mL). To determine the leukocyte contribution to biosynthesis, isolated human PMNs and monocytes (2 × 107 cells/each) were cultured, stimulated, and extracted as described for CRECs. In parallel, isolated PMNs and monocytes (2 × 107 cells/each) were cocultured with CRECs (2 × 107 cells/each). Coculture was stimulated and extracted as described for CRECs to determine biosynthesis through the interplay of CRECs and leukocytes. Cells and medium from each culture were extracted for lipidomic analysis.

**Enzyme-Linked Immunosorbent Assay and Protein Array**

When 80% confluence was reached in DMEM, CRECs (1 × 105 cells/each) were incubated with 50 nM RvE1 or RvD1 for 8 hours, then stimulated with 2 ng/mL IL-1β for 4, 12, and 24 hours. IL-8 and prostaglandin E2 (PGE2) in the supernatants were quantified by ELISA kit (human IL-8 ELISA kit; R&D Systems [Minneapolis, MN]; PGE2 ELISA kit [Cayman]). For 24-hour stimulation, macrophage inflammatory protein-1β (MIP-1β), regulated on activation of normal T cell expressed and secreted (RANTES), IL-6, and TNF-α in the supernatants were quantified with an array kit (Bio-Plex Protein for human; Bio-Rad, Hercules, CA). In another experiment, to mimic in vivo conditions, CRECs (1 × 105 cells/each), PMNs (1 × 105 cells/each), and monocytes (1 × 105 cells/each) were cultured together, treated with 50 nM RvE1 for 8 hours, and stimulated with 2 ng/mL IL-1β for 24 hours. Soluble vascular cell adhesion molecule-1 (sVCAM-1), MIP-1β, RANTES, TNF-α, and MCP-1 in the supernatant from the centrifugation of cell
culture were quantified with the array kit (Bio-Plex Protein for human; Bio-Rad). Cells untreated with resolvins were used as controls.

Western Blot Analysis

Western blot analysis was conducted according to published specifications. Western blot analysis was conducted according to published specifications.33 Briefly, protein extracts were obtained from CRECs (1 \times 10^5 cells/each) treated with RvE1 or RvD1 and IL-1β (H9252). Ten micrograms whole protein per sample was fractionated by electrophoresis on 4% to 15% Tris-HCl ready gel (Bio-Rad) and transferred to polyvinylidene fluoride membrane (Millipore, Billerica, MA). Blots were blocked in blocking buffer (LI-COR, Lincoln, NE) for 1 hour at ambient temperature and then incubated with primary antibody for mouse anti–human cyclooxygenase-2 (COX-2; Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti–human VCAM-1 (membrane-bounded form, Santa Cruz), and mouse anti–human actin (Santa Cruz Biotechnology) in blocking buffer at 4°C overnight. Membranes were then incubated with fluorescent-labeled secondary antibody (LI-COR) in blocking buffer for 1 hour at ambient temperature. Signals were scanned with an imaging system (LI-COR). The densitometry of acquired bands was measured with specialized software (Quantity One; Bio-Rad).

PMN Transmigration across CREC Monolayer Barriers

PMN transmigration was conducted according to published specifications.11,34 Briefly, protein extracts were obtained from CRECs (1 \times 10^5 cells/chamber) treated with RvE1 or RvD1 and IL-1β. Ten micrograms whole protein per sample was fractionated by electrophoresis on 4% to 15% Tris-HCl ready gel (Bio-Rad) and transferred to polyvinylidene fluoride membrane (Millipore, Billerica, MA). Blots were blocked in blocking buffer (LI-COR, Lincoln, NE) for 1 hour at ambient temperature and then incubated with primary antibody for mouse anti–human cyclooxygenase-2 (COX-2; Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti–human VCAM-1 (membrane-bounded form, Santa Cruz), and mouse anti–human actin (Santa Cruz Biotechnology) in blocking buffer at 4°C overnight. Membranes were then incubated with fluorescent-labeled secondary antibody (LI-COR) in blocking buffer for 1 hour at ambient temperature. Signals were scanned with an imaging system (LI-COR). The densitometry of acquired bands was measured with specialized software (Quantity One; Bio-Rad).

Statistical Analysis

Experimental data were analyzed by Student's t-test (P < 0.05; n = 3–6) and were presented as mean ± SD.
RESULTS

Bioynthesis of Resolvin E1 and D1 through Interplay of CRECs and Leukocytes

LC-UV-MS/MS lipidomic analysis revealed that RvE1 was biosynthesized in a coculture of choroid-retinal endothelial cells and leukocytes under inflammatory stimulation (Fig. 1). The MS/MS spectrum and retention time of the LC peak from coculture of CRECs and leukocytes matched the authentic RvE1 standard, indicating that the RvE1 structure was identified. The MS/MS ions 195, 223, 291, 205 [223-H2O] and 217 were the signature ions for the identification. Ions m/z 269 [M-H-2H2O], 295 [M-H-3H2O], 305 [M-H-2H2O], 313 [M-H-H2O], and 349 [M-H] were also consistent with RvE1 structure (Fig. 1A). The MS/MS spectrum for ion m/z 375, acquired from the LC peak with retention time identical with the synthetic authentic RvD1 standard, also matches that of the RvD1 standard. MS/MS ions demonstrating the RvD1 structure are as follows: m/z 141 and m/z 233 were produced by C-C bond α-cleavage from 7-hydroxy and 8-hydroxy; ion m/z 277 was formed from C-C bond α-cleavage of 17-hydroxy; ion m/z 189 was formed from β-cleavage from 8-hydroxy; ions 375 [M-H], 339 [M-H-2H2O], 351 [M-H-2H2O], and 295 [M-H-2H2O] were also consistent with the structure of RvD1. Ion 357 [M-H-H2O] was completely fragmented and was not present in MS/MS spectrum because it was efficiently fragmented by the wide-band activation for the MS/MS (Fig. 1B).

With the use of LC-UV-MS/MS lipomics, RvE1, RvD1, 17-hydroxy-DHA (HDHA), 18-hydroxy-EPE (HEPE), and 5-HEPE were also identified and quantified for the coculture of CRECs and leukocytes (Fig. 1C). In CRECs, 17-HDHA and 18-HEPE were present at much higher levels than were 7-HDHA and 5-HEPE (Fig. 1D). 17-HDHA, 18-HEPE, 7-HDHA, and 5-HEPE are monohydroxy markers of the hydroperoxy intermediates for the biosynthesis of RvE1 and RvD1. Results showed that inflammatory stimulation with TNF-α and IL-1β significantly increased the amounts of RvD1 and RvE1 and of 17-HDHA, 18-HEPE, 7-HDHA, and 5-HEPE in coculture of CRECs and leukocytes (Fig. 1C). It also increased the amounts of 17-HDHA and 18-HEPE in CRECs (Fig. 1D).

There were significantly more 7-HDHA and 5-HEPE than 17-HDHA and 18-HEPE from leukocytes (Fig. 1E). RvE1 and RvD1 were not detected in CRECs and were found in significantly lower amounts in leukocytes than those in the coculture (Figs. 1D, E).

Reduction of Inflammatory Signals from CRECs by RvE1 and RvD1

Chemokines are formed in injured tissues and attract leukocytes moving toward and transmigrating across endothelial cells to injury sites. The release of IL-8 from CRECs increased when the duration of IL-1β stimulation lasted longer (Fig. 2). In comparison with control or nontreatment with RvD1 or RvE1, treatment with RvE1 or RvD1 at 24-hour stimulation reduced CRECs to release IL-8 (Fig. 2A) as well as MIP-1β and RANTES (P < 0.05) (Fig. 2B); it also reduced the releases of IL-6 and TNF-α (P < 0.1; Fig. 2B). PGE2 is an important inflammatory prostaglandin in the inflammation phase. The results showed that the PGE2 concentration in CRECs increased with longer inflammatory IL-1β stimulation. However, CRECs treated with RvD1 after 24-hour stimulation had significantly less PGE2 than those that did not undergo RvD1 treatment. RvE1 treatment did not affect the PGE2 concentration in CRECs (Fig. 2C). COX-2, the inducible pivotal enzyme for the synthesis of prostaglandins, including PGE2, was quantified by Western blot analysis. Treatment with RvE1 or RvD1 did not change COX-2 formation in CRECs (Fig. 3). In fact, treatment with these resolvins downregulated VCAM-1 formation compared with the control (P < 0.05), and the reduction with RvE1 was not significantly different from that with RvD1.

Reduction of Expressions of Inflammatory Factors from Cocultures of CRECs and Leukocytes by RvE1

Leukocytes play critical roles in inflammation. They also interact with endothelial cells to promote inflammation. To mimic in vivo conditions, CRECs, PMNs, and monocytes were cultured together and treated with RvE1 to determine the actions of RvE1 on expressions of inflammatory factors. Results showed that RvE1 significantly decreased the releases of sVCAM-1, MIP-1β, RANTES, and TNF-α but had no effect on the release of MCP-1 (Fig. 4).

Reduction of PMN Transmigration across Monolayer Barriers of CRECs by RvE1 and RvD1

PMN transmigration across CREC monolayer barriers, as illustrated by Figure 5A, represents leukocyte transmigration across
choroidal and retinal vascular endothelium, the critical event of posterior ocular inflammation. Thus, CRECs and PMNs were used as targets for the action of RvE1 and RvD1. When CRECs were treated with RvE1 or RvD1 (50 nM), PMN transmigration was reduced by 22.4% (9.2 ± 0.4 × 10^4 PMNs; P < 0.05) and 27.0% (11.1 ± 0.5 × 10^4 PMNs; P < 0.05), respectively (Fig. 5B). When RvE1 or RvD1 was used to treat PMNs, they inhibited PMN transmigration across the CREC monolayer barriers on a concentration-dependent basis. Reduction rates in PMN transmigration through the treatment of PMNs with 200 nM RvE1 or RvD1 were 37.2% (11.6 ± 1.0 × 10^4 PMNs; P < 0.05) and 28.0% (8.7 ± 0.08 × 10^4 PMNs; P < 0.05), respectively, which were significantly greater than those with 10 nM RvE1 or RvD1 (Fig. 5C).

**DISCUSSION**

RvE1 and RvD1 are potent anti-inflammatory lipid mediators biosynthesized from EPA and DHA, respectively.10-12 The inflammatory stimuli significantly increased the generation of LTB4, which was added to the bottom of the lower chamber. Transmigration lasted 45 minutes. Migrated PMNs were then quantified and presented as the percentage of control transmigration of cells not treated with RvE1 and RvD1. Results are expressed as mean ± SD. *P < 0.05 compared with control.

**FIGURE 3.** RvE1 or RvD1 decreases the formation of VCAM-1 and COX-2 in CRECs. (A) CRECs were treated with RvE1 or RvD1 and then were stimulated with IL-1β for 24 hours. VCAM-1, COX-2, and actin formation was subjected to Western blot analysis with specific immunostaining for each protein. (B) Relative quantities of VCAM-1 and COX-2 compared with actins based on densitometric results from the gels. Results are expressed as mean ± SD. *P < 0.05 compared with control.

**FIGURE 4.** RvE1 regulates the release of cytokines and chemokines by coculture of choroid-retinal endothelial cells, PMNs, and monocytes. Cocultured CRECs, PMNs, and monocytes were pretreated with 50 nM RvE1 (or 0 nM for control) for 8 hours and stimulated with 2 ng/mL IL-1β for 24 hours. Cell culture supernatants were analyzed for the release of VCAM-1, MCP-1, MIP-1β, RANTES, and TNFα by multiplex. RvE1 inhibited the release of VCAM-1, MCP-1, MIP-1β, RANTES, and TNFα. Original magnifications: 100× and 10×, as indicated. Results are expressed as mean ± SD. *P < 0.05 compared with control.

**FIGURE 5.** RvE1 or RvD1 reduces PMN transmigration across monolayer barriers of CRECs. (A) Diagram illustrating the PMN transmigration model. (B) CRECs in the upper chambers of 24-well transwell plates were treated with 50 nM RvE1 or RvD1 for 8 hours and stimulated with 2 ng/mL IL-1β for 24 hours, followed by the addition of isolated fresh PMNs. Control CRECs were treated with IL-1β but not with RvE1 or RvD1. (C) PMNs were treated with 10 to 200 nM RvE1 or RvD1 for 15 minutes and were added to the top of untreated monolayer of CRECs. PMNs transmigrated across the CREC monolayer to LTB4, which was added to the bottom of the lower chamber. Transmigration lasted 45 minutes. Migrated PMNs were then quantified and presented as the percentage of control transmigration of cells not treated with RvE1 and RvD1. Results are expressed as mean ± SD. *P < 0.05 compared with control.
RvE1 and RvD1 by coculture of CRECs and leukocytes (Fig. 1C), which implies a self-protective mechanism by the cells to counteract inflammatory attack by promoting the production of anti-inflammatory RvE1 and RvD1. This was demonstrated by the fact that RvE1 and RvD1 reduced certain inflammatory signaling molecules, including cytokines, PGE2, COX-2, and VCAM-1, induced by inflammatory stimulation of CRECs and leukocytes (Figs. 2–4).

RvE1 and RvD1 were biosynthesized in a coculture of CRECs and leukocytes (Figs. 1A–C) but not in a culture of CRECs (Fig. 1D) and in much lower amounts in leukocytes, which is consistent with the requirement of enzyme 5-lipoxygenase (LO) plus 15-LO for RvD1 biosynthesis and 5-LO plus cytochromes P450 for RvE1 biosynthesis.2 The inflammatory stimulation increased 15-LO and P450 activities, reflected by significantly higher levels of 17-HDHA and 18-HEPE (the 15-LO and P450 products) in CRECs than in nonstimulated controls (Figs. 1C, D). It also enhanced 5-LO activity in leukocytes, reflected by increasing levels of the 5-LO products 7-HDHA and 5-HEPE (Figs. 1C, 6), by inflammatory stimulation on the coculture of leukocytes and CRECs. The enhanced 5-LO activity came from leukocytes because CRECs alone did not generate 5-LO products (Fig. 1D) and leukocytes alone primarily generated 5-LO products. 5-LO is the major lipoxygenase in leukocytes, whereas 15-LO is the major lipoxygenase for the endothelium.39 Thus, RvE1 and RvD1 were primarily generated through the interplay of CRECs and leukocytes. As depicted in Figure 6, DHA is converted by 15-LO activity from CRECs into 17-HpDHA, which is taken by leukocytes and transformed to RvD1 by leukocyte 5-LO activity; Meanwhile, DHA is dioxygenated by 15-LO in CRECs to 17S-HpDHA, which is transformed to and detected as 7S-HpDHA. EPA is transformed by cytochrome P450 dioxygenase in CRECs to 18R-HEPE, which is sequentially converted to RvE1 by 5-LO. 5-LO also generates 5HpEPE (detected as its reduced form, 5-HEPE) from EPA. (B) RvD1 formation: DHA is dioxygenated by 15LO in CRECs to 17S-HpDHA, which is transformed to RvD1 by 5-LO. Meanwhile, DHA is transformed by 5-LO to 7S-HpDHA, which is detected as its reduced form 7-HDHA.

As chemoattractants, chemokines stimulate directional leukocyte migration and activate the expression of integrin on leukocytes such as CD11/CD18 and VLA-4, which increases leukocyte binding to the ligands ICAM-1 and VCAM-1, respectively, on the endothelium.23,24 IL-8 was reported to favor the progress of age-related macular degeneration.43 When IL-8 receptors are genetically deficient, PMN infiltration is reduced significantly.44 VCAM-1, specifically expressed by the endothelium, participates in early inflammation events. It mediates the transient adhesion event, which allows leukocytes to be tethered and subsequently roll along the vessel wall.45 A rapid activating event that increases adhesion and arrests the leukocytes is then executed by VCAM-1 together with ICAM-1. TNF-α is involved in systemic inflammation and stimulates the acute-phase reaction. RvE1 and RvD1 decreased the release of cytokines IL-8, IL-6, MIP-1β, and RANTES and the formation of cell adhesion molecule VCAM-1 by CRECs (Figs. 2B, 3). RvE1 reduced the release of sVCAM-1, MCP-1, MIP-1β, RANTES, and TNFα by coculture of CRECs and leukocytes (Fig. 4). Consistent with these anti-inflammatory activities at the molecular level, RvE1 and RvD1 inhibited PMN transmigration across CREC monolayers when they were used to treat PMNs or CRECs (Fig. 5). PMN transmigration across CRECs is a critical step in PMN recruitment and inflammation.5 Therefore, RvE1 and RvD1 have anti-inflammatory activities on PMNs and CRECs. Earlier reports showed that RvE1 and RvD1 reduced leukocyte infiltration in a peritonitis inflammation model and is proinflammatory in many conditions.37 In our study, the PGE2 level in CRECs increased with the duration of IL-1β stimulation. Because PGE2 leads to enhanced proinflammatory responses in animal models and inhibited neutrophil responses with isolated human cells,41 PGE2 generation may be relevant to the modulation of PMN transmigration and cytokine formation in our experiments. Our experiment showed that both resolvins had no effect on the formation of COX-2 (Fig. 3), indicating that the reduction in PGE2 by RvD1 might have resulted from the regulation on PGE2 synthases downstream of COX in the prostaglandin enzymatic synthesis cascade. These synthases convert the COX product prostaglandin H2 to PGE2.42 More study is needed to further delineate RvD1 action on PGE2 enzymatic synthesis pathways.
PMN transmigration across monolayers of other types of endothelial cells. A recent study shows that RvE1 binds to its receptor ChemR23 and attenuated nuclear factor-κB. Nuclear factor-κB is confirmed to be crucial in the regulation of inflammatory cytokines, including IL-1, IL-2, IL-6, and TNF-α and chemokines such as IL-8, MIP-1α, MCP-1, and RANTES. Thus, it is possible that RvE1 attenuates the nuclear factor-κB signaling pathway to reduce the release of inflammatory mediators. Although the receptor of RvD1 has not been identified, it may be associated with signaling pathways for suppressing the formation of PGE₂ and PGE synthase and may impair nuclear factor-κB signaling pathways. Furthermore, the different effects of RvE1 and RvD1 on PGE₂ production imply that they may have different signaling pathways.

In summary, this study reveals for the first time that the interplay of choroidal retinal endothelial cells and leukocytes under inflammatory conditions transform ω-3 DHA and EPA into resolvins E1 and D1, respectively. These resolvins, in turn, reduce the expression of inflammatory signaling molecules and PMN transmigration across CREC barriers. Diets enriched with DHA and EPA reduce the risk for retinopathy of prematurity and reduce the expression of inflammatory signaling molecules and promote transendothelial neutrophil passage.

References


