J Mol Med
DOI 10.1007/s00109-017-1542-4

JMolMed

REVIEW

The expansive role of oxylipins on platelet biology

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Received: 11 February 2017 / Revised: 29 April 2017 / Accepted: 4 May 2017

Abstract In mammals, three major oxygenases, cyclooxygenases (COXs), lipoxygenases (LOXs), and cytochrome P450 (CYP450), generate an assortment of unique lipid mediators (oxylipins) from polyunsaturated fatty acids (PUFAs) which exhibit pro- or anti-thrombotic activity. Over the years, novel oxylipins generated from the interplay of theoxygenase activity in various cells, such as the specialized pro-resolving mediators (SPMs), have been identified and investigated in inflammatory disease models. Although platelets have been implicated in inflammation, the role and mechanism of these SPMs produced from immune cells on platelet function are still unclear. This review highlights the burgeoning classes of oxylipins that have been found to regulate platelet function; however, their mechanism of action still remains to be elucidated.

Keywords Lipoxygenase · Cyclooxygenase · Oxygenases · Eicosanoids · Prostaglandins · Thrombosis

Introduction

Cardiovascular disease remains the leading the cause of mortality globally accounting for nearly 1 in 3 deaths annually [1]. Platelet activation leading to clot formation and thrombosis is an essential component of both the hemostatic and thrombotic responses in the blood following physiological and pathophysiological disturbance of the endothelium lining the vessel wall [2]. The inability to properly regulate platelet reactivity often leads to atherothrombotic events, including myocardial infarction and stroke. Recent work in the field has uncovered a number of lipid products, eicosanoids, derived from ω-3 or -6 polyunsaturated fatty acids (PUFAs) that significantly regulate and alter platelet function. The PUFAs include arachidonic acid (AA), linoleic acid (LA), eicospentaenoic acid (EPA), docosapentaenoic acid (DPA), docosahexaenoic acid (DHA), and dihomo-γ-linolenic acid (DGLA). Understanding how these newly identified lipids fit into the overall regulation of platelets in the vessel will aid in our understanding of lipid-platelet interactions, often resulting from altered diet or fatty acid supplementation, that play key roles in the ability of the platelet to form a hemostatic "plug" following vascular injury or alternatively form an occlusive thrombus following pathophysiologic insult to the vessel. Finally, understanding how these lipids and lipid products are generated and regulate platelet reactivity should reveal novel targets for therapeutic intervention to prevent thrombosis while limiting the risk for bleeding following vessel injury. Thus, this review will be limited to describing the various lipids and bioactive lipid products shown to regulate platelet function and modulate hemostasis and thrombosis in the vessel.

PUFAs are generally inert and depend on oxygenase activity to generate a wide array of structurally distinct bioactive fatty acids metabolites. The formation of lipid products is typically initiated by stimulation of the cell that results in an increase in intracellular calcium. This calcium flux results in translocation of cytosolic phospholipase A2 ($cPLA_2$) to the lipid membrane where it cleaves the fatty acid from the sn-2 position of the phospholipids to release free fatty acids for oxidation in the cell. Once cleaved from the lipid membrane, the freed fatty acids can be metabolized by cyclooxygenase

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Published online: 20 May 2017

(COX), lipoxygenase (LOX) or cytochrome P450 (CYP450) to form oxidized lipids (oxylipins). Oxylipins have been thought to predominantly function by regulating cellular properties and signaling through one of three pathways. The first involves binding to G protein-coupled receptors (GPCRs) to further propagate intercellular signaling. Secondly, fatty acids or their oxylipins can directly interact with peroxisome proliferator-activated receptors (PPARs) within the cell. While fatty acids are thought to be weak activators of PPARs, when they accumulate in the vicinity of the PPAR, reports have shown their affinity for activating PPAR signaling is significantly increased [3]. The third regulatory mechanism utilized by fatty acids and oxylipins in the platelet is direct inhibition of oxylipin-producing enzymatic pathways or further metabolic transformation of lipids within the cell (Fig. 1). The review will cover the oxygenase pathways, classes of structurally distinct oxylipins, and their biological effects on the platelets.

Cyclooxygenase

Cyclooxygenase (COX) exists in two isoforms, COX-1 and COX-2 in the body; however, the platelet expresses primarily COX-1, and its inhibition is thought to be a primary target for reduction of platelet reactivity in the patients with cardiovascular risk. COX activation primarily results in the generation of prostanoids (prostaglandins (PGs) and thromboxanes (TXs)) derived from PUFAs that are responsible for maintaining either physiological or pathophysiologic states, such as

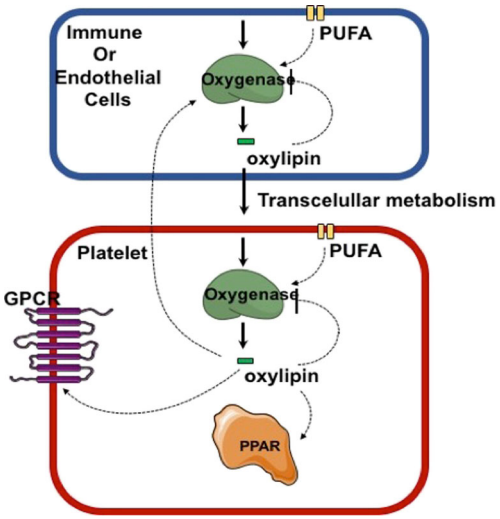

Fig. 1 Polyunsaturated fatty acids (PUFAs) are released from the embedded phospholipid bilayer membrane, which are then converted by intracellular oxygenases (COX, LOX, or CYP450) to generate wide array of oxylipins that can diffuse across the cellular membrane to be further converted by oxygenases, act on intracellular signaling component, peroxisome proliferator-activated receptor (PPAR), or act on receptor to regulate platelet function

inflammation and tumorigenesis [4]. This section describes the select prostanoid lipids generated from the PUFAs through the COX pathway that regulate platelet function.

COX-derived metabolites and their regulatory roles on platelet function

COX transforms AA to series 2 PGs (PGE_2, PGD_2, PGI_2) and thromboxanes (TX) A_2 that can exhibit either pro-thrombotic or anti-thrombotic modulation of platelet function (Table 1) [5, 6]. In terms of thrombosis, TXA_2, when formed, is released and acts through the thromboxanereceptor (TPα), which is coupled to $G\alpha_q$ and $G\alpha_{13}$ and functions to amplify platelet activation leading to enhanced aggregation and thrombosis [7]. In contrast, PGD_2 derived mainly from mast cells, leukocytes, and some platelets [8], had been shown to dampen platelet activation [9–11] through its binding to the DP_1 receptor and subsequent elevation of cAMP [12–14]; however, there are evidence that PGD_2 can also directly activate PPARs [15]. PGD_2 can be further dehydrated to PGJ_2, Δ^{12}-PGJ_2, and 15-deoxy-$\Delta^{12,14}$-PGJ_2, and inhibit platelet activation through a number of signaling pathways including activation of PPARs [16, 17]. While PGD_2 derivatives are known PPAR ligands, the role of PPARs in platelet activation has not been fully elucidated. Similar to PGD_2, PGI_2 (prostacyclin), a well-characterized vasodilator [18], has been shown to activate adenylate cyclase in the platelet via the prostacyclin (IP) receptor and in turn antagonizes platelet aggregation at sites of injury [6, 19]. Although PGI_2 has been shown to exert anti-platelet effects in vivo and is also clinically available to treat cardiovascular related diseases, a major concern of the use is the increased occurrence of hypotension. Moreover, PGE_2 exhibits pleiotropic effects by which it can induce both pro- and anti-platelet responses depending on the concentration [20, 21]through the binding of one of more of its prostaglandin receptors: EP_1, EP_2, EP_3 and EP_4 [22].

DGLA is an ω-6 PUFA that can be acquired through supplementation of γ-linolenic acid (GLA) in the diet. COX converts DGLA to series 1 prostaglandins (PGD_1, PGE_1) and TXA_1 [23, 24], which inhibit platelet function in vitro and in vivo [25]. These DGLA-derived COX prostanoids exerted their anti-platelet action by activating the $G\alpha_s$-coupled GPCRs, prostaglandin (EP_2 and EP_4) or IP receptors.

Similar to DGLA, COX acts on EPA, an ω-3 PUFA, to generate anti-inflammatory [26] lipid mediators, series 3 PGs and TXs [27, 28]. EPA-derived metabolites of COX (PGE_3, PGD_3, PGI_3) inhibit platelet aggregation [29–32] and P-selectin expression induced by platelet activating factor (PAF) as well as inhibiting platelet-rich plasma (PRP) [7]. Evidence for the series 3 PGs receptors is scant. PGE_3 had been suggested to be a partial agonist of the EP receptors in human kidney cells with varying degree of affinity potencies [29], inducing secondary messenger actions. For instance, PGE_3 mediates $G\alpha_q$

Table 1 Oxylipin regulation of platelets

Oxygenase	PUFA	Oxylipin	Actions
COX	AA	PGD_2	Inhibits platelet activation via DP receptor and possibly PPAR
	AA	PGE_2	Exhibits both anti-platelet and pro-platelet activation, depending on concentrations and binding to receptors EP_1-EP_4
	AA	PGI_2	Inhibits platelet function via IP receptor and PPAR
	AA	TXA_2	Activates platelet function via TPα receptor
	DGLA	PGD_1	Inhibits platelet function via EP_2, EP_4 and IP receptors
	DGLA	PGE_1	Inhibits platelet function via EP_2, EP_4 and IP receptors
	DGLA	TXA_1	Inhibits platelet function via EP_2, EP_4 and IP receptors
	EPA	PGD_3	Inhibits platelet function via DP receptor and PPAR
	EPA	PGE_3	Inhibits platelet function via EP_2 and P_4 receptors
	EPA	PGI_3	Inhibits platelet function via IP receptor and PPAR
	EPA	TXA_3	Inhibits platelet function via EP_2 and EP_4 receptors
5-LOX	AA	5-HETE	Exhibits both anti-platelet and pro-platelet activation; unknown mechanism of action
	AA	LTA_4	Unknown function
	AA	LTB_4	Unknown function
	AA	LTC_4	Activates platelet function via $CysLT_2R$
	AA	LTD_4	Potentiation of platelet activation; unknown mechanism of action
	AA	LTE_4	Potentiation of platelet activation; unknown mechanism of action
	AA	5-oxo-ETE	Unknown function
12-LOX	AA	12-HETE	Exhibits both anti-platelet and pro-platelet activation possibly through 12-HETER
	DGLA	12-HETrE	Inhibits platelet through $G\alpha_s$
	DHA	11-HDHA	Inhibits platelet activation; unknown mechanism of action
	DHA	14-HDHA	Inhibits platelet activation; unknown mechanism of action
	DPA	11-HDPA	Inhibits platelet activation possibly through antagonism of COX-1
	DPA	14-HDPA	Inhibits platelet activation possibly through antagonism of COX-1
	EPA	12-HEPE	Inhibits platelet activation; unknown mechanism of action
15-LOX	AA	15-HETE	Exhibits both anti-platelet and pro-platelet activation; unknown mechanism of action
	DGLA	15-HETrE	Exhibits both anti-platelet and pro-platelet activation; unknown mechanism of action
	DHA	17-DHDA	Exhibits both anti-platelet and pro-platelet activation; unknown mechanism of action
	DPA	13-HODE	Exhibits both anti-platelet and pro-platelet activation; unknown mechanism of action
CYP450	AA	5,6-EET	Inhibits platelet activation; unknown mechanism of action
	AA	8,9-EET	Inhibits platelet activation; unknown mechanism of action
	AA	11,12-EET	Inhibits platelet activation; unknown mechanism of action
	AA	14,15-EET	Inhibits platelet activation; unknown mechanism of action
	AA	19-HETE	Inhibits platelet function via IP receptor
	AA	20-HETE	Inhibits human platelet activation; antagonism of PGH_2/TXA_2 receptor or further oxidation to inactive metabolites
	DHA	7,8-EDP	Inhibits platelet activation; unknown mechanism of action
	DHA	10,11-EDP	Inhibits platelet activation; unknown mechanism of action
	DHA	13,14-EDP	Inhibits platelet activation; unknown mechanism of action
	DHA	16,17-EDP	Inhibits platelet activation; unknown mechanism of action
	DHA	19,20-EDP	Inhibits platelet activation; unknown mechanism of action
	EPA	8,9-EEQ	Inhibits platelet activation; unknown mechanism of action
	EPA	11,12-EEQ	Inhibits platelet activation; unknown mechanism of action
	EPA	14,15-EEQ	Inhibits platelet activation; unknown mechanism of action
	EPA	17,18-EEQ	Inhibits platelet activation; unknown mechanism of action
5-LOX, 15-LOX	DHA	Resolvin D_1	Potentiation of platelet activation possibly through GPR32
12-LOX	DHA	MaR1	Exhbits both anti-platelet and pro-platelet activation; unknown mechanism of action
15-LOX, 5-LOX	DHA	Protectin DX	Inhibits platelet activation; unknown mechanism of action

Table 1 (continued)

Oxygenase	PUFA	Oxylipin	Actions
COX-2, 5-LOX, CYP450	EPA	Resolvin E_1	Inhibits platelet activation possibly through ChemR23
15-LOX, 5-LOX, 12-LOX	AA	LXA_4	Modulate neutrophil-platelet aggregation through LXA_4 receptor; however unknown direct effect on platelet function and mechanism of action
	AA	LXB_4	Unknown function

activation and subsequent intracellular calcium release through the EP_1 receptor. While $G\alpha_s$ activation and enhanced cAMP formation were observed in EP_2 and EP_4 overexpressed cells treated with PGE_3, EP_3 ligand binding resulted in reduced cAMP generation and augmented inositol triphosphate (IP_3) formation coupled to $G\alpha_i$ activation [29]. Platelets treated with EP_3, EP_4, IP and receptor antagonists (DG-41, ONO-AE3-208, and CAY10441, respectively) demonstrated that PGE_3 acted as an antagonist to the EP_3 to further inhibit platelet function, but with no effect mediated by the IP receptor agonist on platelet reactivity. In contrast, CAY10441 reversed the ability of PGE_3 to inhibit platelet function [30]. These studies suggest that PGE_3 is also capable of producing multiple and simultaneous effects, resulting in either pro- or anti-thrombotic outcome.

Both PGD_3 and PGI_3 are deemed to behave similarly to PGD_2 and PGI_2 agonists as well as exerting through the same cognate receptors to increase cAMP. PGD_3 is also shown to be further dehydrated PGJ_3, Δ^{12}-PGJ_3, and 15-deoxy-$\Delta^{12,14}$-PGJ_3 [33] and act on DP receptor or possibly function through the PPARs [34]. PGI_3 is an unstable analog of PGI_2 that exerts its action on the IP receptor, but has been observed to activate PPAR [35]. TXA_3 is presumed to act on EP_2 and EP_4 receptors to enhance cAMP [31] to inhibit platelet function similar to TXA_1, but additional cell line and pharmacological models are required to verify this assumption.

Lipoxygenases

Mammalian lipoxygenases (LOXs) constitute the following heterogeneous group of lipid-peroxidizing enzymes that are categorized accordingly to their positional specificity of AA oxygenation: 5-LOX, 12-LOX, and 15-LOX. While these enzymes are expressed in a number of cells, they each produce oxylipins that function in part to regulate platelet activity, hemostasis, and thrombosis.

5-Lipoxygenase (5-LOX)

5-LOX is best known for its ability to produce leukotrienes (LTs) [28]. LTs are synthesized in myeloid cells (eosinophils, neutrophils, mast cells, macrophages, monocytes, dendritic cells, basophils, and B-lymphocytes) that are involved in inflammatory, immune, and allergy responses. 5-LOX also produces non-LT products (5-hydroxyeicosatetraenoic acid (5-HETE)) and two structural forms of LTs, which consist of cysteinyl-free (LTA, LTB) and cysteinyl-LTs (LTC, LTD, LTE, LTF) [36] (Fig. 2, Table 1). The type of PUFA substrate being oxidized dictates the class of LTs formed to modulate inflammatory response and vascular tone. While platelets lack 5-LOX, there are numerous studies suggesting the interplay between leukocytes and platelets through their eicosanoid production [37] by which platelets can modulate immunological response or vice versa [38, 39].

5-LOX-derived metabolites and their regulation of platelet function

Cellular induction by various stimuli (chemotactic agents, immune complexes, bacterial peptides) leads to translocation of

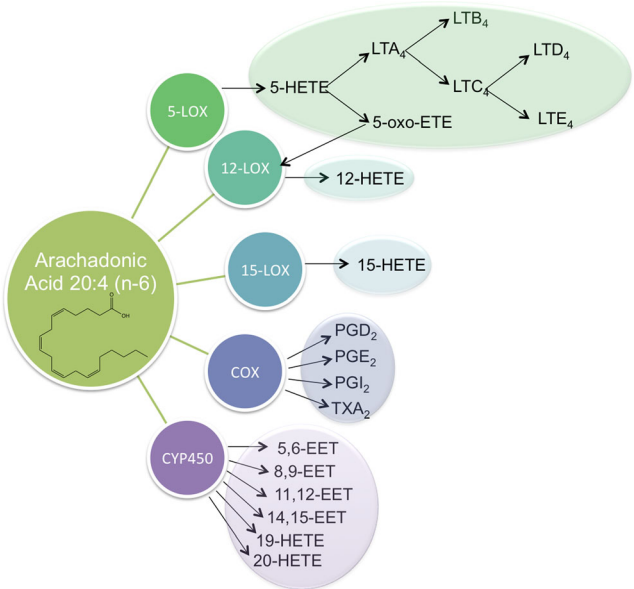

Fig. 2 Arachidonic acid (AA) is oxidized by 5-LOX, 12-LOX, 15-LOX, COX, and CYP450 into their respective classes of oxylipins: 1) non-leukotrienes (LTs) (5-HETE, 5-oxo-ETE) or cysteinyl-free LTs (LTA_4, LTB_4) and cysteinyl-LTs (LTC_4, LTD_4, LTE_4); 2) 12-HETE; 3) series 2 prostaglandins (PGD_2, PGE_2, PGI_2, and TXA_2); 4) epoxyeicosatrienoic acids (EETs) (5,6-EET, 8,9-EET, 11,12-EET, 14,15-EET) and hydroxylates (19-HETE, 20-HETE)

5-LOX to the membrane [40–42] to convert AA to 5-HETE. 5-HETE can further be converted by 5-hydroxyeicosanoid dehydrogenase to form 5-oxo-6,8,11,14-eicatetraenoic acid (5-oxo-ETE) (Fig. 2), or dehydrated to a member of series 4 epoxide intermediate leukotriene, LTA_4 [43].

Previous studies have demonstrated a platelet role in the transcellular metabolism of LTs released by neutrophils, enhancing platelet-neutrophil interactions. The interplay between platelet-adherent leukocyte interactions has been implicated in chronic inflammatory diseases in patients, including aspirin-exacerbated respiratory disease (AERD). While neutrophils lack LTC_4 synthase to convert LTs, platelets have been shown to possess abundant LTC_4 synthase [44–47] to convert unmetabolitzed LTA_4 secreted from neutrophils or monocytes to LTC_4 requiring P-selectin-dependent interaction [48–50]. Through this interaction, transcellular metabolism of LTC_4 is increased, resulting in exacerbated inflammatory response [51–53].

Although the secreted LTs function principally to activate and recruit additional neutrophils to propagate inflammatory responses in allergy or asthma, platelets have also been shown to respond to LTs. Human PRP pretreated with exogenous LTs (LTE_4, LTD_4, LTC_4) for example, showed potentiation in aggregation and TXB_2 production following sub-threshold stimulation with thrombin and epinephrine [54].The effects of LTs potentiation of human PRP aggregation and TXB_2 generation are presumed to be mediated through the platelet cys-LT receptors, type 1 and type 2 ($CysLT_1R$ and $CysLT_2R$) [55], which are involved in chemokine, RANTES, release in inflammation [55–59]. The functional importance of $CysLT_2R$ in platelet function was demonstrated in transgenic mice deficient in $CysLT_2R$ (*Cysltr2*$^{-/-}$) indicating $CysLT_2R$ expression was required for low nanomolar LTC_4 induction of P-selectin, ADP, and TXB_2 release from platelets [60]. On the other hand, LTE_4 and LTD_4 did not augment P-selectin expression in wild-type, *Cysltr1*$^{-/-}$ or *Cysltr2*$^{-/-}$ platelets, suggesting metabolite specificity for these biochemical regulatory steps. Interestingly, LTC_4 induction of P-selectin expression was also observed to be markedly impaired in purinergic receptor $P2Y_{12}$ knockout mouse platelets. Together, these studies suggest that $P2Y_{12}$-targed thienopyridine drugs used for the management of cardiovascular ischemic events may also interfere with theLTC_4/$CysLT_2R$-dependent pathway of platelet activation.

The biological activity of 5-HETE on platelet function has been controversial. In vitro studies showed 5-HETE inhibits the binding of the radiolabeled thromboxane mimetic, [^{125}I] BOP, to the PGH_2/TXA_2 receptor in washed human platelets with IC_{50} values greater than 25 μM. This observation suggests that 5-HETE directly inhibits platelet activation through direct competition with PGH_2/TXA_2 [61]. Conversely, thrombin-induced platelet aggregation and ADP release was shown to be potentiated with 30 μM of 5-HETE [62]. In light of clinical studies being conducted with the use of 5-HETE inhibitors, it will be of high importance to delineate the potential effect of these inhibitory strategies targeting 5-HETE production on platelet reactivity and hemostasis.

12-Lipoxygenase (12-LOX)

Both 12*S*-LOX and 1*2R*-LOX isoenzymes, which generate distinct chiral metabolites from PUFAs, are expressed in selective mammalian tissues and cells. 12-LOX is further classified as platelet, leukocyte, or epithelial-type. Platelet-type 12-LOX is expressed in all mammalian species, whereas the leukocyte-type 12-LOX is found in murine, porcine, and bovine, but not in humans or rabbits [63–65]. Conventionally, 12-LOX is characterized for its ability to convert AA to12-hyderoperoxyeicosatetraenoic acid (12-HpETE), which is rapidly reduced to 12-hydroxyeicosatetraenic acid (12-HETE) (Fig. 2). To date, majority of the platelet related studies have focused on 12*S*-LOX products, since no 12*R*-LOX products have been found to regulate platelet function. Thus, for the purpose of this review, only the *S* configuration metabolites of 12-LOX will be discussed.

Regulation of platelet function by 12-LOX-derived metabolites

The major metabolite of 12-LOX, 12-HETE, has been described to have both anti-thrombotic and pro-thrombotic effects. The anti-thrombotic effect of 12-HETE was first implicated by its direct inhibition of neutrophil PLA_2 activity by which the availability of AA was reduced in vitro [66]. In support, exogenous 12-HETE suppressed collagen-induced liberation of AA in bovine platelets [67]. Platelets from 12-LOX-deficient mice were hyper-responsive to aggregation induced by ADP, and this phenomenon was reversed by 12-HETE treatment [68]. 12-HETE and 12-HpETE were also reported to inhibit PGH_2- and collagen-induced platelet aggregation [69–71] as well as prevent binding of PGH_2 and TXA_2 to their cognate receptors [68].

In stark contrast, 12-HpETE and 12-HETE have been demonstrated to potentiate platelet activation and aggregation. Exogenous 12-HpETE, at nanomolar concentrations, stimulated platelet p38 mitogen-activated protein kinase, as well as phosphorylation of cytosolic PLA_2, increased TXB_2 and dense granule secretion [71–74]. 12-HETE was also shown to potentiate bovine platelet aggregation induced by thrombin as well as inhibiting PGE_1-induced elevation of cAMP. Pharmacological inhibition and genetic ablation of 12-LOX have also demonstrated the importance of 12-HETE in potentiation of platelet activation [74–76].

The pro-thrombotic effect of 12-HETE is thought to be mediated through its esterification into the lipid membrane

following formation in the platelet, which results in enhanced tissue factor-dependent thrombin generation in the vessel [77]. It is also possible that 12-HETE could be mediating its effect on platelet function through high affinity binding to an orphan GPCR, GPR31 (12-HETER) [78], which was originally discovered in cancer cells to promote survival and metastasis as well as neuronal cells that modulate voltage-sensitive calcium channels [79]. Alternatively, 12-HETE had also been shown to enhance and activate peroxisome proliferator-activated receptor γ (PPARγ), a member of the nuclear hormone receptor family of ligand-dependent transcription factors [80]. To date, the expression of 12-HETER in platelets has not been confirmed.

While the predominant metabolite, 12-HETE, has been shown to have contradicting roles, other 12-LOX derived metabolites from EPA, DHA, DPA, and DGLA (12-HEPE, 11/14-hydroxydocosahexaenoic acid (11/14-HDHA), 11/14-hydroxydocosapentaenoic acid (11/14-HDPA), and 12-hydroxyeicosatrienoic acid (12-HETrE)) [70, 76, 81] have been shown to exert anti-platelet or anti-thrombotic in vivo effects. These metabolites vary in potency and ability to be synthesized. For instance, only trace amounts of 11/14-HDHA (Fig. 3) were detected in EPA or DHA pretreatment compared to 12-HEPE and 12-HETE following thrombin-stimulation in platelets, suggesting that higher concentrations of DHA are needed for platelet inhibition [82]. Additionally, DPA is observed to exert its anti-platelet effect [83] through inhibition of COX-1 activity by 11/14-HDPA [84] (Fig. 4).

Though previous studies have implicated the 12-LOX-derived metabolites in cardioprotection through the dampening of platelet activation, there were no direct in vivo evidence to support those claims. More recently, the role of platelet 12-HETrE on thrombosis and underlying mechanisms were investigated in vivo and ex vivo. Mice intravenously administered with 6 mg/kg of 12-HETrE or 50 mg/kg DGLA were protected from thrombus accumulation at the site of arteriole vessel injury [85]. The anti-platelet effects of DGLA in vivo were also shown to be dependent on the presence of functional platelet 12-LOX in mouse platelets. For instance, even though mice lacking 12-LOX (*ALOX12*$^{-/-}$) had attenuated thrombus formation within the vessel following laser injury, DGLA treatment did not further prevent thrombus growth in the *ALOX12*$^{-/-}$ mice compared to the wild-type. This demonstrated that 12-LOX was required for DGLA-mediated inhibition of platelet activation and thrombus formation. Finally, the anti-platelet effect of 12-HETrE was shown to be mediated through a $G\alpha_s$-linked GPCR, which activates adenylyl cyclase and subsequent downstream effectors to inhibit platelet activation.

15-Lipoxygenase (15-LOX)

Two forms of 15-LOX isoforms exist in mammalian tissues, leukocyte-type 15*S*-LOX (15-LOX-1) and epidermis-type 15-LOX type B (15-LOX-2) [86–88]. Tissue distribution of 15-LOX-2 is limited when compared to that of 15-LOX-1. 15-LOX-1 is expressed in eosinophils, leukocytes, reticulocytes, macrophages, dendritic, epithelial cells (bronchial, corneal, and mammary) [89, 90], whereas, 15-LOX-2 is predominantly found in the skin, prostate, lung, and cornea [91].

15-LOX-derived metabolites and regulation of platelet function

While the existence of 15-LOX-1 and -2 in platelets is questionable, platelets have demonstrated the ability to generate the 15-LOX oxylipin products, 15-hydroxyeicosatetraenoic acid (15-HETE), 8,15-dihydroxyeicosatetraenoic acid (8,15-diHETE),

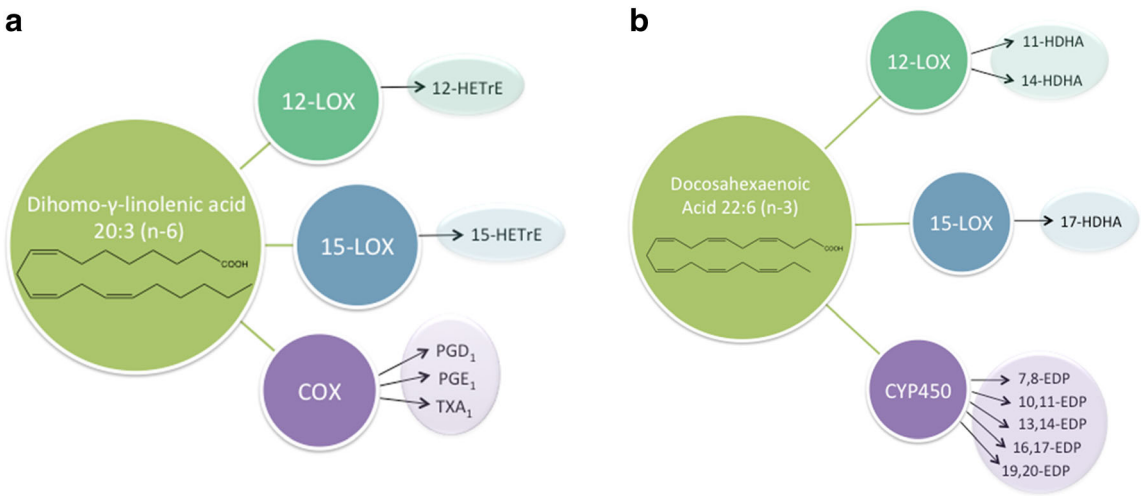

Fig. 3 PUFA oxidation by oxygenases. **a** Dihomo-γ-linolenic acid (DGLA) is oxidized by 12-LOX, 15-LOX, and COX into the corresponding metabolites: 12-HETrE, 15-HETrE, and series 1 prostaglandins (PGD_1, PGE_1, TXA_2). **b** Docosahexaenoic acid (DHA) is also metabolized by the oxygenases into the following: 11- or 14-HDHA by 12-LOX, 17-HDHA by 15-LOX, and epoxydocosapentaenoic acids (EDPs) (7,8-EDP, 10,11-EDP, 13,14-EDP, 16,17-EDP, 19,20-EDP) by CYP450 isoforms

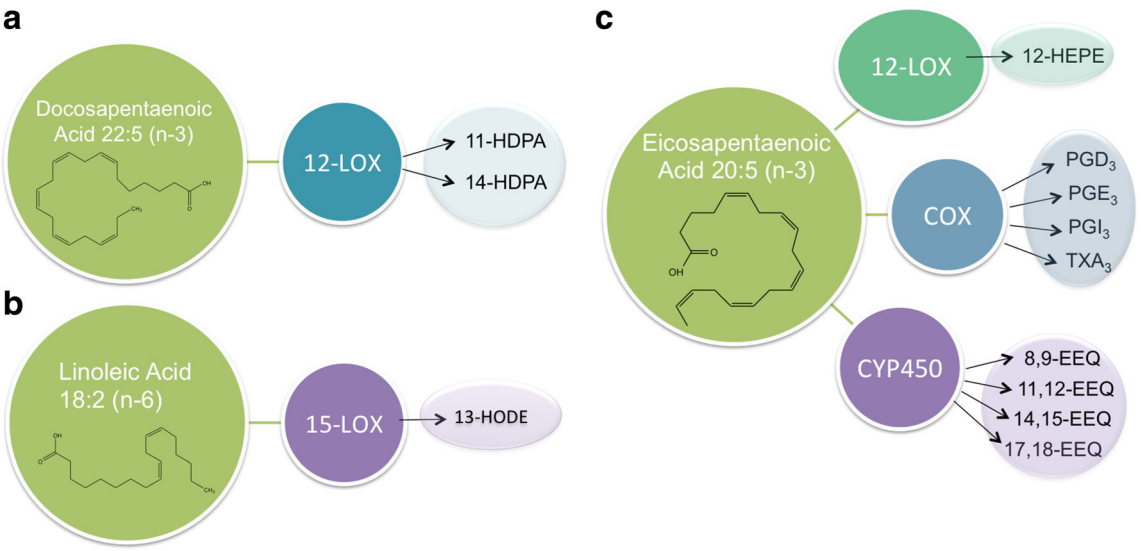

Fig. 4 PUFA oxidation by oxygenases. **a** 12-LOX acts on docosapentaenoic acid (DPA) to convert to 11- or 14-HDPA. **b** Linoleic acid (LA) is metabolitzed by 15-LOX to generate 13-HODE. **c** Eicosapentaenoic acid (EPA) is oxidized by 12-LOX, COX, CYP 450 to 12-HEPE, series 3 prostaglandins (PGD_3, PGE_3, PGI_3, and TXA_3), and epoxyeicosatetraenoic acids (EEQs) (8,9-EEQ, 11,12-EEQ, 14,15-EEQ, and 17,18-EEQ)

and 14,15-dihydroxyeicosatetraenoic acid (14,15-diHETE) from AA (Fig. 2) [87], and 17-hydroxydocosahexaenoic acid (17-HDHA) [92] from DHA (Fig. 3, Table 1). Both 15-HETE and 8,15-diHETE were shown to inhibit platelet aggregation induced by collagen, ADP, epinephrine, AA, or prostaglandin H2 analog [93–95]. Conversely, 15-HETE and 15-HpETE were also demonstrated to enhance whole blood aggregation and thrombin generation in the presence of macrophages [96]. 15-HETE (between 1 and 100 nM) enhanced thrombin-stimulated platelet aggregation, ADP release, and secondary messengers (IP_3, diacylglycerol, and intracellular calcium) production [62]. Similarly, 17-HDHA was shown to potentiate ADP-induced platelet aggregation and spreading, but inhibited α-granule secretion [97]. The latter data suggests that 15-LOX products can function as procoagulant mediators.

Both 13-hydoxyoctadecadienoic acid (13-HODE) and 15-hydroxyeicosatrienoic acid (15-HETrE), major metabolites of 15-LOX derived from linolenic acid (LA) (Fig. 4) and DGLA (Fig. 3), respectively, were shown to inhibit rabbit [98] and human platelet aggregation [99]. Additionally, 13-HODE was demonstrated to inhibit thrombin-induced TXB2 and 12-HETE production in platelets as well as platelet adherence to endothelial cells in vitro [99, 100]. Interestingly, 15-HETrE exhibited biphasic effects on platelet aggregation in which low concentrations potentiated and higher concentrations inhibited platelet aggregation [101].

Cytochrome P450

Cytochrome P450 enzymes (CYP450s) belong to a large group of oxygenases, with at least 57 putatively functional subfamilies in humans and upwards of 102 in mice [102, 103]. CYP450s are expressed primarily in the liver, with some detection in the heart, lung, vasculature, kidney, and gastrointestinal tract. Traditionally, these membrane-bound and heme-containing oxygenases are recognized for their xenobiotic metabolism and detoxification of drugs; however, multifaceted functions have also been uncovered. These enzymes are also involved in the metabolism of eicosanoids from fatty acids, vitamin D3 synthesis, biosynthesis of cholesterol and bile acids, and synthesis and metabolism of steroids [104].

Regulation of platelet function by CYP450 epoxygenase and hydroxylase-derived metabolites

AA can be synthesized by endothelial CYP450 epoxygenase into a number of epoxyeicosatrienoic acids (5,6-epoxyeicosatrienoic acid (5,6-EET), 8,9-epoxyeicosatrienoic acid (8,9-EET), 11,12-epoxyeicosatrienoic acid (11,12-EET), and 14,15-epoxyeicosatrienoic acid (14,15-EET)), which are further catalyzed to dihydroxyeicosatrienoic acids (5,6-diHETrE, 8,9-diHETrE, 11,12-diHETrE, 14,15-diHETrE) by soluble expoxide hydrolase (sEH). Endothelial CYP450 ω-hydroxylase also acts on AA to generate 19-hydroxyeicosatetraenoic acid (19-HETE) and 20-hydroxyeicosatetraenoic acid (20-HETE) to maintain vascular tone and hemostasis (Fig. 2). Early studies demonstrated that many epoxygenase isomers ranging from 1 to 10 μM, regardless of their regiochemical, geometric, and steriochemical structures, were effective at inhibiting human platelet [105–107] or PRP [108] aggregation independent of TXB_2 and cAMP formation [107]. However, more recent reports suggest that 11,12-EET (ranging from 1 to 10 μM) do

not inhibit platelet aggregation stimulated with collagen, ADP or a thrombin-receptor activating peptide [109]. The conflicting observations from several research groups will require further study to determine if this class of EETs is pro-thrombotic, anti-thrombotic, or is bi-functional depending on platelet conditions (PRP or isolated platelets) and agonist stimulation used.

EETs (5,6-EET, 11,12-EET, 8,9-EET, and 14,15-EET) have additionally been demonstrated to hyperpolarize platelets through the activation of calcium potassium channels resulting in decreased ADP-induced P-selectin expression on platelet surfaceas well as platelet adhesion to cultured endothelial cells under physiological shear stress [110]. To further support CYP450-derived products from endothelial cells regulate platelet function, supernatant releasate from bradykinin-stimulated cultured endothelial cells overexpressing CYP2C9 were shown to inhibit platelet adhesion. Finally, in vivo anti-thrombotic effects of CYP2C9-derived metabolites were demonstrated in the arteriolar wall of hamster. Hamsters administered with CYP2CP inhibitor, sulfaphenazole, at doses known to block endothelium-derived hyperpolarizing factor-dependent dilations, significantly enhanced platelet-vessel wall interactions. The firm adhesion of platelets to vessel wall was reversed when superfused with 10 μM of 11,12-EET [111].

The CYP450 ω-hydroxylase product of AA, 19-HETE, was found to be an orthosteric prostacyclin receptor agonist that inhibited mouse platelet aggregation. To verify that 19(*S*)-HETE, and not its regioisomer 19(*R*)-HETE, was responsible for binding to the prostacyclin receptor and inhibiting platelet activity, a megakaryocyte cell line, MEG-01 [112], with intact $G\alpha_s$ expression was shown to enhance cAMP formation following dose-dependent 19(*S*)-HETE treatment. Additionally, COX-1/2 inhibition of COS-1 human IP receptor expressing cells did not interfere with the ability of 19(*S*)-HETE to directly induce cAMP formation in MEG-01. Blocking the IP receptor with the selective prostacyclin receptor inhibitor, Cay104401, prevented 19(*S*)-HETE stimulation of cAMP generation in MEG-01 cells. Similarly, 19(*S*)-HETE was able to displace ^{3}H-iloprost in COS-1 cells expressing IP receptor, demonstrating that 19(*S*)-HETE behaved as a competitive agonist binding to the same domain of the IP receptor as Iliprost (and likely PGI_2). These observations were confirmed when pretreatment of IP deficient mouse (*Ptgir*$^{-/-}$)_platelets with 3 μM 19(*S*)-HETE failed to block thrombin-induced platelet aggregation.

Another eicosanoid found to have potent inhibitory properties against the platelet is 20-HETE. This eicosanoid was found to have a potent effect on inhibiting human platelet aggregation and TXB_2 formation induced by AA, calcium ionohophore, A23187, and the TXB_2 mimetic without affecting thrombin-induced aggregation [113]. The proposed inhibitory effect of 20-HETE on platelet activation was also presumed to be its antagonism of the PGH_2/TxA_2 receptors [113, 114]. Aside from receptors antagonism, 20-HETE was also shown to be further metabolized by COX-1 and 12-LOX to the inactive 11,12-dihydroxyeicosatetraenoic acid (11,12-diHETE) and 12,20-dihydroxyeicosatetraenic acid (12,20-diHETE), respectively, in human platelets [113]. Thus, it is possible that at least some of the observed anti-platelet effect could be attributed to its metabolic transformation to the diHETEs.

Upon dietary supplementation of ω-3 or -6 PUFAs, AA-derived products of CYP450 epoxygenase are partially replaced by EPA and DHA-derived epoxyeicosatetraenoic acids (EEQs) and (epoxydocosapentaenoic acids (EDPs), respectively [115]. In the case of CYP450 epoxygenase-derived metabolites of EPA (8,9-EEQ, 11,12-EEQ, 14,15-EEQ, 17,18-EEQ) (Fig. 4) and DHA (7,8-EDP, 10,11-EDP, 13,14-EDP, 16,17-EDP, and 19,20-EDP) (Fig. 3), all metabolites were shown to inhibit AA-induced platelet aggregation [116] (Table 1). Even though all the diols produced by sEH conversion of the EEQs (8,9-DiHETE, 11,12-DiHETE, 14,15-DiHETE, 17,18- DiHETE) and EDPs (7,8-DiHDPA, 10,11-DiHDPA, 13,14-DiHDPA, 16,17-DiHDPA, 19,20-DiHDPA) inhibited platelet aggregation; they were less potent at inhibiting platelet aggregation than the parent epoxides.

Although several of these studies have demonstrated EET and hydroxylate metabolites are derived from cells with intact CYP450, preformed expoxides and 20-HETE of AA have been found as integral components of human platelet membrane [106]. Thus, it is possible that circulating EETs and their diol products, DHETs, and hydroxylates are avidly taken up by platelets and endothelial cells [117, 118]. These products can be released during receptor-mediated hydrolysis of platelet phospholipids [106] or further metabolized by COX [119] and LOX [113]. For instance, once stimulated, EETs are de-esterified in platelets and released to influence the migration pattern of nearby neutrophils [106, 120]. In contrast, CYP450 ω-hydroxylase inhibitor, HET0016, blocked angiotensin and endothelin-stimulation of 20-HETE secretion from platelets, suggesting that CYP450 isoforms exist in the platelet [115]. Based on study discrepancy, the endogenous expression of CYP450 in the platelet has not been confirmed and will need to be definitively determined before platelet generation of expoxide or hydroxylate products can be assigned to the platelet itself or alternatively if these products are presented to the platelet from other blood cells, including the endothelium and neutrophils.

The interplay of oxygenases and generation of specialized pro-resolving lipid mediators (SPMs)

Over the past decade, studies have focused on the role of specialized pro-resolving lipid mediators (SPMs) on preventing excessive inflammation, infection, and wound repair, through their ability to attenuate or dissipate chemotactic and pro-inflammatory signals. SPMs, which include lipoxins (LX), D and E series resolvins (Rv), (neuro) protectins (PD), and maresins (MaR), are synthesized by the sequential action of LOXs on PUFAs to resolve and restrain inflammation [100] (Fig. 5). Despite the prevalence of platelet involvement in the inflammatory process, little is known on how and whether SPMs play a direct role on regulation of platelet function.

Lipoxins A (LXA_4) and B (LXB_4) were one of the first SPMs to be identified from the combination of 5- and 15-LOX in human leukocytes [43] as well as neutrophil-derived 5-LOX and platelet 12-LOX [121–124] from AA. Although platelets express LXA_4 receptor (ALX) [125], LXA_4 does not directly inhibit platelet aggregation induced by ADP [98] and bacterial infection [98]. Alternatively, aspirin-triggered lipoxin (ATL), 15(*R*)-epi-lipoxin A_4 (15(*R*)-epi-LXA_4)) [126], is indirectly derived from the acetylated COX-2 metabolism of AA. Both LXA_4 and 15(*R*)-epi-LXA_4 had been demonstrated to modulate neutrophil-platelet aggregation through ALX; however, it remains unclear whether these lipoxins can directly regulate platelet function based on limited studies.

Resolvin E_1 (RvE_1), synthesized by acetylated COX-2 or sequential CYP450 and 5-LOX activity of EPA, was demonstrated to inhibit human PRP aggregation stimulated by ADP and TXB_2, but not collagen [127]. RvE_1 was also shown to inhibit P-selectin expression on activated platelets and platelet actin polymerization, without affecting calcium mobilization.

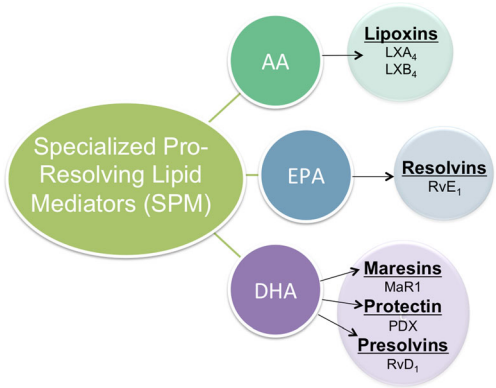

Fig. 5 Specialized pro-resolving lipid mediators (SPMs) constitute a wide array of lipids classes derived from the interplay of oxygenase activity on AA, EPA, and DHA. Lipoxins, (LXA_4, LXB_4) and E series resolvin (RvE_1) are derived from AA and EPA, respectively. DHA can be indirectly converted by the interaction of the oxygenases into MaR1, PDX, or D series resolvin, RvD_1

The observed anti-platelet effects of RvE_1 were shown to act through the ChemR23 receptor on the surface of platelets [128]. In contrast, resolvin D_1 (RvD_1) and its intermediary precursor, 17-HDHA, derived from 15-LOX and 5-LOX synthesis of DHA, potentiated ADP-mediated platelet aggregation and spreading on fibrinogen. ADP-mediated release of α-granules in platelets were not affected by RvD_1 and 17-HDHA; however, thrombin stimulation of α-granules was significantly attenuated by these SPMs [129]. RvD_1 is presumed to exert its effect on its cognate receptor, GPR32, on the platelet surface. Thus far, while data support a role for platelets in the generation of inflammatory markers, the mechanism by which resolvins regulate platelets remains unclear.

Maresin 1 (MaR1) is derived from the biosynthesis of DHA by both neutrophil 15-LOX and platelet 12-LOX [98]. Early studies demonstrated MaR1 anti-inflammatory and pro-resolving properties in lung catabasis. While MaR1 was shown to potentiate platelet aggregation and spreading, it also dampened pro-inflammatory and pro-thrombotic granules, suggesting MaR1 differentially regulates platelet function through a mechanism that has not been fully elucidated to date.

Protectin DX (PDX) belongs to a group of di-oxygenated derivatives of PUFAs, called poxytrins [130]. PDX is an isomer of neuroprotein D1 (PD1) [131], which was originally discovered to attenuate brain ischemia-reperfusion [132, 133]. The PDX isomer was demonstrated to be biologically less potent than PD1 in the resolution of inflammation; however, effective in inhibiting collagen-, AA-, and thromboxane-induced platelet aggregation through inhibition of COX-1, at nanomolar concentrations [134].

Discussion and future implications

Regulation of platelet function is a key step in both physiological and pathological hemostatic processes. While inhibition of platelet activation remains a first-line approach for prevention of myocardial infarction and stroke, morbidity and mortality due to cardiovascular diseases and stroke remain the top causes of death globally. Hence, a greater understanding of the regulators of platelet function in vivo will significantly aid in the development of novel treatments to prevent unwanted clotting and occlusive thrombosis. Lipids and their oxylipins have long been known to regulate platelet function; however, until recently, the breadth of regulators and functions they control in the platelet have not been fully appreciated.

In this review, we have highlighted some of the major breakthroughs in identifying oxylipins we now know have direct effects on the platelet (Fig. 6). The use of genetic manipulation and pharmacological tools in both mouse

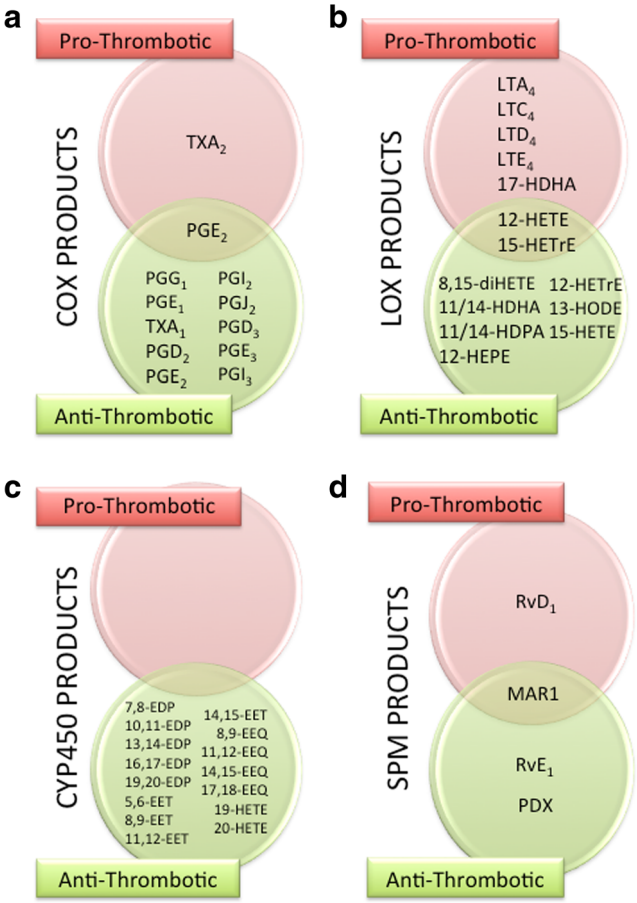

Fig. 6 LOX, COX, CYP 450 derived lipid mediators, and SPMs can be divided into either pro- or anti-thrombotic classes based on their effects on platelet function

and cellular models to determine the oxygenases and their lipid contributions to vascular and platelet functions has progressed considerably over the last two decades. These tools have greatly enhanced our understanding of the varying roles of oxylipins in platelet biology; however, these studies are still limited to another layer of complexity. The difficulty in targeting the precise pathway or oxylipins associated with pathophysiological disease states stems from the source of oxylipins generated by the involvement of multiple oxygenase enzymes localized in different organs and cell types. Even with the use of pharmacological tools to determine the contributions for each oxygenase metabolites to platelet function, selectivity of drug target still remains a major setback. For instance, chemical compounds designed to inhibit CYP epoxygenase enzymes also antagonized CYP hydroxylase enzymes activity. Therefore, interpretation of results with pharmacological inhibitors should be taken with caution. Future work will thus focus on further delineating the full breadth and diversity of oxylipins regulating platelet function ex vivo and in vivo and determining the mechanism(s) by which they exert their regulatory function on the human platelet to develop newer pharmacological approaches for targeting pathways involved in the regulation of platelet to address pathological conditions whereby normal regulation of hemostasis and thrombosis has become dysfunctional.

Compliance with ethical standards

Sources of funding This work was supported in part, by the National Institutes of Health Office of Dietary Supplement, R01 GM105671 (MH), R01 HL114405 (MH), and F31 HL129481 (JY).

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