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The contributions of aspirin and microbial oxygenase to the biosynthesis of anti-inflammatory resolvins: Novel oxygenase products from ω-3 polyunsaturated fatty acids

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Abstract

Resolvins (Rvs) are oxygenated products derived from ω-3 polyunsaturated fatty acids (PUFAs) such as eicosapentaenoic acid (EPA) and docosahexaenoic acid that carry potent protective bioactions present in resolving inflammatory exudates. Resolvin E1 (RvE1) is biosynthesized in vivo from EPA via transcellular biosynthetic routes during cell–cell interactions, and thus RvE1 is formed in vivo during multicellular responses such as inflammation and microbial infections. RvE1 protects tissues from leukocyte-mediated injury and counterregulates proinflammatory gene expression. These newly identified Rvs may underlie the beneficial actions of ω-3 PUFAs especially in chronic disorders where unresolved inflammation is a key mechanism of pathogenesis. Here, we present an overview of the biosynthesis of RvE1, with a focus on the aspirin-triggered and microbial P450-initiated pathways. The generation of RvE1 and its actions appear to dampen acute leukocyte responses and facilitate the resolution of inflammation.

Keywords: ω-3 PUFA; Human leukocytes; Inflammation; Resolution

The protective roles of essential polyunsaturated fatty acids (PUFA) were described as early as 1929 by Burr and Burr [1]. In addition to being important structural components of biological membranes, ω-3 PUFA are precursors of novel lipid mediators with potent actions in tissue homeostasis (recently reviewed in [2]). The essential PUFA include arachidonic acid (AA; C20:4) of the ω-6 series, and eicosapentaenoic acid (EPA; C20:5) and docosahexaenoic acid (DHA; C22:6) from the ω-3 series of PUFA. When enzymatically oxygenated by specific oxygenases, e.g., cyclooxygenase and lipoxygenase, AA is converted to biologically active autacoids, such as prostaglandins (PGs) and leukotrienes (LTs). On the other hand, little was known about potential bioactive products derived from ω-3 EPA and DHA. Although it has been deduced from epidemiological studies, such as the Greenland Eskimos study by Bang et al. [3] late in the 1970s, that fish oil rich in ω-3 EPA and DHA was generally associated with a reduced incidence of cardiovascular disease, the molecular basis responsible for this reduction remained to be established.

Along these lines, the GISSI prevention study more recently also reported a significant benefit associated with taking ω-3 PUFA, namely protection from sudden death by approximately 45% in more than 11,300 cardiovascular patients [4]. Of interest, each patient group in the GISSI study took daily low-dose aspirin, but the contribution of aspirin plus ω-3 PUFA was not taken into consideration in the original data analysis [4] nor in followup studies. In the past several decades, many of

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the clinical assessments of dietary supplementation with ω-3 PUFAs advocate their beneficial impact in a wide range of human diseases (for earlier reviews see [5]). Of special interest are those diseases in which unresolved inflammation could be considered as a key component in disease pathogenesis.

These major ω-3 PUFAs (i.e., EPA and DHA) are widely believed to act by several possible mechanisms to explain their benefits in cardiovascular and inflammatory diseases. These include: (i) preventing conversion of arachidonic acid into proinflammatory eicosanoids such as PGs and LTs via substrate competition, and/or (ii) serving as an alternative substrate to produce less potent 5-series LTs and 3-series thromboxanes [6–10]. To address this, we recently uncovered two new families of oxygenated products derived from ω-3 PUFA, coined resolvins (Rvs) and protectins. Specific compounds within these families possess potent stereoselective, anti-inflammatory, and immunoregulatory actions. Our results suggest new and potentially important roles for these essential fatty acids as precursors for potent local-acting protective mediators [11–14]. The trivial name Resolvin (resolution phase interaction products) was introduced to emphasize their original isolation and production during the resolution phase of acute inflammatory responses and to signify the contribution of transcellular biosynthesis for these potent new mediators (recent review [2]). Here, we consider the role of oxygenases in and the impact of aspirin on the biosynthesis and actions of RvE1.

Formation of ω-3-derived products in vivo using mediator lipidomic analysis

We questioned whether essential ω-3 fatty acids, such as EPA and DHA, were converted to potent lipid mediators, as widely appreciated with arachidonate. Also, based on the beneficial impact of ω-3 PUFAs in human diseases associated with inflammation, a molecular basis was sought to account for their anti-inflammatory and protective actions. In view of qualitatively overlapping beneficial profiles attributed to dietary ω-3 PUFA and to aspirin in clinical studies, evidence was mustered for new signaling pathways that might account for the earlier findings reported for many human studies (see [4,5]). To address this in an experimental setting, we used a dermal acute inflammation model, i.e., the murine dorsal air pouch (Fig. 1). This system is characterized by rapid polymorphonuclear leukocyte (PMN) infiltration within hours of challenge that is followed by a spontaneous resolution phase [15]. We adopted this for systematic analysis of exudates and to develop "mediator lipidomics" employing liquid chromatography–ultraviolet-tandem mass spectrometry (LC–UV-MS/MS) and profiling with gas chromatography–mass spectrometry (GC–MS)-based analyses to evaluate whether novel lipid mediators were indeed generated during the resolution phase of inflammation (Fig. 1). Following administration of ω-3 PUFA and aspirin, inflammatory exudates were collected at intervals, focusing on the period of spontaneous resolution for which lipid mediator profiles were constructed using LC–UV-MS/MS and GC–MS-based analyses.

Inflammatory exudates formed in murine air pouches via intrapouch injections of TNF-α with ω-3 EPA and aspirin on board generated several novel compounds [11], i.e., 18-hydroxy-eicosapentaenoic acid (18-HEPE) as well as some products characterized earlier such as 5*S*-HEPE [10] (Figs. 2A and B). The stereochemistry of the alcohol group at the carbon-18 position was established as predominantly in the *R* configuration using chiral column chromatography and authentic materials. These findings indicated that murine inflammatory exudates exposed in vivo to EPA and aspirin not only blocked prostanoid formation as expected but also produced the 5-lipoxygenase product 5*S*-HEPE as well as a novel 18*R*-HEPE from the precursor EPA. Air pouch inflammatory exudate cells from these EPA and aspirin-treated mice contained predominantly PMN, and these exudates, when activated ex vivo with calcium ionophore A23187, generated 18*R*-HEPE (10.2 ± 4.3 ng/10^6 cells) and 5*S*-HEPE (10.9 ± 2.9 ng/10^6 cells). In addition, evidence for novel trihydroxy-containing products derived from EPA was also obtained in these resolving exudates. MS/MS fragmentation ions were consistent with a trihydroxy-containing product with a parent ion at *m*/*z* 349 and product ions of structural significance at *m*/*z* 291 and 195 that are consistent with fragmentations denoted in the inset (Fig. 2C). A complete stereochemical assignment of this bioactive product termed RvE1 was recently established as 5*S*,12*R*,18*R*-trihydroxy-6*Z*,8*E*,10*E*,14*Z*,16*E*-eicosapentaenoic acid [12], and its route of formation was determined (vide infra, Fig. 5). Also in resolving exudates from mice given DHA, we found novel 17*R*-hydroxy-DHA (17*R*-HDHA) in the presence of aspirin, and 17*S*-HDHA and several related bioactive compounds without aspirin termed Resolvin D series and protectins (see [2,13,14] for details).

Aspirin-triggered pathways for novel lipid mediators

Aspirin is a widely used non-steroidal anti-inflammatory drug (NSAID). It is well documented that aspirin irreversibly inhibits prostaglandin endoperoxide synthase/cyclooxygenase (COX) by acetylation of serine residue and thus blocks the production of prostaglandins [16,17]. Acetylation of COX-1, which is the main form present in platelets, completely blocks the oxygenase activity and thus blocks the production of

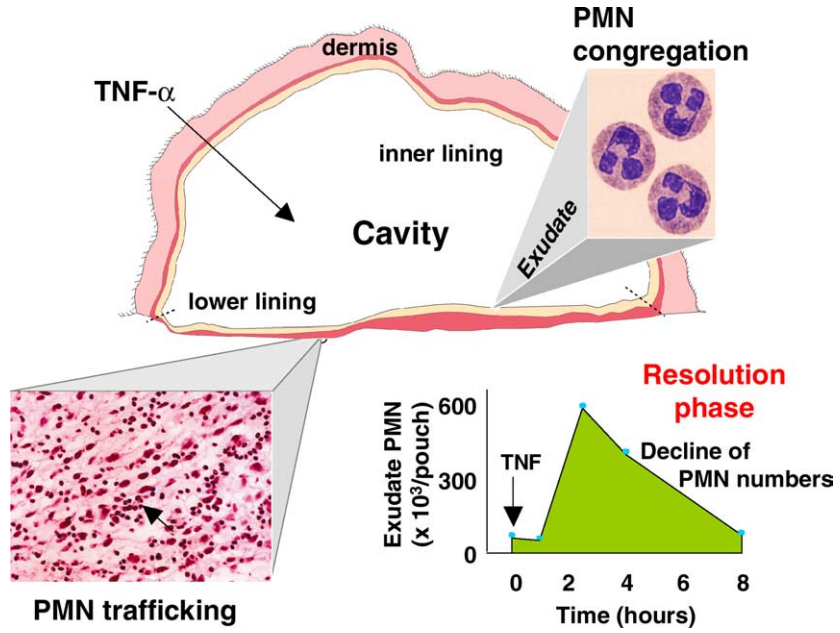

Fig. 1. Murine model of acute inflammation and spontaneous resolution. Murine dorsal air pouches were raised for 6 days and acute inflammation was initiated by TNF-α (10 ng) injection. In this pouch, PMN numbers begin to drop within exudates between 4 and 8 h, the current cellular definition of resolution. Exudates were collected from this spontaneous resolution phase (at 6 h) and lipid mediator profiles were acquired using LC tandem UV-MS/MS.

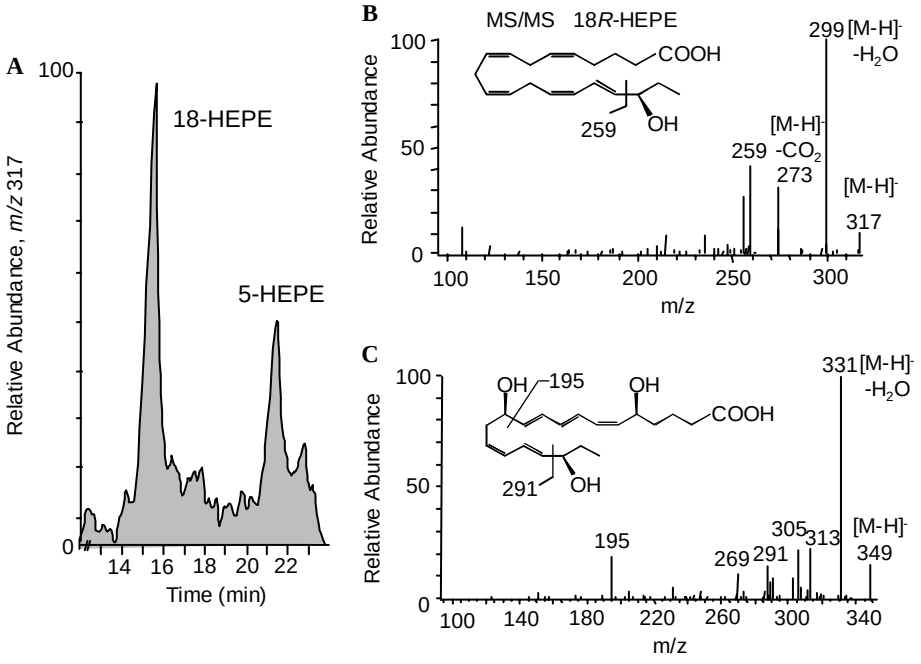

Fig. 2. Mediator lipidomics. Inflammatory exudates from murine dorsal pouches following administration of EPA and aspirin generate novel compounds including 18*R*-HEPE (A,B) and 5,12,18*R*-triHEPE (C). TNF-α-induced leukocyte exudates collected at 6 h from mice given aspirin (3.5 h at 500 μg/pouch) and EPA (4 h at 300 μg/pouch). See Serhan et al. [11] for details.

thromboxane A_2. On the other hand, we and others found that acetylation of COX-2 by aspirin prevents the formation of prostaglandins, but the enzyme remains active in situ to generate 15*R*-hydroxy-5,8,11,13-eicosatetraenoic acid (15*R*-HETE) from arachidonic acid [18–20]; see Table 1). This is likely because the active site of native COX-2 is slightly larger than that of COX-1, which is characterized by a side-pocket extension to the hydrophobic channel [21]. In this regard, our laboratory found that aspirin, in

Table 1
Aspirin's impact on recombinant human COX-2-catalyzed conversion of PUFA: percent differences with acetylated COX-2

PUFA	Products (% difference)		
	ω-2	ω-5	ω-9
Linoleic acid		13-HODE	9-HODE
C18:2 ω-6		−96.2 ± 0.8	−94.6 ± 2.3
Arachidonic acid		**15-HETE**	11-HETE
C20:4 ω-6		2794.6 ± 572.2	−18.0 ± 19.3
Eicosapentaenoic acid	**18-HEPE**	15-HEPE	11-HEPE
C20:5 ω-3	49.7 ± 31.4	68.7 ± 10.8	−92.9 ± 2.7
Docosahexaenoic acid		**17-HDHA**	13-HDHA
C22:6 ω-3		97.8 ± 2.0	−85.7 ± 5.6

Results are means ± SEM, $n = 3$. Aspirin was used at 2 mM with isolated recombinant COX-2 enzyme. Products were extracted, identified, and quantitated using internal standards and LC–MS/MS. Stereochemistry of compounds shown in bold type is predominantly *R* as determined by chiral HPLC analysis (see [11,13]).

addition to its well-appreciated ability to inhibit prostaglandins, can switch on the production of the body's own anti-inflammatory lipid mediators, such as the aspirin-triggered lipoxins [20,22].

Along these lines, human endothelial cells known to induce COX-2 with IL-1β or hypoxia in vitro were pulsed with EPA and aspirin, and the extracted materials were subjected to LC–MS/MS analysis. Selected ion monitoring at *m/z* 259 revealed the formation of 18*R*-HEPE (10.6 ng/10^6 cells) and 15*R*-HEPE (6.0 ng/10^6 cells) from EPA [11].

18*R*-HEPE and 15*R*-HEPE were also generated by isolated recombinant human COX-2 treated with aspirin (Fig. 3). Aspirin acetylates Ser516 in the internal cavity of the COX-2 active site, which causes a shift in the position and chirality of oxygen insertion by a change in the conformation of the omega side chain (Fig. 4 and see [21]). Indeed, aspirin treatment of COX-2 enhanced the production of 15*R*-HETE from AA, 18*R*-HEPE from EPA, and 17*R*-HDHA from DHA. An almost 29-fold increase in 15-HETE production was observed following aspirin treatment, which also blocked PGH_2/prostanoid biosynthesis (Table 1). Hence, together they suggested that aspirin treatment at local sites of inflammation can convert EPA via acetylated COX-2 in vascular endothelial cells to 18*R*-HEPE and 15*R*-HEPE. Of interest, aspirin triggers a novel family of bioactive DHA-derived products, namely 17*R*-series RvDs, the natural epimers of the 17*S*-series Rvs [13]. Human recombinant COX-2 converts DHA to 13-HDHA, and with aspirin this switches to 17*R*-HDHA (Fig. 4, Table 1).

18*R*-HEPE and 15*R*-HEPE, when incubated with activated human PMN engaged in phagocytosis, were converted into two classes of trihydroxy-containing EPE, namely 5*S*,12*R*,18*R*-triHEPE (RvE1) and 5*S*,6*R*,15*R*-triHEPE (15-epi-lipoxin A_5). At local sites of inflammation, interactions between activated PMNs and vascular endothelial cells can initiate the transcellular biosynthesis of lipid mediators. These pathways involve cell–cell interactions within the exudates; for

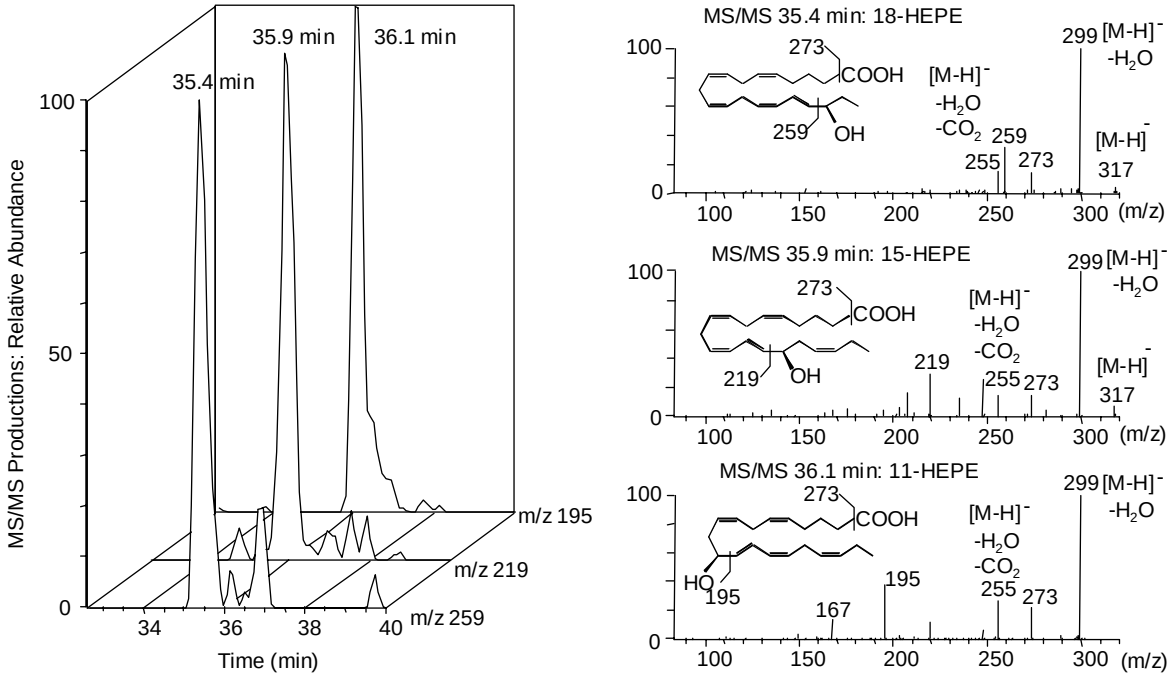

Fig. 3. Aspirin-triggered products generated by aspirin-acetylated COX-2. Human recombinant COX-2 treated with 2 mM aspirin was incubated with EPA (10 μM, 30 min, 37 °C). LC–MS/MS chromatogram of *m/z* 259, 219, and 195 showing the presence of 18-HEPE, 15-HEPE, and 11-HEPE, respectively. The direct precursors to these are likely hydro(peroxy)-containing intermediates that are rapidly reduced to the corresponding alcohol. MS/MS spectra of mono-HEPEs are provided.

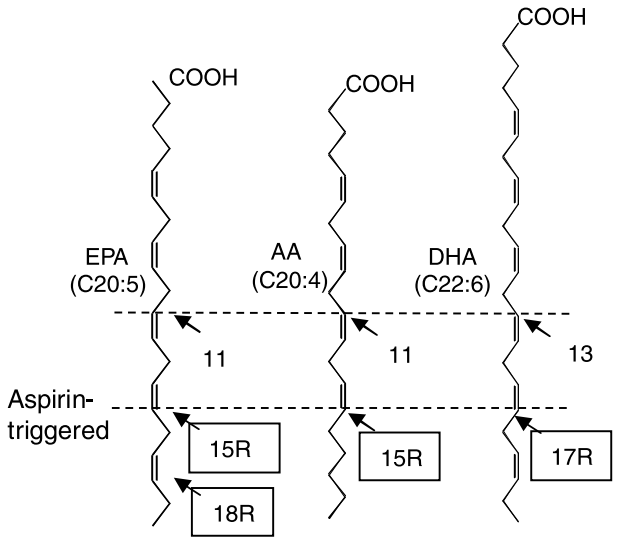

Fig. 4. Summary of positional oxygenation of PUFA by COX-2 versus aspirin-acetylated COX-2. Aspirin acetylation of COX-2 shifts the regioselectivity of oxygen insertion to the positions denoted by the boxed numbers.

example, vascular endothelial cells treated with aspirin convert EPA to 18*R*-HEPE and 15*R*-HEPE that are released [11] and then rapidly converted by activated human PMN by 5-lipoxygenase-like activity to insert molecular oxygen and in subsequent steps through 5(6)epoxide formation to bioactive RvE1 and 15-epi-LXA_5 (Fig. 5).

Microbial P450-initiated pathway

In addition to the aspirin–COX-2 pathway, RvE1 formation can be initiated via novel routes that do not require aspirin. These involve cytochrome P450 monooxygenase (CYP) present in both human cells and microbes. It is known that the CYP family of enzymes play key roles in tissue-specific conversion of natural substrates into biologically active steroids, vitamins, and eicosanoids [23]. For example, oxidation of arachidonic acid in endothelial cells may give rise to 11,12-epoxyeicosatetraenoic acid (11,12-EET) catalyzed by CYP2C8 or CYP2J2. This EET displays potent anti-inflammatory properties because, at micromolar concentrations, it inhibits leukocyte adhesion [24]. Of interest to the present studies, earlier results indicate that microbial P450 enzymes such as *Bacillus megaterium* CYP BM-3 catalyze NADPH-dependent ω-2 hydroxylation of AA to 18*R*-HETE in a regio- and stereoselective fashion [25]. The alcohol configuration at position 18 proved to be >98% *R*. EPA was also converted to 18*R*-HEPE by allylic oxidation (lipoxygenase-like activity) and 17,18-epoxyeicosapentaenoic acid (17,18-EEP) by olefin epoxidation when incubated with *B. megaterium* homogenates (Fig. 6A). However, neither AA nor EPA is a major PUFA of this organism. Along these lines, we found that the microbial P450 could initiate the biosynthetic pathway of RvE1 by generating and providing its precursor 18*R*-HEPE to human leukocytes of the host, which then convert by 5-lipoxygenase-like activity, inserting molecular oxygen at carbon-5 position to form RvE1 (Fig. 5). In addition, this microbial P450 can transform host-derived LTB_5, a 5-lipoxygenase product of EPA [9,10], to generate RvE1 (Fig. 6B). These mechanisms may contribute to RvE1 production especially in gastrointestinal tissues rich in "probiotic" microflora as well as mucosal epithelial cells that contain mammalian P450. These can also contribute to RvE1 formation in other organs and tissues.

Anti-inflammatory actions

RvE1 is a potent regulator of PMN. In vivo at nanomolar levels, RvE1 dramatically reduced PMN infiltration into TNF-α-induced murine dorsal air pouch (dermal inflammation) and zymosan-induced peritonitis [12]. PMN adhesion and migration through the vascular endothelial monolayer is a pivotal event in acute inflammation in vivo [26], and RvE1 inhibited LTB_4-stimulated PMN transendothelial migration with an apparent IC_{50} of 5–50 nM [11].

In addition to PMN, RvE1 carries potent actions with antigen-presenting cells. The *Toxoplasma gondii*-derived pathogen STAg causes activation of splenic dendritic cells (DCs) to mobilize to T-cell enriched areas where they produce high amounts of IL-12. RvE1 blocked this DC migration in vivo and IL-12 production [12]. RvE1 specifically bound to a G-protein coupled receptor ChemR23, and treatment of DCs with small-interfering RNA specific for ChemR23 sharply reduced RvE1 regulation of IL-12.

Moreover, RvE1 protects against severe inflammatory bowel disease induced by 2,4,6-trinitrobenzenesulfonic acid (TNBS) in a mouse model [27]. Mice treated with RvE1 showed increased survival, sustained body weight, and improved histological scores that were important components of the disease. These RvE1-treated mice also had reduced serum anti-TNBS IgG, decreased PMN present in the mucosa, and reduced proinflammatory gene expression including TNF-α, IL-12, and inducible nitric oxide synthase. These results demonstrate novel counterregulatory responses in inflammation initiated via RvE1-receptor activation and provide the first evidence for EPA-derived potent endogenous agonists of anti-inflammation/pro-resolution.

In addition to EPA-derived RvE1, DHA-derived Rv Ds and protectins proved to be potent regulators of PMN infiltration and blocked proinflammatory cytokine production from microglial cells [13,14]. Neuroprotectin D1 (10,17*S*-docosatriene) is protective against stroke

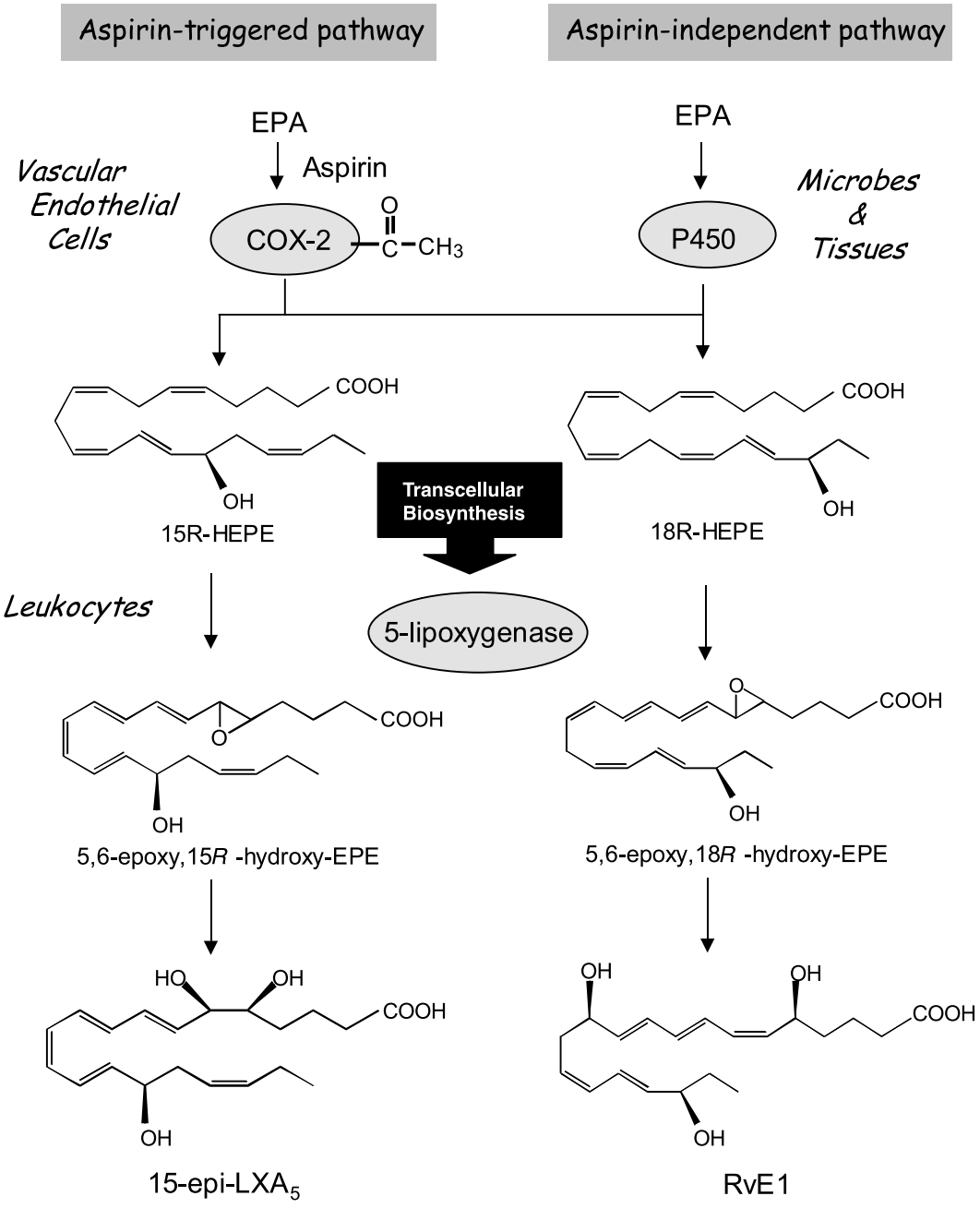

Fig. 5. Transcellular biosynthesis of lipid-derived signals (RvE1 and 15-epi-LXA$_4$) from EPA: aspirin-triggered and independent pathways. Human endothelial cells expressing COX-2 treated with aspirin transform EPA by abstracting hydrogen at C16 or C13 to give *R* insertion of molecular oxygen to yield 18*R*-H(p)EPE and 15*R*-H(p)EPE. Also, microbial cytochrome P450 can monooxygenate EPA to 18*R*-HEPE in an aspirin-independent manner. These intermediates are reduced to alcohols and converted to epoxide intermediates via transcellular biosynthesis by sequential actions of a leukocyte 5-lipoxygenase-like reaction, which leads to the formation of RvE1 and 15-epi-LXA$_4$.

brain injury and retinal pigmented cellular damage [28,29], and promotes epithelial wound healing in cornea [30].

Conclusion

Inflammation is a protective host response to foreign challenge and tissue injury that, if unopposed, could lead to loss of tissue structure as well as function. The notion that inflammatory response by itself generates its own regulators in tandem with proinflammatory mediators makes sense, as it is easier to control with both positive and negative regulatory inputs. Many inflammatory processes are self-limiting and self-resolving systems, suggesting the existence of endogenous regulators during the course of inflammation, and disturbances in such counterregulatory mechanisms

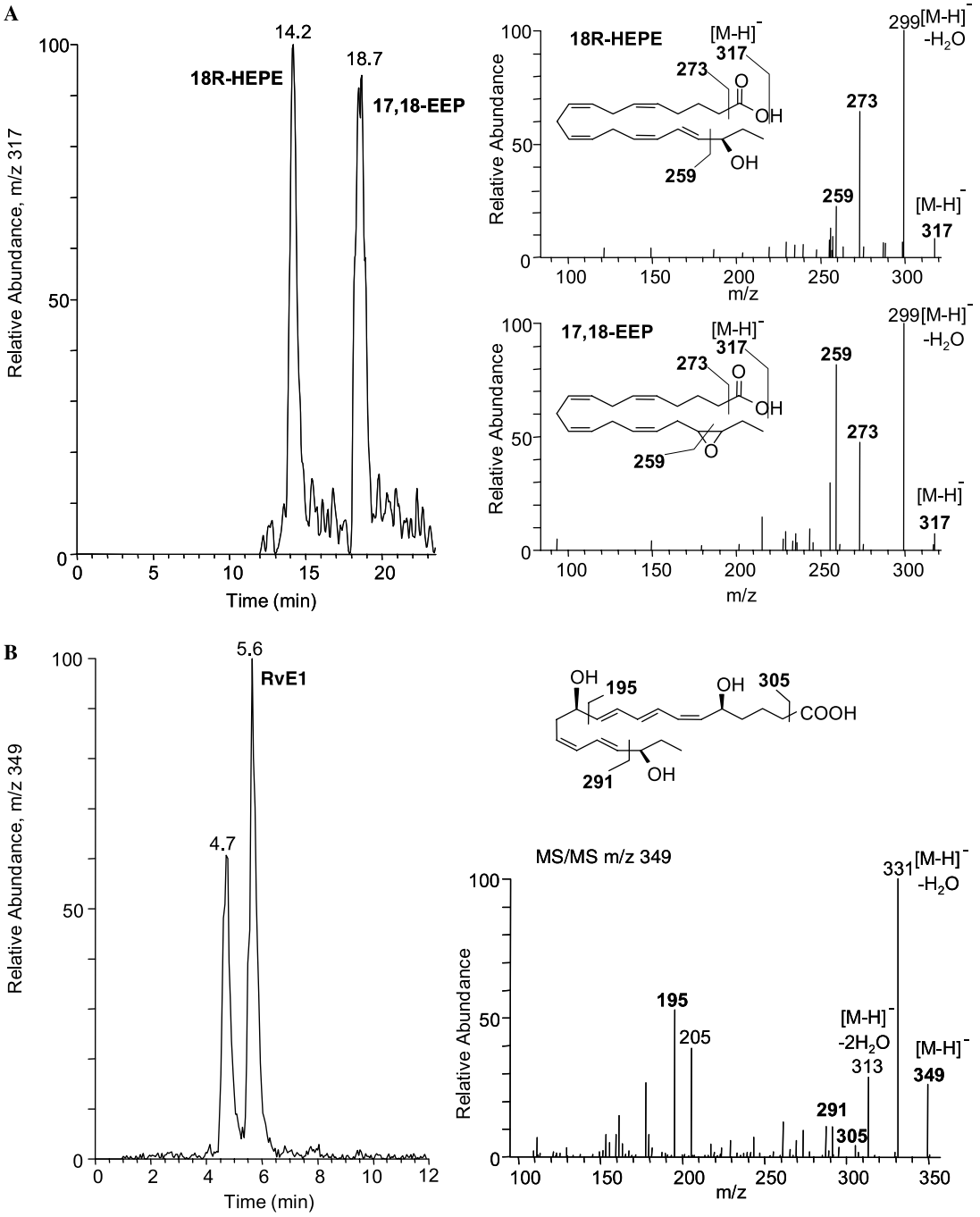

Fig. 6. 18*R*-Oxygenation by *B. megaterium*. Potential for transorganism biosynthesis. (A) Microbial conversion of EPA to 18*R*-HEPE and 17,18-EEP. *B. megaterium* sonicates were incubated with EPA (330 μM) in 2 mM NADPH and 2 M Tris–HCl (pH 8.1). LC–MS/MS chromatogram of *m/z* 317 showing the formation of 18*R*-HEPE and 17,18-EEP. MS/MS spectra are provided (right panels). (B) LTB_5 conversion to RvE1. LTB_5 (15 μM) was incubated with *B. megaterium* sonicates in 2 mM NADPH and 2 M Tris–HCl (pH 8.1). LC–MS/MS chromatogram of *m/z* 349 showing the presence of RvE1 and its isomer. The product eluted at 5.6 min was RvE1, which was confirmed by MS/MS spectrum (right panel) and matching with synthetic isomers of RvE1 (see [12]).

could lead to exacerbated inflammatory responses and tissue damage [31–35].

The discovery of the Rvs offers an endogenous agonist-driven molecular mechanism(s) that could underlie some of the beneficial actions of ω-3 PUFA and aspirin observed in many clinical settings. Given the vast size and surface area of the vasculature as well as its pivotal role in host defense and inflammation, human endothelial cells can contribute substantial amounts of aspirin-triggered lipid mediators generated by acetylated COX-2 with aspirin treatment. In this regard, the vascular endothelium constitutively expresses COX-2 when

exposed to flow conditions as in vivo [36]. This appears to be relevant in healthy humans taking low-dose aspirin [22] as well as when ω-3 supplements are given [12].

Our results also indicate that initiation of RvE1 biosynthesis can also take place in a microbial origin since microbial P450 monooxygenase (aspirin-independent pathway) constitutes a novel means of protection that could be used by probiotic microflora in gastrointestinal tissues to protect against excess immune response. This theme of host-microbial interactions with lipid mediators and transorganism biosynthesis illustrates a much larger domain, because parasites such as *Brugia malayi* (PGE_2, I_2), *Trypanosoma brucei* (PGD_2, $F_{2\alpha}$ synthase), *Plasmodium falciparum* (PGD_2, E_2, $F_{2\alpha}$), *Toxoplasma gondii* (LXA_4 and 15-lipoxygenase), *Pseudomonas aeruginosa* (15-lipoxygenase), and *Mycobacterium tuberculosis* (LXA_4) have also been shown to generate oxygenase-derived eicosanoids. These are generated from host PUFAs that in turn modulate the host immune responses and physiology [37–42]. In summation, our findings help navigate the previously uncharted molecular circuits of inflammation-resolution that are of interest as checkpoint regulators in the pathogenesis of a wide range of human diseases associated with inflammation.

Acknowledgments

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