HHS Public Access

Author manuscript

Prostaglandins Other Lipid Mediat. Author manuscript; available in PMC 2018 September 01.

Published in final edited form as:

Prostaglandins Other Lipid Mediat. 2017 September ; 132: 25–30. doi:10.1016/j.prostaglandins.2017.02.005.

Working Title: Who is the real 12-HETrE?

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Abstract

Oxygenases, including lipoxygenases and cytochrome P450s, generate an array of structurally diverse oxylipins that modulate distinct biological responses in mammals. Depending on the source of tissues and enzymes, distinct oxylipins are generated with inherent cellular function. Here, we report structurally different forms of 12-HETrE, with distinct biological function in tissues as well as their derived enzymatic source.

Keywords

Dihomo-gamma-linolenic acid; Arachidonic acid; Platelet 12-LOX; 12(S)-HETrE; 12(R)-HETrE; Thrombosis

Introduction

A multitude of dietary or essential fatty acids are produced in nature. When humans ingest these fatty acids, they are often elongated or saturated by enzymes in the body. Each of these enzymatic steps creates a new slightly varied fatty acid, which can be enzymatically modified in various tissue beds to function as bioactive lipids (metabolites). The structural differences inherent in these fatty acids contribute to differences in physiological processes by either potentiating or attenuating biochemical steps in a variety of tissues. Predominantly, the fatty acids themselves are inert and rely on catalysis by oxygenases such as cyclooxygenase (COX), lipoxygenase (LOX), or cytochrome P450 (CYP), to modify polyunsaturated fatty acids (PUFAs) to generate potent signaling lipid mediators that play crucial roles in diverse physiological and pathological pathways[1–4].

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Authors' contributions

J. Yeung performed literature search, wrote the manuscript and created figures. M. Holinstat wrote, edited and proofed the manuscript.

Conflict of interest statement

The authors declare no competing financial interest.

One example of this modification is the formation of 12(S)-hydroxy-8,10,14-eicosatrienoic acid (12(S)-HETrE), an endogenous metabolite derived from the platelet-type 12-lipoxygenase (*p*12-LOX) (a non-heme oxygenase) oxidation of the ω-6 PUFA dihomo-γ-linolenic acid (DGLA). This metabolite was recently discovered to provide protection against thrombotic-mediated events *in vivo*[5]. Further, this discovery strongly supports 12(S)-HETrE and possibly other oxidized metabolites as playing potentially therapeutic roles in providing cardioprotection in people. However, the chemical structure of platelet 12(S)-HETrE should not be confused with that of the epithelium-derived 12(R)-hydroxy-5,8,14-eicosatrienoic acid (12(R)-HETrE)[6–11]. While the abbreviation would suggest that these metabolites share the same compound structure and only differ based on tissue source of metabolite origin, they are actually structurally disparate. In addition to their structural difference, the physiological effects elicited by each of these metabolites are unique and in fact opposing in nature. Hence, while published work has described two oxidized lipids with the same shortened abbreviation (12-HETrE), the functional difference between these two metabolites and the difference in tissue expression and biological function are striking. Thus, the primary focus of this review is to compare and contrast the two 12-HETrE compounds described in the literature thus far based on their structural backbones, functions, mechanisms, and involvement in diseased states, for the expressed purpose of defining the physiological and chemical differences inherent to each of these metabolites and delineating the physiological tissues in which each is purported to have a regulatory function.

Formation and function of platelet 12-HETrE

Historically, 12(S)-hydroxy-8,10,14-eicosatrienoic acid (12(S)-HETrE), isolated from human platelets treated with 8,11,14-eicosatrien[1-^{14}C]oic acid, a radiolabeled compound of DGLA[12] (presumably derived from platelet-type 12-LOX oxidation) in 1976, was largely neglected or overlooked due to the preponderance of cyclooxygenase-derived products, prostaglandin E_1 (PGE_1) or thromboxane A_1 (TxA_1) from DGLA[13–20]. However, recent characterization of 12(S)-HETrE and its potential role in platelet function first described in 2012, signifies its revival and importance not only in platelet function[5, 21], but also its potential biological roles in other cellular types.

In agreement with previously postulated formation of 12(S)-HETrE through the *p*12-LOX [12], current studies verify that the *p*12-LOX (which only generates *S*-stereoisomer products)[22], principally found in platelets, keratinocytes, and some tumor cells[23], is important and required for 12(S)-HETrE generation from DGLA [5, 21]. Ikei et al. were also able to demonstrate that the purified *p*12-LOX was just as efficient in transforming DGLA to 12(S)-HETrE as to the predominant substrate, arachidonic acid (AA), converted to 12(S)-hydroxy-5,8,10,14-eicosatetraenoic acid (12(S)-HETE). Similarly to other prostanoids generated in platelets, the role of 12(S)-HETrE was directly assessed via a number of biochemical assays regulating overall platelet function. Following platelet treatment with 12(S)-HETrE, platelet activation endpoints (aggregation, Rap1 and αIIbβ3 integrin activation) were significantly inhibited. Not only was platelet activation potently inhibited, but integrin-dependent platelet-mediated clot retraction was significantly decreased [21]. This strongly suggests that the attenuation of platelet activation through the 12-LOX

oxidation of DGLA to its metabolite could also function in preventing platelet-mediated thrombosis.

More recently, the role of platelet 12(S)-HETrE on thrombosis was investigated *in vivo*. To mimic physiological thrombotic occlusion of the vessels, the cremaster arterioles of male mice were exposed to laser emission, resulting in a vascular insult of the arteriole endothelium exposing the underlying collagen matrix, and fluorescently labeled platelets and fibrin that make up the thrombus were monitored for accumulation at the site of laser injury. Mice that had been intravenously administered 12(S)-HETrE or DGLA were protected from thrombus accumulation at the site of the arteriole injury compared to the wild-type control [5]. The antiplatelet effects of DGLA *in vivo* were also shown to be dependent on the presence of functional *p*12-LOX. The ability of 12(S)-HETrE to inhibit thrombotic occlusion induced by laser injury implicates its potential use as an anti-platelet therapy to treat thrombotic-associated diseases. Determining the extent of stability for 12(S)-HETrE in circulation will be essential as this metabolite is investigated further for its potential in effectively inhibiting thrombosis following IV or possibly oral administration.

Many prostanoids generated by oxygenases elicit their actions through direct binding of receptors on the surface of cells[24–31]. Similar to other prostanoids such as prostacyclin[4], 12(S)-HETrE was shown recently to bind to the $G\alpha_s$-linked G protein-coupled receptors (GPCR) resulting in activation of adenylyl cyclase (AC) and formation of cAMP[5] to activate protein kinase A (PKA). This process was shown to be a key step in 12(S)-HETrE mediated anti-platelet effects (Figure 1). Thus, the inhibitory regulatory signal mediated through the $G\alpha_s$ pathway was shown to be activated by 12(S)-HETrE. cAMP and vasodilator-stimulated phosphoprotein (VASP) phosphorylation were significantly increased in the presence of 12(S)-HETrE as shown in figure 1. These observations strongly support the interaction of 12(S)-HETrE with $G\alpha_s$-linked GPCRs to elicit anti-platelet effects. However, despite evidence of activation of the $G\alpha_s$ signaling pathway in the platelets following 12(S)-HETrE exposure, the identification of 12(S)-HETrE receptor has not yet been determined and remains an area of active investigation.

The physiological role of 12(S)-HETrE in the platelet is however unique to what has been observed for the AA-derived *p*12-LOX product, 12(S)-HETE. 12(S)-HETE was first reported in a cancer cell line to mediate metastasis[32–38] and prolong survival[39–41] through its high affinity binding to an orphan G protein-coupled receptor (GPCR), GPR31 (12-HETER)[42]. In support of G protein-coupled receptor binding, 12(S)-HETE had also been reported to modulate neuronal excitotoxicity through the $G\alpha_{i/o}$-protein-coupled receptor[43] by which adenylyl cyclase activity was inhibited to reduce voltage-sensitive calcium channel activity. However, 12(S)-HETE had also been implicated in neuroprotection through its ability to enhance and activate peroxisome proliferator-activated receptor γ (PPARγ), a member of the nuclear hormone receptor family of ligand-dependent transcription factors[44].

Moreover, 12(S)-HETE has been shown to play a role in coagulation through esterification into the lipid membrane following formation in the platelet, resulting in enhanced tissue factor-dependent thrombin generation in the vessel[45]. The exogenous addition of 12(S)-

HETE to platelets has also been demonstrated to increase dense granule secretion following stimulation of the thrombin receptor pathway in the human platelet[46]. Hence, while *p*12-LOX can form a number of oxidized metabolites in the platelet based on the available PUFA substrates, 12(S)-HETE and 12(S)-HETrE appear to exert opposite effects on platelet activation based in part on differential receptor signaling. For instance, 12(S)-HETE had been reported to augment calcium mobilization and PKC activation[47] as well as Rho, ROCK, and myosin light chain 2 (MLC2) activation through 12-HETER[41]; whereas, 12(S)-HETrE has been recently shown to signal through $G\alpha_s$-GPCR activation pathway, encompassing cAMP formation, and PKA activation through an as-yet-to-be-determined receptor.

Formation and function of 12(R)- and 12(S)-HETrE from *e*12-LOX, 12*R*-LOX, and CYP450

Unlike the 12(S)-HETrE generated from *p*12-LOX, the epidermal, epithelial cells, and neutrophils expressing a combination of epidermis-type 12-LOX (*e*12-LOX)[48, 49], the epidermis *R* form of 12-LOX (12*R*-LOX), uncovered in the late 1990s,[50, 51] and cytochrome P450 (CYP450)[9, 10, 52] are also indirectly involved in the generation of both *R* and *S* stereoisomers of 12-HETrE, which are structurally different from the platelet 12(S)-HETrE. The two chiral forms of 12(R)-HETrE and 12(S)-HETrE, 12(R)-hydroxy-5,8,14-eicosatrienoic acid and 12(S)-hydroxy-5,8,14-eicosatrienoic acid, respectively, derived from AA, were identified in the epithelial, epidermal, and neutrophils of mammals[7, 8, 10, 52–58].

While 12*R*-LOX and *e*12-LOX generally form their respective stereoselective *R* or *S* enantiomeric 12-hydroperoxy-5,8,10,14-eicosatetraenoic acid (12-HpETE) from AA, which is readily reduced to 12-hydroxy-5,8,10,14-eicosatetraenoic acid (12-HETE), CYP450 isoenzymes generate a mixture of *R* and *S* of 12-HETE, with the R enantiomer tending to be the predominate [59, 60]. Both *R* and *S* 12-HETE enantiomeric forms are converted to 12-oxo-5,8,10,14-eicosatetraenoic acid (12-oxo-ETE) by 12-hydroxyeicosanoid dehydrogenase (12-HEDH), an enzyme that is also capable of oxidizing other 12-hydroxyeicosanoids. 12-oxo-ETE is further metabolized by the NADPH-dependent cytosolic enzyme, 12-oxoeicosanoid Δ^{10}-reductase (Δ^{10}-reductase), to 12-oxo-6,8,14-eicosatrienoic acid (12-oxo-ETrE or 10,11-dihydro-12-oxo-ETE) and reduced by 12-ketoreductase (12-KR) to either 12(R)-HETrE or 12(S)-HETrE[55, 56, 61] as shown in figure 2.

Although both 12(R)- and 12(S)-HETrE were found to be biologically active, 12(R)-HETrE appeared to be the major metabolite formed in quantity compared to 12(S)-HETrE[56, 61]. 12(R)-HETrE has been demonstrated to be directly associated with or to increase vasodilation[52] and inflammation in mammals[53, 62], as well as functioning as a potent chemotactic agent for neutrophils[11]. In addition to inflammation, 12(R)-HETrE had been implicated in vascular permeability and neovascularization in the cornea of the rabbit[10] following hypoxia-induction. Enhanced VEGF expression via ERK1/2 activation[63] was observed also to be concomitant with neovascularization in the corneal epithelial cells[64] following 12(R)-HETrE treatment. Treating coronary endothelial cells treated with 12(R)-HETrE also resulted in NF-κB activation as well as increased c-fos, c-jun, and c-myc oncogene expression[65], indicating 12(R)-HETrE's angiogenic-induced process involves

the NF-κB activation pathway. While binding assays of 12(R)-HETrE to the surface and cytoplasm of the endothelial cells had suggested a putative receptor[66], the 12(R)-HETrE receptor has yet to be identified as a new target for inhibiting angiogenesis and inflammation-associated diseases.

Although 12(S)-HETrE is produced by the 12*R*-LOX or CYP450 pathway in lower amounts in human neutrophils, monocytes, and macrophrophages[67, 68] and deemed to be biologically inactive in certain cells compared to 12(R)-HETrE[52], it appeared to be biologically more important in neutrophils than 12(R)-HETrE. 12(S)-HETrE was demonstrated to be about 20 times more potent in stimulating cytosolic calcium release from neutrophils than 12(R)-HETrE[55]. Neutrophils treated with 12(S)-HETrE also displayed enhanced chemotactic activity compared to 12(R)-HETrE.

Conclusion

Platelet 12(S)-HETrE is structurally and functionally unique from the 12(R)- and 12(S)-HETrE produced by 12*R*-LOX, CYP450 isoenzymes, and epithelial 12-LOX. Platelet 12(S)-HETrE had recently been discovered to elicit anti-thrombotic function within the vessels without affecting bleeding. This finding implicates its therapeutic potential in the treatment of cardiovascular diseases. Although platelet 12(S)-HETrE is shown to impinge on the $G\alpha_s$ signaling pathway, the receptor has yet to be identified on the platelets. Current studies are ongoing to identify which $G\alpha_s$-coupled GPCRs on the surface of platelets functions as the 12(S)-HETrE receptor. Several $G\alpha_s$-coupled GPCRs are already known to be expressed on the human platelet, including the IP_2, EP_2, EP_4, and DP_1 receptors. Using pharmacological, genetic, and screening approaches, it is reasonable to presume that the 12(S)-HETrE receptor will be identified in the near future. These approaches will enable *in vivo* studies using 12(S)-HETrE derived from DGLA oxidation by 12-LOX to prove which receptor(s) are essential for 12(S)-HETrE-mediated protection from injury-induced platelet activation and thrombosis in the vessel. Following its identification, it will be worthwhile for investigators to follow up on the contrasting concepts laid out in this review in regards to the multiple forms of 12-HETrE to determine if AA-derived 12-HETrE metabolites are also able to signal platelets (and possibly other cells) through the platelet 12-HETrE receptor. The AA-derived 12(S)-HETrE has been demonstrated to induce calcium release in the neutrophils. Thus, this implicates 12(S)-HETrE derived from CYP450 pathway could impinge on either $G\alpha_q$ or $G\alpha_i$-coupled receptors on leukocytes as well as platelets. Enhanced calcium flux in platelets would potentiate platelet activation in a manner similar to what has been previously published for 12(S)-HETE[46, 69]. It will be of great interest in the future to determine if AA-derived 12(S)-HETrE functions as a procoagulant signal in the human platelet and if so, whether this potential signaling has a physiologically relevant role in regulating platelet reactivity during inflammatory states. Future studies of platelet 12-LOX regulated 12(S)-HETrE formation as well as the other structurally unique forms of 12-HETrE produced by 12R-LOX, CYP450, and epithelial 12-LOX and their receptors will likely uncover a myriad of physiologically relevant signaling events beyond that of cardiovascular health and inflammation.

Acknowledgments

This work was supported in part by the National Institutes of Health (NIH) Office of Dietary Supplement (ODS), GM105671 (M.H.), HL114405 (M.H.), and Ruth L. Kirschstein Institutional National Research Service Awards (NSRA) F31HL129481 (J.Y.).

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Highlights

- Structurally distinct 12-HETrE structures derived from platelet 12-LOX and CYP450
- 12(S)-HETrE derived from platelet 12-LOX oxidation of DGLA is anti-thrombotic
- 12(R)-HETrE derived from CYP450 oxidation of AA is pro-inflammatory

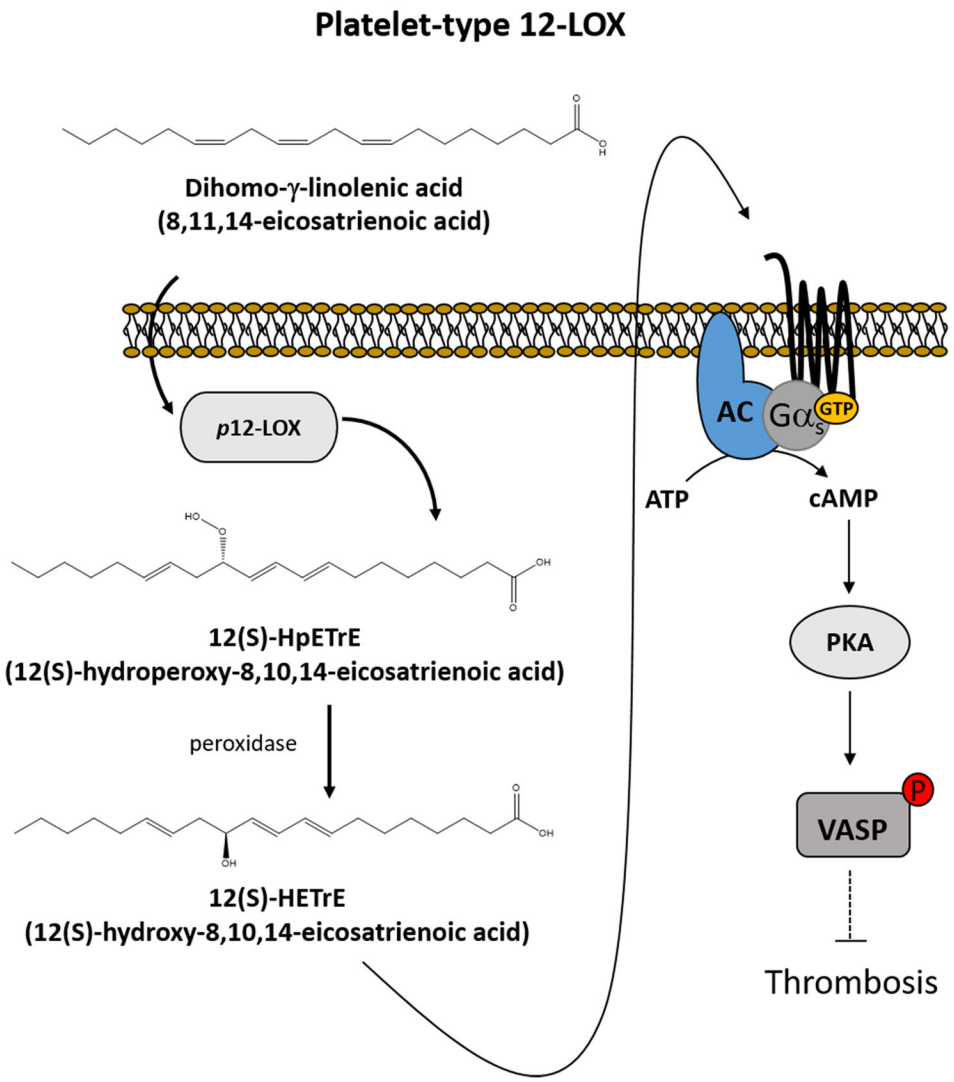

Fig. 1.

Platelet 12-lipoxygenase (*p*12-LOX) generates 12(S)-hydroperoxy-8,10,14-eicosatrienoic acid (12(S)-HpETrE) from dihomo-γ-linolenic acid (DGLA; 8,11,14-eicosatrienoic acid), which is readily reduced to 12(S)-hydroxy-8-10-14-eicosatrienoic acid (12(S)-HETrE)). 12(S)-HETrE is released and acts in an autocrine or paracrine manner to inhibit thrombosis through the Gαs signaling pathway. 12(S)-HETrE directly activates the Gαs signaling pathway, which involves the activation of adenylyl cyclase (AC) to generate cAMP from ATP. cAMP binds and activates protein kinase A (PKA), which phosphorylates vasodilator-stimulated phosphoprotein (VASP) to inhibit platelet-mediated thrombosis.

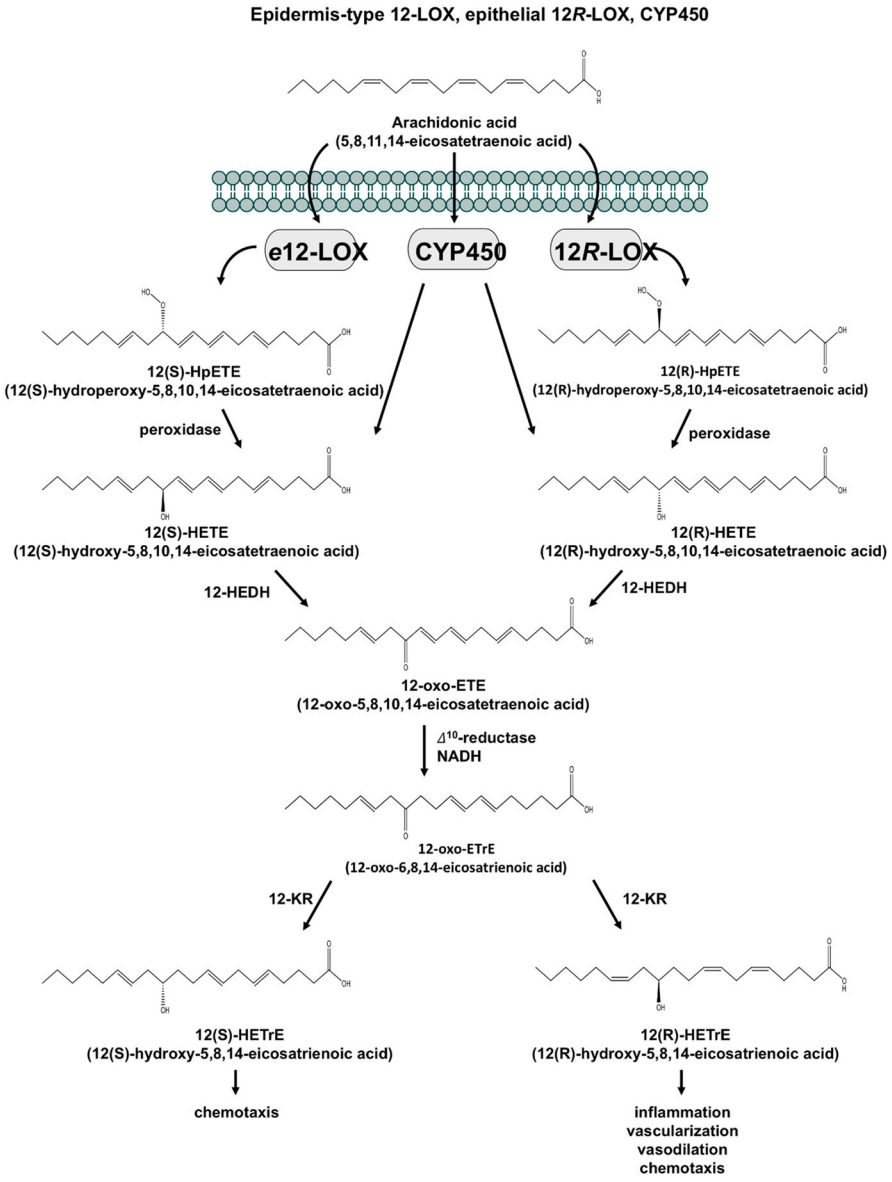

Fig. 2.

Epidermis-type 12-LOX (*e*12-LOX) and epithelial 12*R*-LOX oxidizes arachidonic acid (AA) to their respective *S* and *R* enantiomers 12-hydroperoxy-5,8,10,14-eicosatetraenoic acid (12-HpETE), which are reduced to 12-hydroxy-5,8,10,14-eicosatetraenoic acid (12-HETE). CYP450 isoenzymes generate both *S* and *R* enantiomers of 12-HETE. 12(S)-HETE and 12(R)-HETE are transformed into 12-oxo-5,8,10,14-eicosatetraenoic acid (12-oxo-ETE) by 12-hydroxyeicosanoid dehydrogenase (12-HEDH) and reduced to 12-oxo-6,8,14-eicosatrienoic acid (12-oxo-ETrE) by 12-oxoeicosanoid Δ^{10}-reductase (Δ^{10}-reductase). 12-ketoreductase (12-KR) converts 12-oxo-ETrE to either 12(R)-hydroxy-5,8,14-eicosatrienoic acid (12(R)-HETrE) or 12(S)-12-hydroxy-5,8,14-eicosatrienoic acid (12(S)-HETrE).