Published in final edited form as:
Biochim Biophys Acta. 2015 April ; 1851(4): 397–413. doi:10.1016/j.bbalip.2014.08.006.

Protectins and Maresins: New Pro-Resolving Families of Mediators in Acute Inflammation and Resolution Bioactive Metabolome

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Abstract

Acute inflammatory responses are protective, yet without timely resolution can lead to chronic inflammation and organ fibrosis. A systems approach to investigate self-limited (self-resolving) inflammatory exudates in mice and structural elucidation uncovered novel resolution phase mediators *in vivo* that stimulate endogenous resolution mechanisms in inflammation. Resolving inflammatory exudates and human leukocytes utilize DHA and other n-3 EFA to produce three structurally distinct families of potent di- and trihydroxy-containing products, with several stereospecific potent mediators in each family. Given their potent and stereoselective picogram actions, specific members of these new families of mediators from the DHA metabolome were named D-series resolvins (Resolvin D1 to Resolvin D6), protectins (including protectin D1-neuroprotectin D1), and maresins (MaR1 and MaR2). In this review, we focus on a) biosynthesis of protectins and maresins as anti-inflammatory - pro-resolving mediators; b) their complete stereochemical assignments and actions *in vivo* in disease models. Each pathway involves the biosynthesis of epoxide-containing intermediates produced from hydroperoxy-containing precursors from human leukocytes and within exudates. Also, aspirin triggers an endogenous DHA metabolome that biosynthesizes potent products in inflammatory exudates and human leukocytes, namely aspirin-triggered Neuroprotectin D1/Protectin D1 [AT-(NPD1/PD1)]. Identification and structural elucidation of these new families of bioactive mediators in resolution has opened the possibility of diverse pathophysiologic actions in several processes including infection, inflammatory pain, tissue regeneration, neuroprotection-neurodegenerative disorders, wound healing, and others.

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Disclosure: CNS is an inventor on patents [resolvins] assigned to BWH and licensed to Resolvyx Pharmaceuticals. CNS is a scientific founder of Resolvyx Pharmaceuticals and owns equity in the company. CNS' interests were reviewed and are managed by the Brigham and Women's Hospital and Partners HealthCare in accordance with their conflict of interest policies.

Keywords

Resolvins; leukocytes; LC-MS-MS-based targeted lipid mediator metabolomics; lipid mediators; eicosanoids

Introduction

Docosahexaenoic acid (DHA) is a highly conserved structure, an essential fatty acid in humans, and has physical properties that evolved to impact cellular membrane and neural function [1-4]. Recent results from this laboratory indicate that DHA is also a precursor to potent local autacoids. These includes the D-series resolvins, protectins and maresins that are produced in self-resolving inflammatory exudates in mice [reviewed in ref. 5]. Neutrophils (PMN) are first to arrive at the site of inflammation during the acute inflammatory response and play an important protective role in innate immunity and host defense. However, excessive accumulation of PMN within tissues can lead to tissue damage, amplification of the inflammatory response, injury from within and prolongation of the signs of inflammation [6]. The control of neutrophil infiltration is of wide interest, as new anti-inflammatory agents are needed to control excess neutrophil responses within tissues that can give rise to chronic inflammatory diseases [7]. Along these lines, evidence was sought for the endogenous mechanism(s) controlling PMN infiltration and natural tissue resolution, since protective PMN (i.e. acute inflammatory responses) are programmed to be self-limited and tightly controlled [8-10]. Lipid mediators such as prostaglandins and leukotrienes play pivotal roles in the initiation of acute inflammation [11], whereas resolvins and protectins promote and stimulate active resolution [8, 9, 12]. In excess, prostaglandins and leukotrienes are generally pro-inflammatory [11] and involved in the classic initiation phase of the acute inflammatory response in humans.

Studies in this laboratory uncovered potent new families of n-3 essential fatty acid (EFA)-derived mediators generated during resolution that are antiinflammatory, neuroprotective, and activate novel resolution pathways [9, 10, 13, 14]. Resolution of acute inflammation is a central component of host defense and the return of tissue to homeostasis [15]. It is now well recognized that inflammation plays a key role in many prevalent human diseases including cardiovascular diseases, atherosclerosis, Alzheimer's disease, and cancer [16-18]. Although much is known about the molecular basis of initiating signals and proinflammatory chemical mediators in inflammation [19], it has only recently become apparent that endogenous stop signals are critical at early checkpoints within the temporal events of inflammation [20-22]. In this context, lipid mediators are of considerable interest. The arachidonic acid-derived prostaglandins and leukotrienes are potent pro-inflammatory mediators [23], whereas the lipoxins, biosynthesized from arachidonic acid, are potent anti-inflammatory and proresolving molecules [for reviews see 24, 25, 26]. During the course of inflammation, arachidonatederived eicosanoids switch from prostaglandins and leukotrienes within inflammatory exudates to lipoxins that in turn stop the recruitment of neutrophils to the site. This switch in eicosanoid profiles and biosynthesis is driven, in part, by cyclooxygenase-derived prostaglandin E_2 and prostaglandin D_2, which instruct the transcriptional regulation of enzymes involved in lipoxin biosynthesis [22]. Hence, the appearance of lipoxins within

inflammatory exudates is concomitant with self-limited, or also described in the literature as spontaneous, resolution of inflammation [22], and these chemical mediators are nonphlogistic stimulators of monocyte recruitment and macrophage phagocytosis of apoptotic PMN [27, 28].

Further studies on the endogenous mechanisms of anti-inflammation using a murine model of self-limited resolution demonstrated, for the first time, that resolution is an active biochemical process that involves the generation of specific new families of mediators [for recent reviews, see refs. 29, 30]. During self-limited resolution, cell-cell interactions and transcellular biosynthesis lead to the production of these new families of potent bioactive lipid mediators from ω-3 essential fatty acid precursors and were termed resolvins (resolution phase interaction products derived from DHA, EPA and n-3 DPA) and protectins (docosatrienes derived from DHA) [9, 10, 13, 31, 32]. These novel di- and trihydroxy-containing products from n-3 essential fatty acids are biosynthesized by previously unrecognized enzymatic pathways that include resolvins, protectins and maresins. Specific members of each family display potent anti-inflammatory, immunoregulatory and pro-resolving actions *in vitro* and *in vivo* in murine models of inflammatory diseases [9, 10, 13] (see Figure 1).

The omega-3 polyunsaturated fatty acids were assigned, in 1929, essential roles because their exclusion from the diet gave rise to a new form of deficiency disease [33]. Many more recent reports document the importance of fish oil (omega-3) fatty acids EPA and DHA in human diseases associated with uncontrolled tissue inflammation. In particular, omega-3 DHA and EPA are protective in inflammatory bowel disease and colitis [34], cardiovascular disease [35-38], and Alzheimer's disease [39]. However, the cellular and molecular mechanisms responsible for these now well-documented beneficial actions of omega-3 fatty acids remain an important challenge and public health concern, given the widespread use of n-3 supplements and the many diseases characterized by excessive inflammation. DHA is enriched in neural tissues, where it appears to play functional, not just structural roles [4, 40]. Along these lines, results from earlier studies indicated that DHA was enzymatically converted to products coined docosanoids, whose structures were unknown at the time, that might be linked to retinal protection [41] and potentially neuronal function [42]. The complete structures of those molecules and functions were not established.

Human whole blood, isolated leukocytes, and glial cells each enzymatically convert DHA to 17*S*-hydroxy-containing docosatrienes (dihydroxy products) and 17*S*series resolvins [9, 13]. The new 10,17*S*-docosatriene series displayed potent antiinflammatory actions that included reducing or limiting further PMN numbers in exudates *in vivo* (the cessation process in resolution of inflammation [15]), and down regulating production of proinflammatory lipid mediators and cytokines by glial cells *in vitro* [13]. During the resolution phase of peritonitis, unesterified DHA levels increase and 10,17*S*-docosatriene is generated within the resolving exudates, where it appears to promote catabasis (the return from disease, cf. refs. [8, 43]), namely the return to homeostasis, by shortening the resolution interval [9, 43]. This novel DHA-derived 10,17*S*-docosatriene was next also found to be produced *in vivo* during strokes in murine tissues, studies in collaboration with Nicolas Bazan and colleagues, and limited the entry of leukocytes into the area of neural damage in the brain, thus reducing

the magnitude of tissue injury [44]. Also, in collaboration with Bazan and colleagues, we found that this 10,17*S*-docosatriene is neuroprotective in retinal pigmented cells and introduced the term neuroprotectin D1 for this potent DHA product [14], a member of the protectin family of mediators [9, 31] that accumulates in the ipsilateral hemisphere of the brain following focal ischemia [45].

NPD1 is formed from DHA in cornea in a lipoxygenase-dependent fashion to protect from thermal injury as well as promote wound healing [46]. Importantly, neuroprotectin D1, resolvin D1, and resolvin D5 are produced by trout brain cells from endogenous DHA, suggesting that the structures of these DHA-derived local mediators are highly conserved from fish to humans [47]. Together, these results underscored the need to establish the complete stereochemistry of endogenous biologically active 10,17*S*-docosatriene, namely the configuration of the conjugated double-bond system and chirality of the carbon-10 position alcohol in the potent bioactive molecule. In recognition of its wide scope of formation and actions, protectin D1 (PD1) is used to denote the structure of this chemical mediator and the prefix *neuro* before *protectin D1* notes its neural system origins (i.e. retina, brain) and addresses functional role [46-50].

The chemical signals and mediators produced by macrophages are of wide interest, because macrophages play key roles in innate host responses and local inflammation [51, 52], as well as in neovascularization, resolution of inflammation, and wound healing [51, 53, 54]. Along these lines, we also identified a novel family of potent DHA-derived molecules produced by macrophages, denoted the maresins for macrophage mediators in resolving inflammation, that act directly on phagocytes [54, 55]. MaR1, the first mediator identified in this novel family, is defined as possessing potent anti-inflammatory and proresolving actions both *in vivo* and *in vitro* [54, 55]. The present review will also discuss the approach employed for establishing the function and complete stereochemistry as well as the biological actions of Protectins, Maresins (Figure 1), some of their natural isomers and biosynthetic intermediates.

Protectin Biosynthesis and Stereochemistry

The DHA-derived 10,17-dihydroxy conjugated triene-containing product, termed PD1, is biosynthesized by several human cell types, murine exudates, skin, and brain tissues [9, 13, 14, 44, 48], as well as isolated fish tissues [47]. PD1 displays potent protective and anti-inflammatory actions [10, 13, 14, 44].

PD1 isolated and identified earlier carries alcohol groups at carbon 10 and 17 positions flanking its conjugated triene portion of this molecule, found to be critical for potent functions of this molecule [9, 13]. The stereochemistry of the carbon 17-position alcohol was retained from the precursor predominantly in the *S* configuration when derived from the lipoxygenase product 17*S*-H(p)DHA precursor [9, 13], eliminating isomers from the matching and biosynthetic pathway in Fig. 2 and ref. [50]. The doublebond geometry and stereochemistry of the alcohol group at position 10 were tentatively assigned based on biogenic evidence [9, 48], i.e., the formation of alcohol trapping products in murine brain and human leukocytes as well as identification of two vicinal diols 16,17*S*-diHDHA. The

complete stereochemical assignments and the major biosynthesis route found with isolated human leukocytes (PMN and lymphocytes; refs. [9, 48, 50]) are illustrated in Figure 2.

Each of the double bond isomers likely to be biosynthesized were prepared in view of potential biosynthetic routes involved in PD1 formation, namely the involvement of epoxide-containing intermediates and/or double dioxygenation (Fig. 2) intermediates [9, 13, 14, 48]. The *R* and *S* configuration of the alcohol group at the carbon 10-position were each prepared by stereocontrolled total organic synthesis. Each of the stereocontrolled steps from defined precursors enabled preparation of geometric isomers of the conjugated triene region that were confirmed by NMR [see ref. 50]. Also, for these experiments we prepared dihydroxydocosanoids using isolated plant lipoxygenase(s) to obtain, as in earlier experiments [56], both positional isomers 7,17*S*-diHDHA (termed resolvin D5) [57] and 10,17*S*-diHDHA [9, 13]. The preparation of these using micellar substrate presentations was given in further detail in [56]. These reference compounds prepared by biogenic synthesis with DHA were useful in analyses of biosynthetic routes.

Serhan et al. [50] reported the prominent ions and chromatographic behaviors for each of the double bond and positional isomers prepared by total organic synthesis. Each of the eight isomers gave characteristic UV λ_{max}^{MeOH} for a conjugated triene chromophore with a λ_{max}^{MeOH} at ∼270 nm with shoulders at 260 nm and 282 nm (note that these values reflect the ± 1 nm cutoff of the scanning UV spectrophotometer used in refs. [9, 50]. Each of these isomers gave a specific λ_{max}^{MeOH}, which appeared to reflect the geometry of the double bond system. For example, the Δ15-*trans* isomer in the conjugated triene portion of 10,17-diHDHA gave a UV λ_{max}^{MeOH} of 269 nm [50]. Only one of these products matched the chromatographic behavior using both LC-MS and GC-MS including C values (the relative retention time in GC compared to saturated fatty acid standards) and retention times as well as bioactivities. As expected, each of the major prominent ions for these isomers in both LC-MS-MS and GC-MS-MS were identical (i.e., daughter and parent ions were the same for each, with different retention times).

To determine the complete stereochemical assignment of PD1, we directly compared the physical and biologic properties of DHA-derived PD1 and related 10,17 dihydroxy-docosatriene stereoisomers obtained from human and mouse cells to those prepared by total organic and biogenic synthesis [50]. These included the following: 10*S*17*S*-dihydroxy-docosa-4*Z*,7*Z*,11*E*,13*Z*,15*E*,19*Z*-hexaenoic acid, also known as PDX; 10*R*,17*S*-dihydroxy-docosa-4*Z*,7*Z*,11*E*,13*E*,15*Z*,19*Z*-hexaenoic-acid, NPD1/PD1; 10*S*,17*R*,-dihydroxy-docosa-4*Z*,7*Z*,11*E*,13*E*,15*Z*,19*Z*-hexaenoic-acid; 10*R*,17*S*dihydroxy-docosa-4*Z*,7*Z*,11*E*,13*E*,15*E*,19*Z*-hexaenoic-acid; 10*R*,17*S*-dihydroxy-docosa-4*Z*,7*Z*,11*E*,13*Z*,15*E*,19*Z*-hexaenoic-acid; and 10*S*,17*S*-dihydroxy-docosa-4*Z*,7*Z*,11*E*,13*E*,15*Z*,19*Z*-hexaenoic-acid. The total syntheses of these are reported in refs. [58, 59]. Of interest, 10*S*,17*S*-dihydroxy-docosa-4*Z*,7*Z*,11*E*,13*Z*,15*E*,19*Z*-hexaenoic acid and 10*S*,17*S*-dihydroxy-docosa-4*Z*,7*Z*,11*E*,13*E*,15*Z*,19*Z*-hexaenoic-acid coeluted in this system, as did both compounds 10*R*,17*S*-dihydroxy-docosa-4*Z*,7*Z*,11*E*,13*E*,15*Z*,19*Z*-hexaenoic-acid and 10*S*,17*R*,-dihydroxy-docosa-4*Z*,7*Z*,11*E*,13*E*,15*Z*,19*Z*-hexaenoic-acid [see ref. 50]. It is of interest to note that the mass spectral identification of acid alcohol trapping products in human leukocytes suggested that an epoxide-containing intermediate was involved in NPD1/PD1 biosynthesis in human cells

[48]. The isolation and identification of alcohol trapping products indicated the involvement of an epoxide intermediate in the conversion of DHA to 10,17*S*-diHDHA, a docosatriene containing a characteristic conjugated triene structure involving three of the six double bonds present in this compound. The role of a 16(17)epoxide intermediate generated from the 17*S*hydroperoxy-DHA precursor was further supported by the identification of two vicinal diols, i.e., 16,17*S*-dihydroxydocosatrienes present in these LC-MS-MS profiles also generated from DHA. The 16(17)epoxy-DHA could open *via* non-enzymatic hydrolysis to a racemic mixture, i.e., 16*R/S*,17*S*-diHDHA, or to a single 16,17*S*-vicinol alcohol by the actions of an appropriate epoxide hydrolase in a reaction similar to that demonstrated earlier in the biosynthesis of LXA_4 [60-62]. Hence, the biosynthesis of PD1 from a 16(17)epoxide intermediate would require an enzymatic reaction to move the double bond configuration to set the triene geometry to 11*E*,13*E*,15*Z* and direct the attack of H_2O and insertion of its oxygen into the carbon 10 position of PD1 determined to be in the 10*R* stereochemical configuration, which also proved critical for potent bioactions.

The chirality of the alcohols and double bond geometry of the triene were systematically addressed in these experiments [see ref. 50]. The MS-MS spectrum of PD1 obtained from murine peritonitis (4 h) biosynthesized *in vivo* upon challenge with zymosan gave the mass spectrum that was recorded using the same instrument settings and conditions that matched synthetic 10*R*,17*S*-dihydroxy-docosa-4*Z*,7*Z*,11*E*,13*E*,15*Z*,19*Z*-hexaenoic acid. To obtain additional evidence for matching, GC-MS analyses were also performed. The chromatographic behavior and prominent ions in two separate mass spectrometry systems (LC-MS-MS and GC-MS), together with biological activity [50], permitted criteria for assignment of the physical properties of PD1 and related isomers. Because the parent and daughter ions were the same for each related natural isomer, retention time in two chromatographic systems and bioactivity were key requirements for assigning the stereochemistry of the endogenous NPD1/PD1.

10*S*,17*S*-dihydroxy-docosa-4*Z*,7*Z*,11*E*,13*Z*,15*E*,19*Z*-hexaenoic acid, also coined PDX, is consistently identified in profiles obtained from murine peritonitis [50]. It is, however, not the major product of isolated human cells nor does it carry potent actions of NPD1/PD1, also reported earlier [9, 13, 63]. Consistent with its biosynthesis (Fig. 2), the appearance of this double dioxygenation product (namely formed by consecutive sequential actions of two lipoxygenase reactions on the 1,4-cis-penta-dienes) was timedependent both *in vivo* and *in vitro* [50]. Stereoselective insertion of oxygen from H_2O was expected to give rise to a 10*R* configuration when attacking the carbonium cation intermediate to form PD1; proposed in refs. [13, 48]. Double bond geometry in the triene portion of the double dioxygenation product, i.e. 10*S*,17*S*-diHDHA or PDX [64], was not consistent with the biosynthesis of the triene of PD1 in that the *trans*, *cis*, *trans* configuration is present [48, 50]. Since these and other lipid mediators are highly conserved structures found in many species from fish to human [47], a species difference between mouse and human in PD1 structure is not evident, although the role of these products in lower organisms remains of interest.

The biosynthesis of Resolvin D5 (7,17-diHDHA) in the resolving inflammatory exudates and infectious exudates [9, 10, 57] and its formation from DHA or 17-hydroxy-DHA with isolated human neutrophils suggested that the biosynthesis of this mediator involved

sequential lipoxygenation, also known as double dioxygenation [10]. That is, in addition to using molecular oxygen for insertion at the 17-position, lipoxygenation (5-LOX and/or 15-LOX; vide infra) could also insert molecular oxygen at the 7-position in sequential fashion. The identification of this novel from DHA and the sequential lipoxygenation events in its formation [9, 13] appeared to be similar to that of 5*S*,15*S*diHETE generated from arachidonic acid [65, 66]. Hence, it was unexpected in earlier studies [56] when sequential actions of potato 5-lipoxygenase and/or 15-lipoxygenase with the substrates in micellar configuration were discovered to produce both 7,17-diHDHA and 10,17-diHDHA positional isomers as major products as well as multiple geometric isomers as minor products following hydrolysis of enzymatically generated epoxides *in vitro* [cf. 9, 13]. The formation of these isomers is apparently dependent on substrate, pH, and enzyme concentrations [56].

Oxygen Incorporation in Biosynthesis of Protectins

To study the role of sequential lipoxygenase actions in the proposed mechanism of PD1 formation [9, 50] (Figure 2) and its related positional isomer 10*S*,17*S*-diHDHA (also known as 10*S*,17*S*-DiHDoHE, PDX; https://www.caymanchem.com), we carried out incubations in an atmosphere enriched in isotope $^{18}O_2$ with 17-hydroperoxy-DHA as substrate and isolated potato 5-lipoxygenase [50]. Note that PD1 and PDX differ in both chirality at carbon 10 and geometry of their respective triene double-bond configuration (Fig. 2) [64]. Following extraction and isolation, the product profiles, GC-MS and LCMS-MS results indicated that ^{18}O was incorporated [50] in the carbon 10-position in 10*S*,17*S*-diHDHA (Fig. 2). Chromatographic separation of 10*S*,17*S*-diHDHA gave prominent ions with MS-MS, indicating on average >75% incorporation of ^{18}O originating from molecular oxygen in the carbon 10-position with a range of 51.4 to 91.8% increase in diagnostic ions. Since these enzymes use molecular oxygen as a substrate, it is not possible, under these conditions, to completely replace enzymeassociated ^{16}O for the ^{18}O isotope as calculated earlier for lipoxin A_4 in refs. [60, 61]. The extent of ^{18}O present in diagnostic ions was determined for m/z 181/183, 261/263, 289/291, 297/299, 315/317, 323/325, 341/343, and 359/361, and the ratio of ^{16}O to ^{18}O calculated from ion intensities and averaged. These results indicate that 10*S*,17*S*diHDHA can be produced *via* double lipoxygenation in biological systems and *in vitro* with isolated enzymes.

Results from matching studies indicated that the double bond geometry for the conjugated triene portion of this molecule was in the *trans,cis,trans* configuration [50]. Double dioxygenation to form 10*S*,17*S*-diHDHA was also a mechanism to generate this compound *in vivo*, since it is a prominent product in murine exudates from peritonitis, suspensions of human leukocytes incubated with DHA [13] and murine brain [9, 10], as well as trout leukocytes and brain [47]. Figure 2 outlines the proposed scheme and proposed role for double dioxygenation and its product 10*S*,17*S*-diHDHA, a member of the protectins that may be an independent pathway marker of cell-cell interactions and DHA consumption *in vivo*. The double bond geometry in the conjugated triene portion of the molecule (*trans,cis,trans)* is consistent with oxygenation using molecular oxygen with two sequential lipoxygenation steps [13, 50]. This product is also known as PDX [67]. Given the biological actions, chromatographic and physical properties of PD1 as well as the results from epoxide trapping experiments with human PMN and the isolation of two vicinal diol 16,17*S*-

docosatrienes as minor products [13], it is likely that, once the 16,17-epoxide [48, 50] is generated *in situ* (as illustrated in Figure 2), an enzymatic reaction is needed to produce PD1 carrying the 10*R*,17*S*-dihydroxy-*trans*,*trans*,*cis* configuration arising from an epoxide intermediate as depicted in Figure 2 that is produced [48].

Actions of Protectin D1 *in vivo*

Keep in mind that resolution mechanisms need to proceed in all organs from head to toe, where some organ-dependent differences, i.e. kinetics, may occur [8, 68]. As indicated above, the complete stereochemical assignment for synthetic PD1 also relied on determining biological action of the related isomers. Earlier results indicated that PD1's anti-inflammatory properties were comprised of limiting leukocyte infiltration in murine systems [9, 13, 44, 56]. Synthetic PD1 reduced human PMN transmigration in response to leukotriene B_4 and T cells [12, 48, 69]. Amounts as small as 1.0 nM gave 30% inhibition in leukocyte infiltration. The Δ15-*trans* isomer of PD1, where the conjugated triene portion of the molecule is in the *trans* configuration, did not block PMN transmigration *in vitro* [50].

These PMN transmigration experiments were carried out in parallel with murine acute inflammation. In these, acute peritonitis was initiated by challenge with the microbial isolate zymosan A and the actions of five isomers were assessed *in vivo*. Two compounds (10*S*, 17*R*,-dihydroxy-docosa-4*Z*,7*Z*,11*E*,13*E*,15*Z*,19*Z*-hexaenoic-acid and 10*S*,17*S*-dihydroxy-docosa-4*Z*,7*Z*,11*E*,13*E*,15*Z*,19*Z*-hexaenoic-acid; see ref. [50]) were excluded from matching with PD1 because the physical retention times on LC and GCMS [50] and biosynthetic considerations indicated that they were not likely candidates for endogenous human PD1 or isomers produced. It is noteworthy that PD1 at doses as low as 1 ng per mouse gave striking inhibition of PMN infiltration within the exudates. In these studies, the double dioxygenation product 10*S*,17*S*-docosatriene (PDX) was substantially less potent. In this context, the double dioxygenation product PDX was not active at 0.1 ng compared directly to synthetic PD1. At higher doses, 10*S*,17*S*-HDHA blocked PMN infiltration, but it was less potent than PD1. The 10*R* version of the double dioxygenation products, was essentially equipotent at a 1 ng dose but did not increase potency in a dose-dependent fashion at 10 ng and 100 ng doses [50]. The Δ15-*trans* isomer of PD1 was, at equal doses of 1 ng/mouse, substantially less potent. Of interest, 10*S*,17*S*-dihydroxy-docosa-4*Z*,7*Z*,11*E*,13*E*,15*Z*,19*Z*-hexaenoic-acid was the most potent of this series of isomers *in vivo*. However, only trace amounts were obtained in isolated human PMN extracts. Hence, a rank order of potency at the 0.1 ng dose of these 10,17-diHDHA isomers was 10*S*,17*S*-dihydroxy-docosa-4*Z*,7*Z*,11*E*,13*E*,15*Z*,19*Z*-hexaenoic- acid ≫ PD1 > 10*S*,17*S*-DiHDHA (also known as PDX, the double dioxygenation product [67]) > the Δ15-*trans*-PD1 ≫ 10*S*,17*R*,-dihydroxy-docosa-4*Z*,7*Z*,11*E*,13*E*,15*Z*,19*Z*-hexaenoic-acid. In these, we also assessed the carboxy methyl ester of PD1 versus the native synthetic PD1. The carbon 1-position carboxy-methyl ester was similar in its ability to block *in vivo* the hallmark of acute inflammation, namely PMN infiltration. The methyl ester of 10*S*,17*S*-dihydroxy-docosa-4*Z*,7*Z*,11*E*,13*E*,15*Z*,19*Z*-hexaenoic-acid also proved to be a potent regulator of PMN infiltration and diminished pain *in vivo* [70]. One of the protectins, 10,17-docosatriene, is also produced in murine ischemic stroke and is a potent regulator of PMN infiltration, reducing stroke-mediated tissue damage [44], and in ocular systems [71]. Given its potent protective actions in the retina and brain,

we initially termed this bioactive mediator neuroprotectin D1 (NPD1) [12, 14, 72]. Since this potent chemical mediator has a broader range of activities in the immune, cardiovascular and renal systems, for nonneuronal local biosynthesis and actions we used the name protectin D1 (PD1) for the first in this family of mediators. The complete stereochemistry and anti-inflammatory actions of PD1 (10*R*,17*S*-dihydroxy-docosa-4Z,7Z, 11E,13E,15Z,19Z-hexaenoic acid) were established [50] and its biosynthesis in lymphocytes and immunoregulatory roles also demonstrated [8, 48, 69]. PD1 is also renoprotective [73], induces corneal nerve regeneration [74] and stimulates cardiac and neural stem cell differentiation at nanomolar potencies [75]; see Table 1.

In earlier experiments with mice, when administered i.v., 10,17*S*-docosatriene (Tables 1 and 2) was found to be more potent than indomethacin in reducing PMN infiltration in peritonitis, i.e., ∼40% inhibition at 100 ng/mouse [13]. Synthetic PD1 in as small a dose as 1 ng/mouse gave ∼40% inhibition of PMN infiltration that was maintained at the 10 ng and 100 ng doses. Thus, PD1 is a potent regulator of human and mouse PMN infiltration but does not completely block PMN recruitment, which is consistent with its autacoid actions and apparently does not compromise host defense *via* immune suppression of effector cell function [76]. This was also the case with human PMN transmigration, which required 10-100 nM PD1 to reduce PMN transmigration by 35-45% *in vitro*. Thus, although PD1 stereoselectively reduces PMN transmigration *in vitro*, given its potent actions *in vivo*, PD1 likely targets additional cell types *in vivo* to evoke its potent anti-inflammatory actions *in vivo* (Table 1). Alternatively, these potencies *in vivo* vs. isolated human cells might also reflect species differences. As an inducer of peritonitis, zymosan stimulates the initial formation of many endogenous chemoattractants for PMN, i.e., LTB_4, the complement component C5a, chemokines and cytokines [56]. Since PD1 stops PMN recruitment *in vivo*. it counteracts these several different sets of PMN chemoattractants that regulate trafficking of these cells *in vivo* [56]. Given the inherent chemical labilities of PD1, we also prepared and tested a more chemically stable form denoted 15,16-dehydro-PD1. In general, acetylenic triple bonds are more stable than conjugated double bond systems, which are prone to isomerization. Although less potent, chemical stabilization of the conjugated double bonds with an acetylenic form proved useful as the molecule retained activity *in vivo* (Tables 1 and 2). These results are consistent with the ∼40% inhibition obtained with a 4,5-acetylenic analog of PD1 [56].

On identification and structural elucidation of 10,17-diHDHA in resolving inflammatory exudates [9, 48, 56] and its potent anti-inflammatory actions, it was critical to establish its biosynthesis from DHA. To address this, we also studied isolated human PMN, whole blood, microglial cells, and murine exudates and tissues for the purpose of direct comparison [9, 13].

In addition to PD1, which carried potent bioactions, an isomer of PD1, 10*S*,17*S*diHDHA (PDX), was also identified for the first time (Fig. 2). This compound proved to be a double dioxygenation product and was also formed from DHA but in a reaction that required two sequential lipoxygenation steps and oxygen incorporation that was directed at the 10*S* position from molecular oxygen (i.e., $^{18}O_2$ in an enriched atmosphere *in vitro)*. This reaction to products of 10*S*,17*S*-diHDHA is markedly different from the proposed enzymatic

hydrolysis of the epoxide intermediate. The double dioxygenation product formed *in vivo* is different from PD1 in at least three key ways: a) PD1 carbon 10 position alcohol is in the 10*R* configuration while the dioxygenation product is in the 10*S* configuration; b) the double bond structure of PD1 conjugated triene is in the 11*E*,13*E*,15*Z* configuration; the 10*S*,17*S*-dioxygenation product is in the 11*E*,13*Z*,15*E* configuration; c) most importantly, PD1 is more potent: PD1 ≫ 10S,17S-diHDHA; and d) PD1 is generated by isolated human leukocytes and tissues.

Also in support of the stereospecific basis of these DHA-derived products in human and murine systems is the bioaction of the Δ15-trans-PD1 isomer, which can arise *via* workup-induced isomerization of PD1 and possesses little bioactivity *in vitro* or *in vivo* within the dose or concentration range (Fig. 2) observed with biogenic or synthetic PD1. Also, 1*0R*, 17*S*-dihydroxy-docosa-4*Z*,7*Z*,1*1E*,13*Z*,1*5E*,19*Z*-hexaenoic-acid, identified in human leukocytes (Fig. 2) and which differed from 10*S*,17*S*-dihydroxy-docosa-4*Z*,7*Z*,11*E*,13*Z*,15*E*, 19*Z*-hexaenoic acid at the *10R* position and carried the same double bond geometry, was essentially equipotent at a 1 ng dose (Fig. 2). Hence, the biosynthesis of PD1 from DHA appears to require stereoselective enzymatic steps to evoke bioactions. This requirement for stereoselective enzymatic reactions is widely appreciated in the biosynthesis of eicosanoids (9, 44). Of interest, human leukocytes specifically bind ^{3}H-labeled NPD1/PD1 [77]. Thus the fidelity of the enzyme that produces PD1 from the proposed carbonium cation intermediate [13] in its ability to direct insertion of H_2O-derived alcohol at carbon 10 exclusively in the 10*R* with apparently trace amounts of 10*S* as in 10*S*,17*S*-dihydroxy-docosa-4*Z*,7*Z*,11*E*,13*E*,15*Z*,19*Z*-hexaenoic-acid is an intriguing point for further studies. The GPCR for NPD1/PD1 is currently not known, whereas the GPCRs for RvE1, RvD1 and LXA_4 are known and were recently reviewed in Serhan and Chiang [78].

Earlier results indicated that DHA, which does not appear to be a natural substrate for potato 5-lipoxygenase, is converted to 10-HDHA by this enzyme and the double dioxygenation product 10,20-diHDHA [79]. Of interest, Whelan et al. [80] demonstrated that this plant lipoxygenase is highly versatile with DHA as a substrate and identified several monohydroxy-DHA products at carbon positions 4, 7, 8, 11, 13, 14, 16, 17 to give positional isomers of HDHA. Each of these was an enzymatic product of this flexible enzyme. As with other lipoxygenases, we found that potato 5-LOX as well as the soybean 15-lipoxygenase gave specific diHDHA profiles of products that were dependent on pH, enzyme, and substrate concentrations used in the incubations [9]. When the substrates were presented in micellar configuration with the enzymes, hydroperoxy intermediates were converted to epoxides that, on hydrolysis, gave many of the isomers as relatively minor products but were nonetheless in quantities useful for *in vitro* and *in vivo* studies [56]. These results were advantageous in the preparation of key pathway intermediates (i.e., 7,17-diHDHA, Resolvin D5, 17*S*-HDHA, and 17 *S*-H(p)DHA) to mammalian systems used in biosynthesis studies and determining the identity and actions of enzymatic products generated *in vivo* as well as by isolated human cells from DHA [9, 13, 48].

Both DHA potato 5-LOX products 10,20-diDHA and 10-HDHA were originally reported [Ref 79; doctoral thesis]. The classic steric analysis of 10*S*-HDHA and the formation of 10,20-diHDHA and 17-H(p)DHA were reportedly optimized for the plant LOs [81]. In our

experiments, the double dioxygenation product prepared, matched, and identified in both human PMN and *in vivo* during peritonitis carries its alcohols as expected in the 10*S*,17*S* configuration in this diHDHA (10*S*,17*S*-dihydroxy-docosa-4*Z*,7*Z*,11*E*,13*Z*,15*E*,19*Z*-hexaenoic acid). Hence, this natural isomer of PD1 formed *in vivo* from DHA has its Δ13 position double bond in the *cis* configuration (i.e., 13*Z*) and its Δ11 in the trans configuration within the conjugated triene portion of the molecule (11*E*,13*Z*,15*E*) and possesses some anti-inflammatory activity *in vivo*. albeit proved to be much less potent than natural or synthetic PD1. In humans in vivo, PD1 level may not be apparent on chromatographic analysis with LC-MS-MS, but the endogenous biosynthetic isomers Δ15-trans-PD1, 10-epi-15-trans-PD1, etc. can be identified in substantial amounts that signify the activation of the pathways *in situ* [82].

In addition to this lipoxygenase-initiated route of biosynthesis for PD1, an aspirintriggered route with a *17R* epimer of PD1 *(17R* series) is apparent with human cells *via* acetylated COX-2 and subsequent reactions [9, 44]; the complete stereochemistry of this bioactive epimer is now determined; see below. It is of interest to note that 10*S*,17*R*-dihydroxy-docosa-4*Z*,7*Z*,11*E*,13*E*,15*Z*,19*Z*-hexaenoic acid [50] was essentially inactive *in vivo*.

Aspirin-Triggered Mediators

Aspirin-triggered resolvins and related ω-3 -derived mediators were first identified in self-limited resolving murine exudates as well as brain, and their production documented with isolated human cells (i.e., leukocytes, microglia and vascular cells) [9] and in stroke [44]. The complete stereochemistry and bioactions of this aspirin-triggered 10,17-docosatriene was recently established [49]. The complete stereochemistry of the aspirin-triggered protectin pathway from murine exudates and human PMN is shown in Figure 3. AT-(NPD1/PD1) displays potent protective bioactions comparable to NPD1/PD1 both *in vitro* and *in vivo*. reducing both PMN infiltration and enhancing the removal of apoptotic PMN by macrophages, and hence signals the cardinal signs of resolution.

Aspirin is unique in its mode of action. Unlike many of the current antiinflammatories that delay complete resolution and are considered toxic to this vital process (i.e., resolution toxic) [76, 83], aspirin jump-starts resolution by novel previously unrecognized mechanisms that involve the biosynthesis of aspirin-triggered (AT) lipid mediators [84, 85]. Along these lines, aspirin-triggered lipoxins were the first aspirintriggered lipid mediators uncovered [86]. Aspirin is well appreciated for its ability to inhibit COX-1 and inactivate this enzyme, blocking both prostaglandin and thromboxane production in cells that possess these biosynthetic pathways [87, 88]. The mechanism of aspirin's action involves acetylation of COX within the enzyme's catalytic region. This prevents alignment of the substrate arachidonic acid for oxygenation within the catalytic center that produces the prostaglandin endoperoxide intermediate (PGG2) required for the biosynthesis of both thromboxanes and prostaglandins *via* COX-1.

Aspirin's action within cells that possess COX-2 appears different. The catalytic region of COX-2 is larger than that of COX-1 and when acetylated by aspirin the biosynthesis of endoperoxide is blocked. Yet, unlike COX-1, acetylated COX-2 remains active producing lipoxygenase-like products such as 15-HETE from arachidonic acid [10] but with the

oxygen insertion in the *R* configuration rather than *S* as is the case with lipoxygenases [86, 89]. The COX-2-produced 15R-HETE is converted to the potent bioactive aspirin-triggered lipoxins that retain the carbon 15 position alcohol in the *R* configuration as in AT-15*R*-(epi)-lipoxin A_4, which is produced *in vivo* in humans [90]. AT-LXA_4 is longer acting *in vivo* than LXA_4 because the 15*R* alcohol is less efficiently enzymatically converted to the inactive 15-oxo-LXA_4 metabolite [91]. This aspirintriggered pathway was recently demonstrated in humans, where low-dose aspirin is antiinflammatory and triggers the endogenous biosynthesis of AT-LXA_4, which in turn stops PMN infiltration in skin blisters [Ref. 92 and references within].

AT-(NPD1/PD1) was identified in resolving murine exudates treated with aspirin [9]. Thus, biologic AT-(NPD1/PD1) was obtained from resolving murine exudates *in vivo*. To this end, peritonitis was initiated *via* intra-peritoneal (i.p.) administration of zymosan A (1 mg/mouse), and exudates were harvested 24 hours post injection (i.e. within the resolution phase [43]). Exudates were taken for LC-UV-MS-MS-based mediator lipidomics [49]. Among the candidates, synthetic AT-(NPD1/PD1) matched peak I, by co-injection. Synthetic AT-(NPD1/PD1) gave a UV spectrum and tandem mass spectrum that was essentially identical to the material beneath, which further confirmed the matching. AT-(NPD1/PD1) was also isolated and identified from activated human leukocytes, namely, aspirin treated human PMN. By matching biologic materials from both human and murine exudates AT-(NPD1/PD1) with synthetic candidates, the results established the complete stereochemistry of endogenous AT-(NPD1/PD1) as 10R,17Rdihydroxydocosa-4Z,7Z,10E,12E,14Z,19Z-hexaenoic acid [Ref 49 and see Figure 3].

AT-NPD1/PD1 Stops Neutrophil Infiltration: PMN Cessation *in vivo*

The complete stereochemical assignment for AT-(NPD1/PD1) also relied on determining in parallel its biological actions [49]. Earlier results indicated that NPD1/PD1 exerted potent anti-inflammatory actions regulating leukocyte trafficking in murine systems [9, 13, 50]. In these studies, we compared directly the bioactions of NPD1/PD1 to that of AT-(NPD1/PD1) carried out in parallel with the physical matching experiments. Synthetic AT-(NPD1/PD1) limited PMN infiltration into the peritoneum in TNFα-stimulated peritonitis. Both NPD1/PD1 (0.1 ng-10 ng) and AT-(NPD1/PD1) (0.01ng-10.0 ng) proved to be significant regulators of TNFα-stimulated leukocyte infiltration into the peritoneum (Tables 1 and 2). AT-(NPD1/PD1) reduced total leukocyte population of the exudate including PMN, monocyte and lymphocyte infiltrates, reaching a maximal reduction at 1 ng/mouse by as much as 50.4%. Also, both PD1 and AT-NPD1/PD1 reduced PMN infiltration in response to zymosan [49].

We studied whether PD1 or AT-NPD1 impacts PMN transendothelial migration using isolated human cells because this is the first committed step of leukocytes in acute inflammation. AT-(NPD1/PD1) and NPD1/PD1 (0.1-10.0 nM) each reduced (∼30% and ∼50%, respectively) PMN transendothelial migration induced by LTB_4 [49]. In comparison, equal concentrations of the Δ^{15}-trans isomer of AT-(NPD1/PD1) where the conjugated triene portion of the molecule was in the *trans* rather than *cis* configuration did not significantly reduce PMN transendothelial migration. Again in this system, the precursor

DHA did not reduce LTB_4-stimulated PMN-transendothelial migration at these concentrations. To corroborate these findings, we next used an electric cell-substrate impedance sensing system (ECIS) that sensitively quantitates cellular responses in two cell systems by real-time monitoring of barrier impedance [93]. Both AT-(NPD1/PD1) and NPD1/PD1 (1nM) decreased LTB_4-stimulated PMN-transendothelial migration by ∼40 and 30%. AT-(NPD1/PD1) also enhanced the uptake of apoptotic human PMN by human macrophages at concentrations as low as 0.1 nM, as did NPD1/PD1 when compared directly [49].

This novel aspirin-triggered DHA metabolome impacts inflammation and resolution of cellular processes. On the basis of physical and biological properties in matching results with material from human cells, murine exudates and total organic syntheses, the complete stereochemistry of AT-(NPD1/PD1) proved to be *10R,17R*dihydroxydocosa-4Z,7Z,11E,13E, 15Z,19Z-hexaenoic acid. The new aspirin-triggered product demonstrated potent regulatory actions with leukocytes *in vivo* and reduced human PMN transendothelial migration and enhances efferocytosis [49]. This matching approach was deemed necessary because typically less than nanogram amounts of a given lipid mediator are produced *in vivo* and >100 μg of isolated product is required for direct NMR determinations of conjugated double bonds that might give assignments for stereochemistry [50, 94-96] (see Figure 3).

Similar to the inversion of stereochemistry observed in the aspirin-triggered lipoxin and resolvin pathways, we anticipated that AT-(NPD1/PD1) was likely to possess the carbon 17R stereochemistry [9], rather than the 17S chirality present in NPD1/PD1 produced *via* a lipoxygenase reaction [12]. Aspirin acetylation of COX-2 gives rise to a catalytic activity that converts DHA to 17*R*-hydroxy-containing products [9]. It is noteworthy to point out that the chirality at the C-10 position and the double bond geometry of the triene system could not be reliably predicted based on LC-MS-MS lipidomics alone. As proposed for other potent polyunsaturated fatty acid-derived local mediators, it is likely that the aspirin-triggered protectin pathway involves the conversion of the hydroperoxide 17*R*-HpDHA to an epoxide intermediate (Figure 3). Given the significant conformational differences anticipated for these DHA-derived products, the stereochemical mode of the enzymatic epoxide opening and the resulting triene geometry needed to be verified by matching with isomers of known stereochemistry. In order to achieve the complete stereochemical assignments for the bioactive product(s), therefore, we prepared several geometric isomers of the 10,17*R*-dihydroxydocosa-4Z,7Z,11,13,15,19Z-hexaenoic acid with specific changes in R/S and Z/E configurations, and confirmed their stereochemistry using NMR spectroscopy. These synthetic materials were also used for the direct matching and comparison with biologically produced AT-(NPD1/PD1) [96].

Matching results indicate that in this aspirin-triggered DHA metabolome, the stereochemical configuration of AT-(NPD1/PD1) differs from NPD1/PD1 only at carbon C-17, so the two are diastereomers. Results from earlier investigations have shown that the differences in the MS and UV spectra between lipid mediator diastereomers are minimum if any [86, 94, 95]. The tandem mass spectra of the AT-17*R*-containing product and NPD1/PD1 were essentially identical, and both compounds displayed essentially the same triplet-band of absorbance-shaped UV spectra with λ_{max}^{MeOH} at 271 nm (Figure 3), characteristic of “ol-triene-ol”

structures [50, 97]. Some diastereomers can possess different physical properties [98] by which they can be physically differentiated. Indeed, NPD1/PD1 and its aspirin-triggered isoform were well separated using reverse phase lipid chromatography conditions. Diastereomers can give different biological properties, such as LTB_4 and its natural isomer 12-epi-6-trans-LTB_4, which is log orders of magnitude less active [97, 99].

TNFα is a well-known cytokine for its role in host defense, however aberrant or uncontrolled TNFα responses are associated with several inflammatory disorders [7]. Although anti-TNF therapies are widely available and in clinical use, it is becoming increasingly clear that a complete blockade of TNFα may promote adverse side effects [100], thus indicating limitations in this approach [101]. Importantly, AT-(NPD1/PD1) is a potent regulator of PMN infiltration *in vivo* yet did not completely block PMN recruitment, and hence is not immunosuppressive. This is consistent with the unique counterregulatory actions of resolvins and specialized proresolving mediators that do not compromise host defense *via* immune suppression of effector cell function [95]. In human PMN transmigration, 1-10 nM amounts of AT-(NPD1/PD1) reduced LTB_4-stimulated PMN transmigration, and the Δ^{15}-trans isomer of AT-(NPD1/PD1) was less potent, underscoring the stereoselectivity of AT-(NPD1/PD1) and the counterregulatory yet not immunosuppressive actions of pro-resolving mediators.

This assignment implies that the postulated enzyme-catalyzed opening of the 16R,17R epoxide precursor of AT-(NPD1/PD1) would proceed, in theory, *via* the introduction of the C-10 hydroxyl from the same side as the epoxide oxygen atom to give the 10R,17R configuration, likely from water (Fig. 3). This addition would be opposite of the observed opening of the 16S,17S epoxide-containing intermediate precursor of NPD1/PD1 that leads to the 10R,17S configuration. This difference suggests that the C-10 hydroxyl group is introduced *via* an enzyme-catalyzed process, and that the enzyme involved has a strong preference for the formation of the 10R isomer, rather than the stereochemical inversion of the epoxide precursor. These observations are consistent with the formation of a rigid cationic-type intermediate at the enzyme active site, which undergoes stereospecific attack by a water molecule from the same direction to afford the 10R product. Also, the well known antarafacial hydrogen abstraction in LTA_4 biosynthesis in this case to produce the 16,17-epoxide-containing intermediate from DHA might be similar to the biosynthetic mechanism of LTA_4 from 5-HpETE as carried out by the 5-LOX [102, 103]. It is possible in this scheme (Figure 3) that the 17-hydroperoxide intermediate is converted to an epoxide at C16-C17 position that could give to *cis* or *trans* epoxides that require enzymatic processing *in situ* to give the potent active stereochemical structure of AT-(NPD1/PD1).

In humans, low-dose aspirin triggers 15-epi-lipoxin production that appears in plasma of healthy subjects [90]. At low dose, aspirin increases 15-epi-LXA_4 in skin blisters, which reduces PMN infiltration to the skin blister, demonstrating the antiinflammatory actions of low-dose aspirin in humans [92]. Aspirin treatment reduces mortality from colorectal cancer in long-term studies with daily use [104]. Omega-3 EFA (EPA and DHA) at ∼1g/day reduce cardiovascular mortality [105]. Thus, the combination of aspirin with omega-3 EFA and the aspirin-triggered lipid mediators (i.e., AT-LX, ATRv) may have a beneficial impact in many diseases associated with ongoing inflammation and host-mediated local tissue injury.

Indeed, in a recent clinical study, the combination of both aspirin (81 mg/day) and omega-3 EPA and DHA significantly reduced periodontal disease in patients with chronic periodontitis [106], presumably by jump-starting resolution of periodontal inflammation *via* production of aspirin-triggered mediators and inhibition of prostanoids [107]. Hence, it is likely that the aspirin inhibition of prostanoids and concomitant biosynthesis of aspirin-triggered lipid mediators such as AT-lipoxins, AT-resolvins and AT-protectins, including the AT-(NPD1/PD1) pathway (Figs. 1 and 3), may each contribute to some of these beneficial outcomes, which remains to be established in humans taking aspirin long term.

Maresins

Macrophages play key roles in regulating the innate host response to local inflammation and tissue regeneration. These cells are also central in orchestrating other processes including neovascularization and wound healing [108-110]. A new family of DHA-derived pro-resolving mediators from macrophages was recently described and coined *maresins* (macrophage mediators in resolving inflammation). The first members of this family exert potent phagocyte-directed actions that include inhibition of neutrophil recruitment and stimulation of macrophage efferocytosis by the dihydroxy-containing products in this pathway [111]. The biosynthesis of maresins is initiated in macrophages by the 14-lipoxygenation of DHA producing the 14*S*-hydro(peroxy)-docosa- 4*Z*,7*Z*,10*Z*,12*E*,16*Z*,19*Z*-hexaenoic acid (14*S*-HpDHA; Figure 4). Profiling of LM using LC-MS-MS of murine self-resolving exudates demonstrates that 14*S*-HDHA levels, the maresin pathway marker, peak late into the resolution phase, suggesting a role for these mediators in re-establishing tissue homeostasis [111]. The maresin pathway is also present in human macrophages [111, 112] and was identified in human synovial fluids from patients with arthritis [113].

Maresin 1 (MaR1) is the first member identified from this family of macrophagederived pro-resolving mediators. As demonstrated by the identification of alcohol trapping products, the biosynthesis of MaR1 is proposed to involve a 13,14-epoxide intermediate as the MaR1 precursor. This epoxide intermediate is then proposed to be enzymatically hydrolyzed (see Figure 5) *via* an acid-catalyzed nucleophilic attack by water at carbon 7, resulting in the introduction of a hydroxyl group at that position and double-bond rearrangement to form the stereochemistry of bioactive MaR1 [111]. The complete stereochemistry of MaR1 proved to be 7*R*,14*S*-dihydroxy-docosa-4*Z*,8*E*,10*E*,12*Z*,16*Z*,19*Z*-hexaenoic acid, that was shown to possess potent bioactions [114]. MaR1 exerts stereospecific leukocyte-directed actions, characteristic of the maresin family. This pro-resolving mediator also exerts potent cancer-induced antinociceptive and tissue regenerative actions in wound healing in planaria (8).

These findings and the biosynthesis of MaR1 in flatworms suggested that maresin structure and function are conserved in evolution [114]. Given the potent actions of MaR1 it was deemed critical to establish the complete stereochemistry and stereospecific conversion of the proposed epoxide intermediate to MaR1. Also, using stereochemically pure material obtained by total organic synthesis we uncovered novel bioactions of this key epoxy-containing intermediate that regulates leukotriene B_4 production *via* directly inhibiting LTA_4 hydrolase and provides novel mechanisms for phagocytes to stimulate the tissues'

return to homeostasis [115]. Recently, MaR2 was elucidated and named because it also possesses potent bioactions *in vivo* [116].

Maresin Biosynthesis and Structural Elucidation

Using material obtained by stereo-controlled organic synthesis in combination with NMR spectroscopy and LM metabololipidomics, we establish the complete stereochemistry of this 13*S*,14*S*–epoxy-docosahexaenoic acid as 13*S*,14*S*-epoxy-docosa-4*Z*,7*Z*,9*E*,11*E*,16*Z*,19*Z*-hexaenoic acid (Fig. 4) and demonstrate its precursor role in conversion to MaR1 by human macrophages. Also, incubation of this epoxide intermediate with isolated human recombinant LTA_4 hydrolase (LTA_4H) inhibited LTB_4 production from LTA_4. Moreover, incubation of human macrophage LOX with 13*S*,14*S*epoxy-DHA selectively inhibited AA conversion to 12-HpETE. Together, these findings demonstrate novel bioactions within the maresin pathway in controlling proinflammatory LM (i.e. LTB_4) as well as establish the stereochemistry of the epoxide intermediate in MaR1 biosynthesis.

Macrophages are key regulators of the inflammatory response with distinct macrophage subtypes linked with the propagation versus resolution of inflammation [112, 117]. In this context there are two broad categories of macrophages: M1 macrophages or classical macrophages are considered as pro-inflammatory, while M2 macrophages are linked with the re-establishment of homeostasis, wound healing and tissue regeneration [108, 110, 117]. A third macrophage subtype referred to as resolution phase macrophages was identified in resolving exudates, with these macrophages possessing characteristics of both M1 and M2 cells [108, 109], and each human subpopulation expresses 12-LOX [112, 116]. Recent results indicate that DHA and DHAderived pro-resolving mediators, including RvD1, can stimulate a switch in macrophage phenotype from pro-inflammatory to a pro-resolving M2-like phenotype [118, 119]. We recently found that human M2 macrophages are associated with higher MaR1 levels [112], a finding that is in line with the homeostatic and tissue regenerative actions of this pro-resolving mediator [111, 114]. Recently, we demonstrated that this is, at least in part, a result of enhanced ability of this macrophage subtype to convert the 13S,14S epoxide intermediate to MaR1 [115].

To establish the biosynthetic pathways for LM biosynthesis, it was deemed essential to determine the presence and stereochemistry of their intermediates [111, 115]. In the biosynthesis of MaR1 (Figure 4), DHA undergoes 14*S*-lipoxygenation to give 14-HpDHA, following H abstraction, that is converted to 13*S*,14*S*-epoxy-DHA, a proposed intermediate that is then further transformed to 7*R*,14*S*-dihydroxydocosa-4*Z*,8*E*,10*E*,12*Z*,16*Z*,19*Z*-hexaenoic acid, MaR1 [111]. Recently, we found that this 13,14-epoxide is produced by isolated human macrophage lipoxygenase, as demonstrated by the identification of the aqueous hydrolysis products of 13*S*,14*S*-epoxy-DHA along with the corresponding acid methanol trapping products from DHA [115] (Figure 1). Of note in these incubations we also identified the double dioxygenation isomer of MaR1, 7*S*,14*S*-dihydroxydocosa-4*Z*,8*E*,10*Z*,12*E*,16*Z*,19*Z*-hexaenoic acid [cf. 111, 114], suggesting that this macrophage 12-LOX also oxygenates DHA at C7. Although this double dioxygenation product (7*S*,14*S*-diHDHA) possesses some bioactivity [111, 114], within the context of inflammation it displays lower anti-inflammatory and pro-resolving actions than MaR1. Along these lines, the 14-HpDHA

intermediate can also undergo another double dioxygenation at the penultimate carbon (omega-1) to give the 14*S*,21*R*dihydroxydocosa-4*Z*,7*Z*,10*Z*,12*E*,16*Z*,19*Z*-hexaenoic acid [120]. This maresin-related pathway product displays tissue protective actions promoting wound healing in diabetic models and following renal ischemia reperfusion injury by restoring mesenchymal stem cell function [121, 122].

Biosynthesis of lipid mediators occurs at sites of inflammation and tissue injury whereby the intermediates are either rapidly transformed to bioactive mediators *via* stereospecific enzyme-mediated conversion or non-enzymatically hydrolyzed to virtually inactive products [96, 123, 124]. Therefore, tissue levels of these intermediates are transient and hence do not reach quantities that can be isolated for direct stereochemical determination by NMR spectroscopy [96]. To this end, we used direct matching of material obtained by total organic synthesis (which allows for the generation of compounds with known absolute stereochemistry) with biological materials [115]. This approach again permitted both the scale-up and confirmation of the potent antiinflammatory and pro-resolving actions of resolvins, protectins and MaR1 [reviewed in refs. 96, 114] as well as their unambiguous identification in biological tissues *via* targeted lipidomics (LC-MS-MS-based). The ability to identify resolvins revealed their remarkable protective actions including their ability to enhance immune vigilance [125] and to stimulate host immune responses, lowering antibiotic requirements during infection [126].

Using total organic synthesis, we established the absolute stereochemistry of the novel 13*S*, 14*S*-epoxy-containing intermediate, which proved to be 13*S*,14*S*-epoxy-4*Z*,7*Z*,9*E*,11*E*,16*Z*, 19*Z*-hexaenoic acid (Figure 4). When incubated with activated human macrophages this synthetic compound was readily converted to MaR1 (Figure 4). In contrast, incubation of this synthetic epoxide with heat-inactivated cells gave no conversion to MaR1, since the epoxide was instead converted to its corresponding nonenzymatic hydrolysis products (Figure 4). Hence, in the proposed biosynthesis of MaR1, the LOX first abstracts a hydrogen from the methylene group at C12 and inserts molecular oxygen at C14 to yield 14*S*-HpDHA.

In macrophages lacking the murine homologue of this enzyme (12/15-LOX), the conversion of DHA to 14-HpDHA was greatly reduced (>95%), indicating the role of the murine orthologue of 12-LOX in initiating MaR1 biosynthesis in macrophages [111] (Table 2). This HpDHA is then converted to the 13*S*,14*S*-epoxide by the same enzyme following a second hydrogen abstraction from the methylene group at C9. These results together indicate that in addition to catalyzing the 14-lipoxygenation of DHA this same enzyme is also an epoxidase producing the novel maresin epoxide intermediate (see scheme in Figure 4). This type of reaction has been demonstrated for 5-LOX, which converts arachidonic acid to 5-HpETE and then to LTA_4 [102]. The conversion of the 13*S*,14*S*-epoxide to MaR1 then proceeds *via* an enzyme-mediated hydrolysis that converts the double bond geometry at the carbon 8 position from Z to E and at the carbon 12 position from Z to E, giving a final geometry around the triene region of MaR1 corresponding to 8E,10E,12Z (see Figure 4), as well as directs the insertion of a hydroxyl group (from H_2O) at C7 [111]. Thus, the epoxide intermediate to MaR1 requires an enzyme-mediated hydrolysis and Z/E reconfiguration in order to give the conversion of the double bond geometry at Δ^{12} from *trans* to *cis* orientation, giving a final geometry of MaR1 (i.e. 7*R*,14*S*-dihydroxy-docosa-4Z,8*E*,10*E*,

12*Z*,16*Z*,19*Z*-hexaenoic acid). In the absence of this enzyme, the 13*S*,14*S*-epoxide intermediate decays to aqueous hydrolysis products 7*R/S*,14*S*-dihydroxy-docosa-4*Z*,8*E*,10*E*,12*E*,16*Z*,19*Z*-hexaenoic acid and 13*R/S*,14*S*-dihydroxy-docosa-4*Z*,7*Z*,9*E*,11*E*,16*Z*,19*Z*-hexaenoic acid [115].

Because human LTA_4H catalyzes LTA_4 conversion to LTB_4 [127] and is involved in the conversion of EPA to RvE1 in the E-series resolvins [128], we next assessed conversion of 13*S*,14*S*-epoxy-DHA with LTA_4H, which did not result in MaR1 production [115]. These results rule out a role for LTA_4H in converting the 13*S*,14*S*epoxide intermediate to MaR1 (Figure 4). Leukocytes and in particular phagocytes are key cellular players regulating the onset and resolution of inflammation [129]. MaR1 is produced by macrophages during inflammation-resolution and exerts phagocyte-directed actions [111, 114].

Action of Maresins

When administered *in vivo*. MaR1 at concentrations as low as 0.1ng per mouse significantly reduced PMN accumulation in response to sterile injury [111, 114]. MaR1 is also a potent pro-phagocytic stimulus enhancing human macrophage phagocytosis as well as efferocytosis of apoptotic cells [111, 114]. Apart from leukocyte-directed actions, this pro-resolving mediator also exerts tissue-directed actions, whereby administration of MaR1 to surgically injured planaria accelerates tissue regeneration at the site of injury [114]. These results emphasize the role of MaR1 in regulating both local inflammatory responses and potentially stem cell function(s). One mechanism by which MaR1 might regulate stem cell responses is by promoting stem cell differentiation as recently described for another pro-resolving mediator, protectin D1 [130]. MaR1 also displays potent analgesic actions controlling local inflammation resolution and associated inflammatory pain, as well as neuropathic pain, *via* blocking TRPV1-mediated responses [114].

Activation of the maresin pathway can also impact inflammation-resolution by selectively reducing LTB_4 production *via* direct inhibition of LTA_4H (Figure 5). This is a regulatory mechanism that the 13*S*,14*S*-epoxide intermediate shares with LTA_4 [131] and that would be operative in the conversion of DHA to MaR1. The ability of the 13S,14Sepoxy-DHA to directly inactivate LTA_4H is not shared with MaR1; therefore conversion of the epoxide to MaR1 could result in a reduction of its LTA_4H inhibition. We also found a novel regulatory mechanism in eicosanoid biosynthesis, whereby this 13*S*,14*S*epoxy-maresin selectively inhibited AA conversion by 12-LOX [115]. Of interest, both MaR1 and 13S-14S-epoxy-DHA regulate macrophage phenotype skewing the macrophage profile towards a homeostatic and tissue protective phenotype. These findings point to novel anti-inflammatory mechanisms by the MaR1 biosynthetic pathway, and utilization of DHA can regulate pro-inflammatory eicosanoids *via* direct enzyme interactions with the potent autacoid actions of MaR1 and related products themselves as well as changing the macrophage phenotype.

Anti-Inflammatory and Tissue-Protective Roles of Mouse 12/15-Lipoxygenase, Human 15-Lipoxygenase Type 1 and Their Respective Products

Human 15-LOX type I (15-LOX-1) is essential for biosynthesis of lipoxins, protectins and D-series resolvins (Figure 1). In acute periodontitis, transgenic (TG) rabbits overexpressing human 15-LOX-1 markedly reduced bone loss and local inflammation compared to WT animals [132]. Enhanced LX production was associated with an increased anti-inflammatory status of 15-LO TG rabbits. These results indicate that overexpression of 15-LO type 1 and LXA_4 is associated with reduced PMN-mediated tissue degradation and bone loss. On the other hand, ALOX12/15- (mouse orthologue of human 15-LOX-1) deficient mice exhibited exacerbated inflammation in numerous disease models as well as in cornea wound healing, associated with reduced endogenous levels of LXA_4 (see Table 2 and references within). In addition, in cornea thermal injury, LXA_4 rescues excessive inflammation and impaired wound healing in Alox15-deficient mice. Moreover, in ALOX12/15 deficient mice, 14-HDHA levels were greatly reduced, indicating the key role of ALOX12/15 in MaR1 biosynthesis pathway [55]. Thus, 12/15-lipoxygenase in mice shows both pro-inflammatory phenotype and anti-inflammatory phenotype [133] that appear to depend on cell type specificity and diet. For human cells, *in vitro* shRNA knockdown of human 15-LOX-1, PD1 and resolvin levels were also significantly reduced in isolated Th2 lymphocytes, macrophages [48, 134] and epithelial cells [135] (Table 2).

Concluding Remarks

Identification of novel lipid mediators involved in the active resolution of inflammation will lead to future innovative therapeutic strategies toward many inflammatory diseases. Therefore, each SPM has to be fully documented in terms of structure, which is intrinsically related to its functions. Our group first reported PD1 in murine exudates and human cells [9] as well as murine brains [13], and we established stereochemistry of the bioactive molecule and related products [50]. Using total organic synthesis, PD1 stereochemistry was unambiguously assigned and separated from its double oxygenation isomer 10S,17S-diHDHA [48, 50], which was also confirmed and named PDX by Chen *et al.* [64]. PDX belongs to the Poxytrins family (for PUFA oxygenated trienes), which regroup lipid mediators produced by a double lipoxygenation process leading to a specific *E,Z,E* motif [136] without the requirement for an epoxide formation (such as PD1). These two bioactive lipid mediators from DHA, even though belonging to the protectin family [50], have very distinct structures and biosynthesis, and therefore very distinct potency, bioactivity and target cell types of action. While investigating the potential biological actions of the protectins (i.e. PD1 and PDX), it is important to remember that, despite their common name, their presence reflects different biosynthetic pathways; therefore, their production is likely to be differentially regulated depending on the cell trafficking.

Figure 5 illustrates a comparison in the mechanism of protectin and maresin biosynthesis; each is initiated *via* H abstraction from the precursor DHA that is catalyzed by distinct lipoxygenase enzymes in a stereospecific manner. Maresin biosynthesis is initiated by

human macrophage 12-lipoxygenase and converts DHA to an epoxide intermediate 13S, 14S-eMAR, which itself displays anti-inflammatory actions and promotes macrophage phenotype switching. 13S,14S-eMaR in turn is enzymatically converted to MaR1, which displays potent pro-resolving, anti-nociceptive and tissue regenerative actions. The complete stereochemistry of this mediator, several of its biosynthetic isomers as well as that of 13S, 14S-eMaR have now been elucidated, thus providing novel tools for investigating the biological roles of maresins in host responses and re-establishment of homeostasis.

In conclusion, the acute inflammatory response is the first line of protection in response to both sterile and infectious insults. Within this, mediators produced by cellular traffic at the site of inflammation are critical in determining the amplitude and duration of this response. Classic arachidonic acid-derived pro-inflammatory eicosanoids promote vascular leakage, pain, thrombosis and the amplification of leukocyte recruitment. The specialized pro-resolving mediators, including protectins and maresins, actively counterregulate the actions of these pro-inflammatory mediators. Resolution mediators evoke the cardinal signs of resolution, namely, they: a) limit further neutrophil recruitment, b) promote macrophage clearance of debris and bacteria, c) stimulate tissue regeneration and d) reduce pain. The complete stereochemistry and biosynthetic pathways have now been established for each of the main pro-resolving mediators, in addition to their total confirmation by organic synthesis. These open new directions in understanding their roles in pathobiology and provide novel avenues for treatment of conditions where resolution pathways may be defective.

Acknowledgments

We thank Mary H. Small for expert assistance in manuscript preparation. This work was supported in part by a research grant from the Mérieux Foundation and NIH grant nos. R01GM038765 and P01GM095467.

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Abbreviations used in this paper

5*S*-HETE	5*S*-hydroxy-6E,8Z,11Z,14Z-eicosatetraenoic acid
7*S*	14*S*-diHDHA, 7S,14S-dihydroxydocosa-4Z,8E,10Z,12E,16Z,19Z-hexaenoic acid
10S	17S-diHDHA, 10S,17S-dihydroxy-docosa-4Z,7Z,11E,13Z,15E,19Z-hexaenoic acid (the dioxygenation product)
12-HETE	12-hydroxy-5Z,8Z,10E,14Z-eicosatetraenoic acid
14*S*-HDHA	14*S*-hydroxy-4Z,7Z,10Z,12E,16Z,19Z-docosahexaenoic acid
14*S*HDHA	14*S*-hydroxy-4Z,7Z,10Z,12E,16Z,19Z-docosahexaenoic acid
14*S*-HpDHA	14*S*-hydro(peroxy)-4Z,7Z,10Z,12E,16Z,19Z-docosahexaenoic acid
17S-H(p)DHA	17Shydro(peroxy)-DHA

DHA, C22	6, docosahexaenoic acid
EPA, C20	5, eicosapentaenoic acid
GC	gas chromatography
NMR	nuclear magnetic resonance
hm12-LOX	human macrophage 12-lipoxygenase
LC	liquid chromatography
LOX	lipoxygenase
LM	lipid mediator
LT	leukotriene
LTB_4	5S,12R-dihydroxy-6Z,8E,10E,14Z-eicosatetraenoic acid
LX	lipoxin
Maresin	*ma*crophage mediator in *res*olving *in*flammation
MaR1	maresin 1 (7*R*, 14*S*-dihydroxy-docosa-4*Z*, 8*E*,10*E*, 12*Z*,16*Z*, 19*Z*-hexaenoic acid)
MSMS	tandem mass spectrometry
NPD1	neuroprotectin D1
PMN	polymorphonuclear leukocyte
PD1	protectin D1/neuroprotectin D1,10*R*,17*S*-dihydroxydocosa-4*Z*,7*Z*,11*E*, 13*E*,15*Z*,19*Z*-hexaenoic acid (*neuro* prefix is added to denote the tissue and functional role of protectin D1)
pt5LOX	5-LOX from potato
RvD1	Resolvin D1 (7*S*, 8*R*, 17*S*-trihydroxy-docosa-4*Z*, 9*E*, 11*E*, 13*Z*, 15*E*, 19*Z*-hexaenoic acid)
RvD2	Resolvin D2 (7*S*, 16*R*, 17*S*-trihydroxy-docosa-4*Z*, 8*E*, 10*Z*, 12*E*, 14*E*, 19*Z*-hexaenoic acid)
RvD3	Resolvin D3 (4*S*, 11*R*, 17*S*- trihydroxy-docosa-5*Z*, 7*E*, 9*E*, 13*Z*, 15*E*, 19*Z*-hexaenoic acid)
RvE1	Resolvin E1, 5*S*,12*R*,18*R*-trihydroxy-6*Z*,8*E*,10*E*,14*Z*,16*E*-eicosapentaenoic acid
STZ	serum-treated zymosan

Highlights

- Proresolving mediators are biosynthesized during resolution phase of acute inflammation
- Resolution is a biosynthetically active process
- Protectins and maresins are two structurally distinct families of potent local mediators
- NPD1/PD1 reduces neural inflammation, stimulates resolution and reduces pain
- MaR1 stimulates tissue regeneration and resolution mechanisms, is anti-inflammatory and reduces pain.

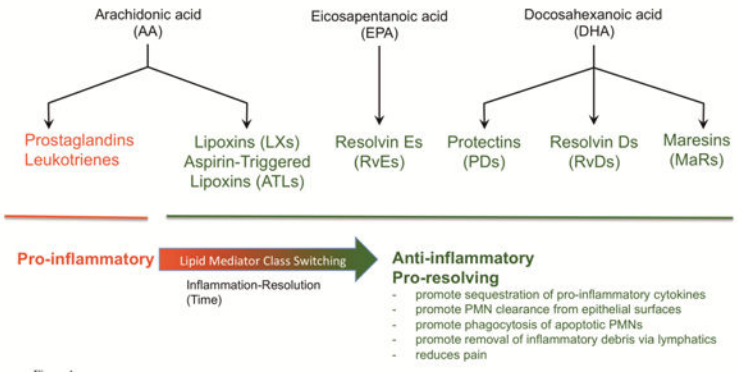

Figure 1. Specialized Pro-Resolving Lipid Mediator Families Biosynthesized From Their Parent Polyunsaturated Fatty Acids

Resolving exudates utilize essential fatty acids such as DHA to form several structurally distinct groups of specialized proresolving mediators that promote clearance and resolution.

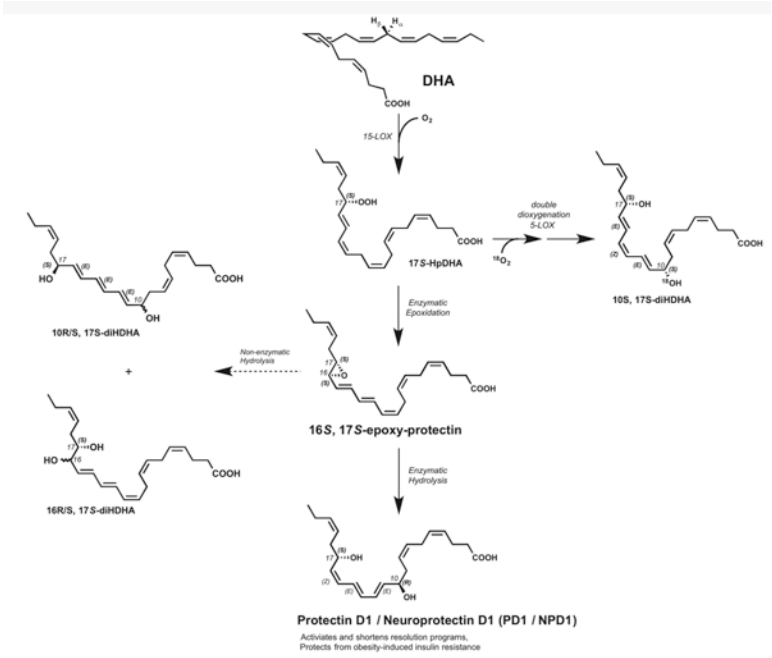

Figure 2. Protectin Biosynthetic Pathway

PD1 produced enzymatically from the 16S, 17S epoxy protectin (pictured in a tentative double bond geometry) was confirmed by epoxide trapping experiments. Non-enzymatic hydrolysis of this epoxide intermediate formed two minor products, the all-trans-triene isomer and vicinal 16, 17*S* diol.

The 10S, 17S double dioxygenation pathway and product occur by sequential actions of two lipoxygenation enzymes as demonstrated in cell incubation and isolated enzymes carried out under an enriched atmosphere of $^{18}O_2$. See text for further details.

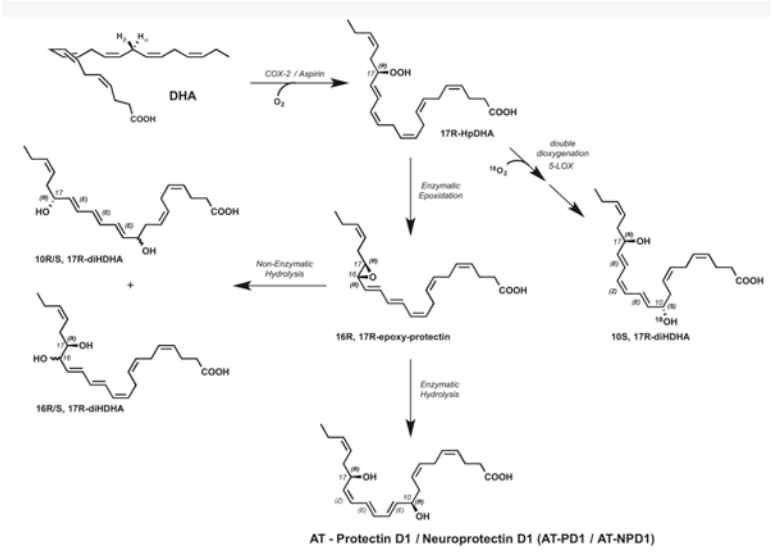

Figure 3. Aspirin-Triggered (AT) Protectin Biosynthetic Pathway

An additional pathway *via* aspirin-acetylated COX-2 enzyme gives rise to inversion of stereochemistry at the 17 position to afford the 17*R* hydroxyl-containing products. The 17*R* hydroperoxide precursor can undergo enzymatic epoxidation to the 16*R*, 17*R* epoxy protectin. This epoxide is then enzymatically hydrolyzed to the 10*R*, 17*R* AT-(NPD1/PD1).

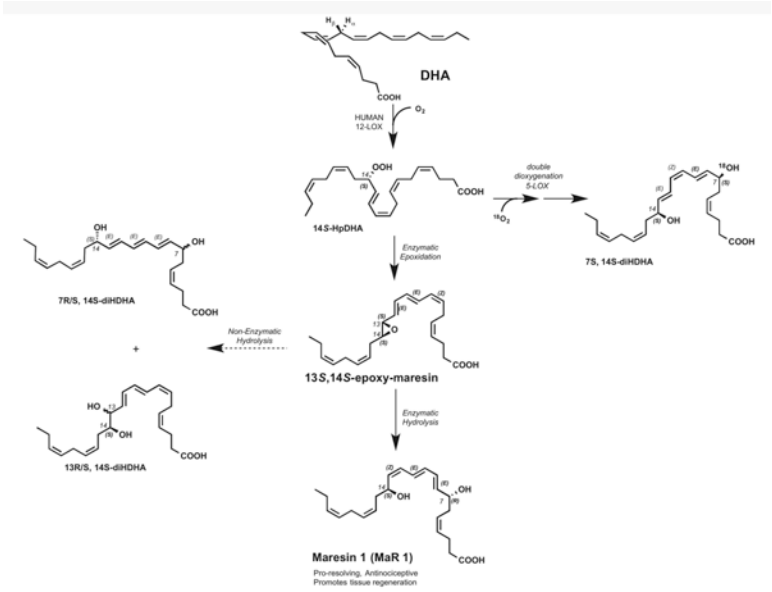

Figure 4. Maresin Biosynthetic Pathway

The biosynthesis of MaR1 is formed through 13, 14 epoxide intermediate confirmed by alcohol trapping products. In addition, several isomers formed through alternative pathways were confirmed by matching studies of synthetic compounds made in a stereo-controlled manner. See text for further details.

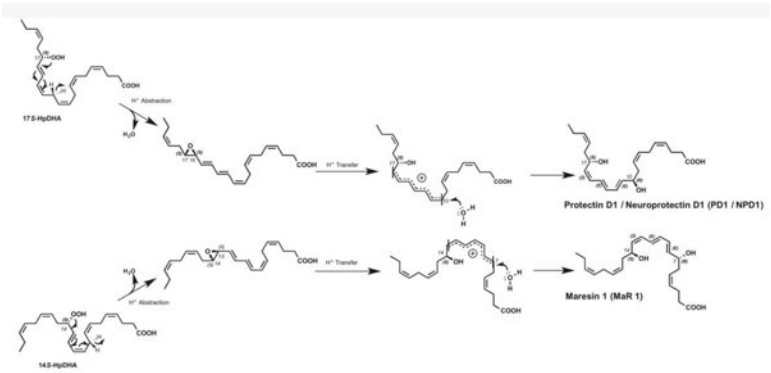

Figure 5. Comparison of Mechanisms in Protectin and Maresin Biosynthesis

Proton abstraction of the 17S hydroperoxide pictured above or the 14S hydroperoxide pictured below forms the 16*S*, 17*S* epoxide and 13*S*, 14*S* epoxide respectively. Enzymecatalyzed formation of a rigid carbonium cation intermediate from the respective epoxides undergoes a stereospecific attack by oxygen from a water molecule. This enzyme-catalyzed hydrolysis affords predominantly the *R* insertion of oxygen from water at the 10 position in the upper protectin pathway and at the 7 position in the lower maresin pathway.

Table 1

Production and actions of Protectins and Maresins

(A) Protectins

Mediator	Species /SystemInvestigated	Concentration / Amount	Action(s)	Function	In vivo Production
NPD1 / PD1	Mouse/ Peritonitis	300 ng / mouse	Inhibits neutrophil recruitment; regulates chemokine/ cytokine production [43, 50]		
		300 ng / mouse	Promotes lymphatic removal of phagocytes [76]		
		100 ng / mouse	Regulates T-cell migration [48]		
	Mouse/Asthma	2-200 ng / mouse / 4 days	Protects from lung damage, airway inflammation and hyperresponsiveness [137]		
		100 ng / mouse	Accelerates catabasis of Th2-mediated inflammation [138]		
		11 pg / mouse			Toll-like receptor triggers production of PD1 [138]
	Human/Asthma	~2 ng			PD1 is generated in human asthma [137]
	Human Eosinophils / Asthma	10 ng / 10^6 cells in *healthy subjects*			Dysregulation of PD1 in eosinophils from asthma patients [139]
	Mouse / Eosinophils	5 μg / mouse	Rescues delayed resolution in eosinophil depleted mice [140]		
	Mouse/Kidney ischemia -reperfusion	3.5 - 35μg / mouse	Protects from ischemia-reperfusion-induced kidney damage and loss of function; regulates macrophages [73, 141]		
		160 pg / kidney			Endogenous formation in kidney increased for ω-3 PUFA [73]
	Mouse/ Retinopathy	10 ng / mouse	Protects against neovascularization[71]		
	Ischemic stroke	100 ng / mouse	Inhibits leukocyte infiltration, NF and cyclooxygenase-2 induction [14, 44]		
	Human/Alzheimer's disease	5 pmol/mg protein			Diminished PD1production in human Alzheimer's disease [142]
	Mouse/Liver Injury	100 nM	Protects from necro-inflammatory liver injury [143]		
	Rabbit/Corneal Injury	100 ng / rabbit	Promotes regeneration of corneal nerves following surgery [144]		
	Mouse / Stem Cells	50 nM	Protects stem cells from oxidative stress [75]		

(A) Protectins

Mediator	Species /SystemInvestigated	Concentration / Amount	Action(s)	Function	In vivo Production
	Mouse / Lyme Disease	0.1 pg / mg of tissue			PD1 produced during the resolution of Lyme disease [145]
	Rat / Optic Nerve Injury	5 μg / rat	Inhibits cell ganglion death [146]		
	Mouse / obesity	100 nM	Regulates adiponectin in adipose tissue of obese (ob/ob) mice [147]		
		~150 pg / mouse			Generated in adipose tissue [147]
	Mouse /neuronal plasticity	1 ng/mL	Reduces TRPV1-mediated inflammatory pain [70]		
	Mouse /neuronal plasticity	300 ng / mouse	Antinociceptive without associated tolerance [148]		
	Mouse / Herpes simplex virus 1	300 ng / mouse	Reduction of stromal keratitis and neovascularization [149]		
	Rats & Mouse / Epilepsy	50 nM	Attenuates evoked hippocampal seizures [150]		
	Fat-1 Mouse / colitis	0.2 ng / mg colon tissue			Physiologically active levels produced in colon of transgenic mice [151]
	Human / ARPE-19 cells	50 nM	Anti-apoptotic for ARPE-19 cell survival [152]		
	Human MΦ PMN / Phagocytosis	0.1-10 nM		Promotes bacterial clearance [57]	
	Mouse / *E. coli* peritonitis	50 ng/mouse	Protects from hypothermia [57] and reduces bacterial titers		
		1 ng / mouse			Lower levels in higher bacterial challenge [57]
10S, 17S diHDHA (PDX)	Mouse/ Peritonitis	300 ng / mouse	Inhibits neutrophil recruitment; regulates chemokine/ cytokine production [50]		
	Mouse/H1N1	100 ng/ mouse		Antiviral; attenuates translocation [153]	
	Human/ Platelets	1 μM	Inhibits platelet aggregation [64, 136]		
	Mouse / Acute Lung Injury	200 μg / mouse	Enhances neutrophil apoptosis [154]		
AT-PD1	Mouse/Diabetes	1 μg/mouse	Stimulates skeletal muscle IL-6 release; improves insulin sensitivity [67]		
	Mouse / Peritonitis		Regulates PMN infiltration and promote efferocytosis [49]		
	Rat / Stroke	333 μg / kg		Reduces brain infarction and	

(A) Protectins

Mediator	Species /SystemInvestigated	Concentration / Amount	Action(s)	Function	In vivo Production
				edema in stroke [	
				edema in stroke [	

(B) Maresins

Mediator	Species / System Investigated	Concentration / Amount	Action(s)	Function	In vivo Production
Maresins	Mouse / Peritonitis	0.1 ng / mouse	Inhibits neutrophil recruitment; enhances phagocyte clearance [55]		
	Planaria / regeneration	100 nM		Stimulates tissue regeneration in planaria [54]	
	Mouse / pain Human/Rheuma-toid Arthritis	10ng /mouse7 pg / 50 μL SF	Antinociceptive [54]		Identified in synovial fluid extracts [113]
	Mouse/ Peritonitis	26 pg / mouse			Elevated levels with CO inhalation [134]
	Human MΦand neutrophils / phagocytosis and efferocytosis	0.1-10 nM	Inhibits proinflammatory mediator production by LTA_4H and AA conversion [55, 115]		
		24 pg / 2×10^5MΦ43 pg / 3×10^5PMN			Macrophage and macrophage of apoptotic PMN [112]
	Mouse/Colitis	300 ng / mouse	Reduced PMN infiltration and cytokine expression as well as NF-κB activation [156]		
	Mouse/Sepsis	10 nM	Promotes efferocytosis [157]		
	Mouse/Acute Lung Injury	100 nM	Inhibits neutrophil recruitment and promotes clearance [158]		

Table 2

Anti-inflammatory and tissue-protective roles: 12/15-lipoxygenase, 15-lipoxygenase type I and their products

(A) *In vivo* modification

Animal models	Alox15 gene modification	Actions/Phenotypes	References
Periodontitis in rabbit	Alox15 transgenic	↓PMN-mediated tissue degradation and bone loss	[132]
Cornea injury	Alox12/15 deficient mice	↑Inflammation ↓ Wound healing ↓ Corneal re-epithelialization ↓ Endogenous LXA_4 production ↓ Heme-oxygenase 1 ***LXA_4 rescues exacerbated inflammation and impaired wound healing in Alox15 deficient mice***	[46][159]
Atherosclerosis	Alox12/15 transgenic mice	Delayed atherosclerosis ↓ IL-17, CCL5, PGE2 ↑ PD1	[133]
	Alox12/15 deficient mice	Accelerated atherosclerosis ↑ IL-12p40, CCL5 ↑ LXA_4 production in macrophages	
Peritonitis	Alox12/15 deficient mice	↓14-HDHA	[55]
Arthritis	Alox12/15 deficient mice	↑ Inflammatory gene expression ↓ LXA_4 in inflamed synovia and macrophages	[160]
Collagen-induced arthritis		COX-2 product PGE_2 induces Alox12/15. PGE_2 stimulates resolution via LXA_4	[161]
Suture-induced chronic cornea Injury	Alox12/15 deficient mice	↑ Inflammatory neovascularization	[162]
Peritonitis	Alox12/15 deficient mice	Resolution deficit caused by eosinophil depletion was rescued by eosinophil restoration or the administration of PD1. Eosinophils from Alox12/15 deficient mice could not rescue the resolution phenotype	[140]
Dermal fibrosis	Alox12/15 deficient mice	↑ TGF-b stimulated MAPK pathway LXA4 counters TGF-b stimulated fibroblast activation	[163]
Endometriosis	Alox12/15 deficient mice	EPA administration decreased the number of lesions in controls but not in Alox12/15 deficient mice. Reduced levels of RvE3 after EPA administration in Alox12/15 deficient mice compared to control	[164]
Airway inflammation	Alox12/15 deficient mice	↓ TLR7-mediated resolution of airway inflammation	[138]
Peritonitis	Alox12/15 deficient mice	Low dose inhaled CO treatment reduces PMN infiltration in WT control, but failed to regulate PMN in Alox12/15 deficient mice	[134]

(B) *In vitro* modification

Cellular systems	Alox15 gene modification	Actions/Phenotypes	References
Isolated Th2 cells	Alox15 type 1 siRNA knockdown	↓ PD1 production	[69]
Retinal pigment epithelial cells	Alox15 type 1 siRNA knockdown	↑ Sensitivity to oxidative stress-induced apoptosis ***NPD1 rescues Alox15-silenced cells from oxidative stress-induced apoptosis***	[135]
Human macrophages	Alox15 type 1 siRNA knockdown	↓ CO-induced HO-1 expression ↓RvD1 and RvD5	[134]