12(S)-HETrE, a 12-Lipoxygenase Oxylipin of Dihomo-γ-Linolenic Acid, Inhibits Thrombosis via Gαs Signaling in Platelets

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Objective—Dietary supplementation with polyunsaturated fatty acids has been widely used for primary and secondary prevention of cardiovascular disease in individuals at risk; however, the cardioprotective benefits of polyunsaturated fatty acids remain controversial because of lack of mechanistic and in vivo evidence. We present direct evidence that an omega-6 polyunsaturated fatty acid, dihomo-γ-linolenic acid (DGLA), exhibits in vivo cardioprotection through 12-lipoxygenase (12-LOX) oxidation of DGLA to its reduced oxidized lipid form, 12(S)-hydroxy-8Z,10E,14Z-eicosatrienoic acid (12(S)-HETrE), inhibiting platelet activation and thrombosis.

Approach and Results—DGLA inhibited ex vivo platelet aggregation and Rap1 activation in wild-type mice, but not in mice lacking 12-LOX expression (12-LOX−/−). Similarly, wild-type mice treated with DGLA were able to reduce thrombus growth (platelet and fibrin accumulation) after laser-induced injury of the arteriole of the cremaster muscle, but not 12-LOX−/− mice, supporting a 12-LOX requirement for mediating the inhibitory effects of DGLA on platelet-mediated thrombus formation. Platelet activation and thrombus formation were also suppressed when directly treated with 12(S)-HETrE. Importantly, 2 hemostatic models, tail bleeding and arteriole rupture of the cremaster muscle, showed no alteration in hemostasis after 12(S)-HETrE treatment. Finally, the mechanism for 12(S)-HETrE protection was shown to be mediated via a Gαs-linked G-protein–coupled receptor pathway in human platelets.

Conclusions—This study provides the direct evidence that an omega-6 polyunsaturated fatty acid, DGLA, inhibits injury-induced thrombosis through its 12-LOX oxylipin, 12(S)-HETrE, which strongly supports the potential cardioprotective benefits of DGLA supplementation through its regulation of platelet function. Furthermore, this is the first evidence of a 12-LOX oxylipin regulating platelet function in a Gαs subunit–linked G-protein–coupled receptor–dependent manner. (Arterioscler Thromb Vasc Biol. 2016;36:2068-2077. DOI: 10.1161/ATVBAHA.116.308050.)

Key Words: blood platelets eicosanoids fibrin lipoxigenase platelet activation thrombosis

Platelet activation plays a critical role in the thrombotic complications associated with life-threatening cardiovascular ischemic events, such as myocardial infarction and stroke. Inhibiting platelet activation in individuals at risk for thrombotic events through the use of aspirin and P2Y12 receptor antagonists has significantly decreased morbidity and mortality associated with these debilitating conditions. Nonetheless, the fact that the rate of ischemic events still remains high in individuals on antiplatelet agents stresses the need to investigate alternative therapies that reduce occlusive thrombotic events without promoting an increased risk of bleeding. Dietary supplementation with polyunsaturated fatty acids (PUFAs) are commonly used for their potential cardioprotective effects including their antiplatelet effects, but the evidence supporting this claim in vivo remains unclear.

Dihomo-γ-linolenic acid (DGLA), an ω-6 PUFA, has been suggested to play a role in inhibiting platelet aggregation ex vivo. Although these studies support DGLA as a potential inhibitor of platelet function, the underlying mechanism by which DGLA elicits its antiplatelet effect and the in vivo relevance of this inhibition have remained elusive. PUFAs are primarily thought to exert their regulatory effects on platelet function through their conversion into bioactive lipids (oxylipins) by oxygenases. In platelets, DGLA can be oxidized by cyclooxygenase-1 (COX-1) or platelet 12-lipoxygenase (12-LOX) after its release from the phospholipid bilayer predominately through the actions of cytosolic phospholipase A2. Although both COX-1 and 12-LOX are able to oxidize

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DGLA to their respective metabolites, the relative contributions of these oxylipin products to the inhibitory effects of DGLA on platelet function remain unclear. Furthermore, the antiplatelet effects of DGLA have been primarily attributed to the COX-1–derived metabolites that have been shown to inhibit platelet activation. However, the DGLA-derived products of COX-1 (TXA<sub>2</sub> and prostaglandin E1) are liable and produced in low amounts in platelets. In addition, a recent study demonstrated that 12(S)-hydroxy-8Z,10E,14Z-eicosatrienoic acid (12(S)-HET<sub>E</sub>), the 12-LOX–derived oxylipin of DGLA, exhibits a potential antiplatelet effect ex vivo. Hence, it is important to delineate whether 12-LOX is required for DGLA-mediated inhibition of platelet function in vivo and whether DGLA and 12(S)-HET<sub>E</sub> play an essential role in regulation of thrombosis.

This study showed that an ω-6 PUFA, DGLA, inhibited platelet thrombus formation in vivo after an insult to the vessel wall. Interestingly, DGLA was unable to inhibit thrombus formation in 12-LOX<sup>−/−</sup> mice, suggesting that the antithrombotic effects of DGLA were mediated by 12-LOX. The 12-LOX–derived oxylipin of DGLA, 12(S)-HET<sub>E</sub>, potently inhibited thrombus formation after vessel injury irrespective of 12-LOX expression. Importantly, the antithrombotic effects of 12(S)-HET<sub>E</sub> did not disrupt primary hemostasis or result in increased bleeding. Finally, the antiplatelet effect of 12(S)-HET<sub>E</sub> was delineated here and shown to inhibit platelet function through the activation of the Gs α subunit (Gs<sub>α</sub>) signaling pathway leading to the formation of cAMP and protein kinase A (PKA) activation in the platelet. Hence, these findings demonstrate an antithrombotic role of DGLA 12(S)-HET<sub>E</sub> at both the mechanistic and in vivo levels.

Materials and Methods
Materials and Methods are available in the online-only Data Supplement.

Results
DGLA Inhibits Platelet Aggregation and Thrombus Growth in a 12-LOX–Dependent Manner
Treatment of human platelets with either DGLA<sup>4–6</sup> or its 12-LOX–derived metabolite, 12(S)-HET<sub>E</sub>, potently inhibited platelet aggregation<sup>6</sup>, however, the relative contribution of 12-LOX to DGLA-mediated inhibition of platelet activation was unclear. To assess the role of 12-LOX in DGLA-mediated platelet inhibition, washed platelets from WT or 12-LOX<sup>−/−</sup> mice were stimulated with an EC<sub>80</sub> concentration of either protease-activated receptor-4–activating peptide (PAR4-AP) or collagen in the presence or absence of DGLA. As previously reported, platelets from 12-LOX<sup>−/−</sup> mice were hypoactive compared with platelets from WT mice, hence, requiring a higher concentration of agonist to reach EC<sub>80</sub>. Pretreatment of platelets from WT mice with DGLA resulted in significant inhibition of aggregation compared with DMSO-treated platelets in response to PAR4-AP or collagen stimulation (Figure 1A and 1B). Conversely, DGLA treatment of platelets from 12-LOX<sup>−/−</sup> mice failed to inhibit platelet aggregation in response to PAR4-AP or collagen stimulation (Figure 1A and 1B). To fairly compare the concentrations of the higher PAR4-AP concentration used on 12-LOX<sup>−/−</sup> platelets, WT platelets treated with DGLA were also stimulated with higher PAR4-AP concentration, resulting in significant inhibition of platelet aggregation compared to vehicle control (data not shown). As the observed DGLA-mediated inhibition of aggregation may be because of the modification of the lipid membrane structure thus affecting platelet signaling or activation, other PUFAs including linoleic acid and arachidonic acid (AA) were used as controls to rule out a lipid membrane insulating effect in platelet activation. Pretreatment of platelets with either linoleic acid or AA had no inhibitory effect on PAR4-AP or collagen-mediated platelet aggregation compared with vehicle alone (Figure 1A and 1B).

To determine whether DGLA inhibited platelet aggregation by impinging on intracellular signaling, the activation of Rap1, a common signaling effector required for integrin α<sub>IIb</sub>β<sub>3</sub> activation was assessed in DGLA-treated platelets stimulated with PAR4-AP<sup>19–21</sup>. In platelets isolated from WT mice, DGLA inhibited Rap1 activation at all concentrations of PAR4-AP tested (Figure 1C). Because DGLA was unable to inhibit platelet aggregation in 12-LOX<sup>−/−</sup> mice, we assessed whether 12-LOX was also necessary for DGLA inhibition of Rap1 activation in platelets. Consistent with the platelet aggregation data, DGLA was unable to inhibit Rap1 activation in platelets from 12-LOX<sup>−/−</sup> mice at any of the concentrations of PAR4-AP tested (Figure 1C). Together, these data demonstrate that the antiplatelet effects mediated by DGLA require 12-LOX.

To determine whether the antiplatelet effects of DGLA observed ex vivo could contribute to the inhibition of platelet thrombus formation in vivo, a laser-induced cremaster arteriole thrombosis model was used to examine thrombus formation (platelet and fibrin) in WT mice<sup>22</sup> (Figure 1D through 1F). Mice were intravenously injected with either vehicle control (DMSO) or 50 mg/kg of DGLA 10 minutes before the initiation of thrombosis by laser injury. After vessel injury of vehicle control-treated WT mice, fluorescently labeled platelets rapidly accumulated at the site of vascular injury then drastically diminished in size as the clot was resolved (Figure 1D and 1E; Movie I in the online-only Data Supplement). Simultaneously, fibrin formation can be seen at the base of the developing thrombus of vehicle control-treated WT mice (Figure 1D and 1E; Movie I in the online-only Data Supplement). WT mice

Nonstandard Abbreviations and Acronyms

| 12(S)-HEPE | 12(S)-hydroxy-5Z,8Z,10E,14Z-eicosatrienoic acid |
| 12(S)-HETE | 12(S)-hydroxy-8Z,10E,14Z-eicosatrienoic acid |
| 12-LOX | 12-Lipoxygenase |
| AA | arachidonic acid |
| AC | adenylyl cyclase |
| COX-1 | cyclooxygenase-1 |
| DGLA | dihomoy-γ-linolenic acid |
| GPCR | G-protein–coupled receptor |
| Gsγ | Gs α subunit |
| PKA | protein kinase A |
| PUFA | polyunsaturated fatty acid |
| VASP | vasodilator-stimulated phosphoprotein |
Figure 1. 12-lipoxygenase (12-LOX) is required for dihomo-γ-linolenic acid (DGLA) inhibition of platelet aggregation and thrombus formation. Representative tracings and combined aggregation data of (A) wild-type (WT) (n=4) or (B) 12-LOX−/− (n=4) platelets stimulated with EC80 concentration of protease-activated receptor-4-activating peptide (PAR4-AP) (WT 100 μmol/L; 12-LOX−/− 200 μmol/L) or collagen (WT 5 μg/mL; 12-LOX−/− 2 or 5 μg/mL) in the presence or absence of 10 μmol/L of polyunsaturated fatty acids (DGLA, arachidonic acid [AA], or linoleic acid [LA]). Aggregation was monitored for 10 min. Data represent mean±SEM. *P<0.05 2-tailed unpaired t test. (Continued)
Figure 1 Continued. C, Active Rap1 (Rap1-GTP) was selectively precipitated from the lysates of platelets isolated from WT or 12-LOX−/− mice incubated with vehicle control or 10 μmol/L DGLA (n= 3–4 mice) before stimulation with increasing concentrations of PAR4-AP (50, 100, and 200 μmol/L). Active Rap1 was normalized to the total amount of Rap1 in each sample, and each bar graph represents a percent-age of vehicle control for each PAR4-AP concentration. Data represent mean±SEM. **P<0.01, ***P<0.001 2-tailed unpaired t test.

D, Representative images of laser-induced injury of the cremaster arterioles, platelet (green), and fibrin (red) accumulation monitored in real-time to assess thrombus growth in the WT vehicle control (n=3 mice, 10–15 thrombi per mouse), DGLA-treated group (n=3 mice, 10–15 thrombi per mouse), 12-LOX−/− vehicle control (n=3 mice, 10–15 thrombi per mouse), and 12-LOX−/− treated with DGLA (n=3 mice, 10–15 thrombi per mouse). Scale bar, 40 μm. Mean fluorescence intensity (MFI) of platelet and fibrin accumulation at the site of injury were recorded over time in WT (E) and 12-LOX−/− mice. Data represent mean±SEM; 2-way ANOVA. NS indicates not significant; and RFI, relative fluorescence intensity.

12-LOX Oxylipin, 12(S)-HETrE, Inhibits Platelet Aggregation and Thrombus Growth

To confirm that 12(S)-HETrE was the 12-LOX product of DGLA mediating the inhibitory effects observed in Figure 1, washed platelets from either WT or 12-LOX−/− mice were treated with 12(S)-HETrE followed by stimulation with either PAR4-AP or collagen. Notably, 12(S)-HETrE (25 μmol/L) inhibited the aggregation of platelets from WT and 12-LOX−/− mice similar in response to PAR4-AP or collagen (Figure 1B and 1A). As expected, no decrease in collagen- or PAR4-AP-mediated platelet aggregation was observed in either WT or 12-LOX−/− platelets pretreated with 12(S)-hydroxy-5Z,8Z,10E,14Z-eicosatetraenoic acid (12-HETE), the prothrombotic 12-LOX–derived oxylipin of AA, compared with vehicle control. In addition, incubation of platelets with 12(S)-hydroxy-5Z,8Z,10E,14Z,17Z-eicosapentaenoic acid, a 12-LOX–derived oxylipin of eicosapentaenoic acid with no known effects on aggregation,16,23,24 did not inhibit collagen- or PAR4-AP–mediated aggregation in platelets from either WT or 12-LOX−/− mice.

To determine whether 12(S)-HETrE inhibited intracellular signaling, the activation of Rap1 was measured in PAR4-AP–stimulated platelets in the presence of 12(S)-HETrE or vehicle control. 12(S)-HETrE suppressed Rap1 activation compared with vehicle control in platelets from either WT or 12-LOX−/− mice (Figure 2C). Thus, 12(S)-HETrE was able to inhibit platelet aggregation and Rap1 activity independent of 12-LOX expression.

Although 12(S)-HETrE significantly attenuated platelet activation, it remained unclear whether 12(S)-HETrE could directly inhibit platelet thrombus formation in vivo. To evaluate the effects of 12(S)-HETrE on thrombus formation, the size and kinetics of the growing arterial thrombus were assessed after laser-induced injury of the cremaster muscle arterioles in WT and 12-LOX−/− mice treated with vehicle control or 6 mg/kg of 12(S)-HETrE (Figure 1D and 2D). After injury, platelets and fibrin were observed to rapidly accumulate at the injured arteriole wall in WT control mice (Figure 2D; Movie I in the online-only Data Supplement). In contrast, WT mice treated with 12(S)-HETrE had significantly smaller and less stable thrombi in response to laser injury as assessed by both platelet and fibrin accumulation (Figure 2D and 2E; Movie V in the online-only Data Supplement). 12-LOX−/− mice treated with vehicle control exhibited a significant decrease in thrombus formation (platelet and fibrin accumulation) (Figure 2D and 2F; Movie III in the online-only Data Supplement) compared with WT control mice after injury (Figure 2D and 2E; Movie I in the online-only Data Supplement). In addition, 12-LOX−/− mice treated with 12(S)-HETrE exhibited significant inhibition of platelet accumulation compared with 12-LOX−/− alone (Figure 2F; Movie VI in the online-only Data Supplement). However, no difference in fibrin accumulation was observed between vehicle control and 12(S)-HETrE treatment of 12-LOX−/−.

DGLA-Induced Oxylipin Production

Endogenously, only minute amounts of DGLA metabolites are produced by COX-1 (prostaglandin E1 and thromboxane B1) or 12-LOX (12(S)-HETrE) because of the low abundance of DGLA in the platelet plasma membrane.26 To determine whether the exogenous addition of DGLA (10 μmol/L) increases the production of 12-LOX and COX-1 metabolites, the lipid releasate from platelets stimulated with PAR4-AP in the presence of vehicle control or DGLA was measured by liquid chromatography/tandem mass spectrometry (LC/MS/ MS). As expected, the amount of DGLA-dependent COX-1 and 12-LOX oxylipins was significantly potentiated in the DGLA-treated group compared with the DMSO control group (Figure 3A and 3B). The amount of AA-dependent metabolites from either 12-LOX (12(S)-HETE) or COX-1 (thromboxane B2 or prostaglandin E2) was unaltered in platelets incubated with DGLA (Figure 3A and 3B), supporting 12-LOX being in excess such that competition for the substrate is not necessary. This is a reasonable presumption based on previously published work showing that in the human platelet transcriptome, the mRNA for 12-LOX, ALOX12, is expressed in the top 8% of all transcripts in the platelet.26
Figure 2. 12(S)-hydroxy-8Z,10E,14Z-eicosatrienoic acid (12(S)-HETrE) inhibits platelet aggregation and thrombus formation. Representative tracings and combined aggregation data of washed platelets from (A) wild-type (WT; n=4) or (B) 12-lipoxygenase (12-LOX−/−) (n=4) mice pretreated with 25 μmol/L 12-LOX oxylipins (12(S)-HETrE, 12(S)-hydroxy-5Z,8Z,10E,14Z-eicosatetraenoic acid [12-HETE], or 12(S)-hydroxy-5Z,8Z,10E,14Z,17Z-eicosapentaenoic acid [12(S)-HEPE]) for 10 min before stimulation with an EC80 concentration of (Continued)
Figure 2 Continued. protease-activated receptor-4-activating peptide (PAR4-AP; WT 100 μmol/L; 12-LOX−/− 200 μmol/L) or collagen (WT 5 μg/mL; 12-LOX−/− 2 or 5 μg/mL) in an aggregometer. Data represent mean±SEM. *P<0.05 2-tailed unpaired t test. C, Active Rap1 (Rap1-GTP) was selectively precipitated from the lysates of platelets isolated from WT or 12-LOX−/− mice incubated with vehicle control or 25 μmol/L 12(S)-HETE (n=3–4 mice) before stimulation with increasing concentrations of PAR4-AP (50, 100, and 200 μmol/L). Active Rap1 was normalized to the total amount of Rap1 in each sample, and each bar graph represents a percentage of vehicle control for each PAR4-AP concentration. Data represent mean±SEM. *P<0.05, **P<0.01, ***P<0.001 2-tailed unpaired t test. D, Representative images of laser-induced injury of the cremaster arterioles, platelet (green), and fibrin (red) accumulation monitored in real-time to assess thrombi growth in the WT vehicle control (n=3 mice, 10–15 thrombi per mouse), 12(S)-HETE-treated group (n=3 mice, 10–15 thrombi per mouse, 10–15 thrombi per mouse), 12-LOX−/− vehicle control (n=3–4 mice, 10–15 thrombi per mouse), and 12-LOX−/− treated with 12(S)-HETE (n=3 mice, 10–15 thrombi per mouse). 12-LOX−/− vehicle control data are the same set as 12-LOX−/− vehicle control used for 12-LOX−/− 10–15 thrombi per mouse), 12-LOX−/− vehicle control (n=3–4 mice, 10–15 thrombi per mouse), and 12-LOX−/− treated with 12(S)-HETE (n=3 mice, 10–15 thrombi per mouse). 12-LOX−/− vehicle control data are the same set as 12-LOX−/− vehicle control used for 12-LOX−/− 10–15 thrombi per mouse). Mean fluorescence intensity platelet and fibrin accumulation at the site of injury were recorded over time in (E) WT and (F) 12-LOX−/− mice. Data represent means±SEM; 2-way ANOVA. NS indicates not significant; and RFI, relative fluorescence intensity.

12(S)-HETE Does Not Disrupt Hemostasis

Because 12(S)-HETE potently attenuated platelet accumulation in the laser-induced cremaster arteriole injury model of thrombosis, it is possible that 12(S)-HETE also alters hemostasis resulting in increased bleeding. To determine whether 12(S)-HETE treatment resulted in an increased bleeding diathesis, 2 hemostatic models were used to assess the impact of 12(S)-HETE on bleeding. Initially, the tail-bleeding time assay was utilized to determine the effects of 12(S)-HETE on primary hemostasis. 12(S)-HETE–treated mice showed no significant difference in tail-bleeding time compared with the control mice after excision of the distal segment (5 mm) of the tail (Figure 4A). To confirm this assay was accurately reporting bleeding risk, heparin-treated mice were also assayed for bleeding time and observed to have a severe bleeding diathesis (data not shown). A second hemostatic model was used to confirm hemostasis was not significantly altered after treatment with 12(S)-HETE. This model involved arteriole puncture of the cremaster muscle induced by severe laser injury to monitor the cessation time of red blood cell leakage from the punctured arteriole wall (Figure 4B; Movies VII and VIII in the online-only Data Supplement). No significant difference in the duration of red blood cell leakage was observed between 12(S)-HETE and control-treated mice. In both the control and 12(S)-HETE–treated mice, a stable, nonocclusive clot formed in response to laser puncture of the vessel wall, resulting in cessation of red blood cell leakage from the vessel. Both distinct hemostatic models suggested that 12(S)-HETE did not disrupt hemostasis.

12(S)-HETE Inhibits Platelets in a Gαs-Linked G-Protein–Coupled Receptor–Dependent Manner

COX-derived oxylipins that inhibit platelet function primarily exert their inhibition through the activation of a G-protein–coupled receptor (GPCR) coupled to Gαs, resulting in adenyl cyclase (AC) activation and the generation of cAMP.26–34 To determine whether the DGLA-derived 12-LOX oxylipin 12(S)-HETE could be regulating platelet reactivity in a similar manner, cAMP formation was measured in washed human platelets stimulated with 12(S)-HETE or 12(S)-hydroperoxy-8Z,10E,14Z-eicosatrienoic acid (12(S)-HPETE), a peroxided, labile precursor of 12(S)-HETE. After a 1-minute stimulation with 12(S)-HETE or 12(S)-HPETE, human platelets exhibited a significant increase in the level of intracellular cAMP compared with vehicle-treated (DMSO) platelets (Figure 5A). As expected, platelets stimulated with forskolin, a direct activator of AC, also showed an increase in cAMP levels. 12(S)-HETE–induced cAMP production was supportive of 12(S)-HETE inhibiting platelets through the activation of AC. To assess whether 12(S)-HETE inhibited platelet aggregation in an AC-dependent manner, platelets were pretreated with SQ 22536, an AC inhibitor, before incubation with 12(S)-HETE or iloprost, a prostacyclin receptor agonist known to signal through AC.36–37 Iloprost and 12(S)-HETE were unable to inhibit PAR4-AP–mediated platelet aggregation in platelets pretreated with SQ 22536 (Figure 5B), supporting an AC-dependent mechanism of platelet inhibition by 12(S)-HETE.

The cAMP-activated kinase, PKA, phosphorylates multiple proteins in platelets including vasodilator-stimulated...
phosphoprotein (VASP). Because serine 157 (S157) in VASP is a known PKA substrate, VASP phosphorylation was used as a surrogate readout for PKA activation. Washed human platelets treated with DGLA or its 12-LOX metabolites (12(S)-HETrE or 12(S)-HpETrE) for 1 minute had enhanced VASP phosphorylation compared with DMSO-treated platelets (Figure 5C). As expected, forskolin-treated platelets also had an increase in VASP phosphorylation. These data demonstrate that the

Figure 4. Hemostasis is not affected by 12(S)-hydroxy-8Z,10E,14Z-eicosatrienoic acid (12(S)-HETrE). Mice were retro-orbitally injected with DMSO or 12(S)-HETrE dissolved in saline before tail-bleeding. A, Mean tail-bleeding time of control (n=12) or 12(S)-HETrE (n=13)–treated mice is denoted by the horizontal line. Arterial hemostasis induced by laser-induced puncturing of the cremaster muscle arterioles was performed to assess the kinetics of hemostatic plug formation. B, Representative images of hemostatic plug formation, composed of platelet (green) and fibrin (red) were acquired over time. Blue arrows denote the site of vessel rupture and leakage of red blood cells (RBCs). C, Time to form hemostatic plug in control (n=7) and 12(S)-HETrE (n=6) mice as assessed by RBC leakage. Data represent mean±SEM; 2-tailed unpaired t test.

Figure 5. 12(S)-hydroxy-8Z,10E,14Z-eicosatrienoic acid (12(S)-HETrE) activates adenylyl cyclase–mediated signaling in platelets. A, Mass spectrometry quantification of cAMP was performed on lysed washed human platelets (n=5) incubated with DMSO, 12(S)-HETrE (25 μmol/L), 12(S)-hydroperoxy-8Z,10E,14Z-eicosatrienoic acid (12(S)-HpETrE; 25 μmol/L), or forskolin (0.5 μmol/L) for 1 min. B, Washed human platelets (n=4) were pretreated with an adenylyl cyclase inhibitor, SQ22536 (25 μmol/L), or DMSO for 20 min and then incubated with 12(S)-HETrE (7.5–25 μmol/L) or iloprost (0.2 to 0.4 nmol/L) for 1 min before stimulation. Platelet aggregation induced by an EC80 concentration of protease-activated receptor-4-activating peptide (PAR4-AP; 35–50 μmol/L) was measured for 10 min. Representative tracings of aggregation are shown on the left, and bar graphs of the final aggregation of 4 independent experiments are shown on the right. C, Vasodilator-stimulated phosphoprotein (VASP) phosphorylation was measured by Western blot analysis on lysates from washed human platelets (n=8) incubated with DMSO, dihomo-γ-linolenic acid (DGLA; 10 μmol/L), 12(S)-HETrE (25 μmol/L), 12(S)-hydroperoxy-8Z,10E,14Z-eicosatrienoic acid (12(S)-HpETrE; 25 μmol/L), or forskolin (0.5 μmol/L) for 1 min using antibodies specific for phospho-VASP (p157 VASP) or total VASP. Phosphorylated VASP was normalized to total VASP or 12(S)-HpETrE for 1 minute had enhanced VASP phosphorylation compared with DMSO-treated platelets (Figure 5C). As expected, forskolin-treated platelets also had an increase in VASP phosphorylation. These data demonstrate that the
cAMP produced in platelets after exposure to 12(S)-HETE is capable of eliciting physiological effects.

The activation of a GPCR coupled to G\(\alpha\) leads to the dissociation of GDP and the subsequent binding of GTP to G\(\alpha\), initiating a well-established signaling cascade resulting in increases in cAMP levels through the activation of AC.\(^{39,40}\) Because 12(S)-HETE was shown to induce cAMP formation and inhibit platelet activation in an AC-dependent manner, we sought to determine whether 12(S)-HETE could activate G\(\alpha\). Activation of G\(\alpha\) was assessed by measuring the incorporation of the radiolabeled, nonhydrolyzable analog, [\(^{35}\)S]GTP\(\gamma\)S, to G\(\alpha\) immunoprecipitated from isolated human platelet membranes after treatment with vehicle control (DMSO), 12(S)-HETE, 12(S)-HpETE, or iloprost. Treatment of human platelet membranes with 12(S)-HETE, 12(S)-HpETE, and iloprost elicited a significant increase in [\(^{35}\)S]GTP\(\gamma\)S binding to immunoprecipitated G\(\alpha\) compared with platelet membranes incubated with DMSO (Figure 5D). Activation of PAR4, a receptor that is known to selectively activate G\(\alpha\)q, was assessed by measuring the incorporation of [\(^{35}\)S]GTP\(\gamma\)S binding confirming the selectivity for G\(\alpha\)q activation in the assay.

**Discussion**

Advances in antiplatelet therapy have significantly decreased the risk for morbidity and mortality because of thrombosis. However, even with the current standard-of-care antiplatelet therapies available, myocardial infarction and stroke caused by occlusive thrombotic events remain one of the primary causes of morbidity and mortality globally. Therefore, identification of novel therapies remains an unmet clinical need. One potential approach to reduce thrombus formation is the dietary intake of DGLA, a naturally occurring ω-6 PUFA, which has been shown to attenuate platelet aggregation ex vivo.\(^{44}\) However, to date, the mechanism by which this inhibition is regulated has not been elucidated. Recently, our laboratory identified a 12-LOX–derived oxylipin of DGLA, 12(S)-HETE, which inhibits human platelet activation.\(^{16}\) In the current study, we sought to determine the relative contribution of 12-LOX–derived metabolites in DGLA-mediated inhibition of platelet function and thrombosis in vivo. In contrast to the previously reported dependence of DGLA-mediated inhibition of platelet function on COX-derived metabolites,\(^{5,6,11}\) we show here that DGLA, but not 12(S)-HETE, was unable to inhibit platelet aggregation in 12-LOX\(^{-/-}\) mice suggesting that 12-LOX plays a key role in facilitating DGLA’s antiplatelet effects.

In mice, 12-LOX was required for DGLA to impinge on platelet activation (Figures 1 and 2), suggesting that 12-LOX metabolites are responsible for the predominance of DGLA inhibitory effects in mice. Interestingly, human platelets stimulated with DGLA had higher VASP phosphorylation than those treated with 12(S)-HETE (Figure 5C), indicating that other DGLA metabolites, such as COX-1–derived oxylipins (Figure 3B), may also contribute to VASP phosphorylation. This observation is supported by previous data that COX-1 or 12-LOX inhibitors partially suppresses the ability of DGLA to inhibit human platelet aggregation.\(^{16}\)

The proposed inhibitory effect mediated through 12-LOX seems paradoxical based on previous work in our laboratory and others showing that 12-LOX is a positive mediator of platelet function.\(^{17,21,42,43}\) However, because of the fact that 12-LOX is an enzyme whose function is to add an oxygen to a free fatty acid to produce a bioactive oxylipin, it is reasonable to conclude from the data presented here and elsewhere\(^{7,8}\) that the substrate for 12-LOX is the determining factor in its effect on platelets and ultimately thrombosis. This conclusion is supported by work in COX that shows that oxidation of AA results in a prothrombotic milieu of oxylipins,\(^{44,45}\) whereas other substrates such as DGLA can result in production of antiplatelet oxylipins.\(^{1,6,8,46}\)

The potent inhibition of thrombus formation by both DGLA and 12(S)-HETE raises the potential that 12(S)-HETE will cause excessive bleeding similar to other antiplatelet agents.\(^{52–49}\) Two hemostatic assays, the tail-bleeding assay, and a second hemostatic model recently developed, the laser-induced cremaster arteriole puncture model, were used in this study to determine whether the DGLA metabolite 12(S)-HETE prolonged bleeding after vascular injury. The mouse tail-bleeding assay for hemostasis is a physiological model, involving the measurement of cessation of bleeding after the excision of 5 mm of the distal tail, showed no prolonged bleed times in mice treated with 12(S)-HETE compared with the control. However, prolonged bleeding was observed in tail-bleeding after heparin administration (data not shown), supporting the sensitivity of the tail-bleeding assay approach. A second hemostatic assessment, the laser-induced cremaster arteriole puncture model was performed by using the laser to puncture a hole through the cremaster.
arterial wall followed by measurement of the time required for fibrin and platelet plug formation in the mice. Similar to the tail-bleeding assay, no difference in bleeding times between control and 12(S)-HETErE–treated mice was observed, supporting the hypothesis that 12(S)-HETErE exerts an antithrombotic effect, while at the same time maintaining primary hemostasis. These data support 12(S)-HETErE either given directly or formed through ω-6 DGLA supplementation as a viable approach for prevention of thrombosis without creating a bleeding diathesis.

12(S)-HETErE was shown here to directly activate a yet to be determined Gt<sub>c</sub>-coupled GPCR. Direct addition of 12(S)-HETErE to purified platelet membranes was shown to increase the binding of [35S]GTP<sub>S</sub> to the Gt<sub>c</sub>-subunit resulting in cAMP formation, activation of PKA, and phosphorylation of VASP (Figure 6). Further studies are required to identify whether 12(S)-HETErE is binding to a novel receptor or a previously characterized Gt<sub>c</sub>-coupled GPCR on the human and mouse platelet. Identification of this receptor will be essential to determine whether the mechanism of action elicited by 12(S)-HETErE is mediated through binding the GPCR in an allosteric or orthosteric manner and how this binding compares with other previously identified oxylipins shown to signal the platelet in a Gt<sub>c</sub>-dependent manner.

The discovery of 12(S)-HETErE regulation of platelet function at both the ex vivo and in vivo levels and the delineation of the mechanism of action through the Gt<sub>c</sub>-coupled GPCR establishes this oxylipin as an important eicosanoid in platelet biology. Beyond the platelet, it is also possible that 12(S)-HETErE plays an important role in the regulatory function of other vascular cells similar to what is observed with other key oxylipins produced in the platelet, such as prostacyclin, prostaglandin, PGD, and thromboxane. Furthermore, this study describes how an omega-6 essential PUFA, such as DGLA, can be used to alter the platelet signalosome to attenuate unwanted platelet activation and occlusive thrombus formation common in atherothrombotic diseases often leading to myocardial infarction and stroke. Additional studies will seek to understand more fully how this newly discovered regulatory pathway limits platelet function and thrombotic risk while minimizing the risk of bleeding. This study fully supports future efforts to target the 12(S)-HETErE pathway through the identification of the 12(S)-HETErE receptor as a first-in-class antiplatelet therapeutic with minimal risk of bleeding.

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Disclosures

None.

References

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Highlights

- DGLA, an ω-6 polyunsaturated fatty acid inhibits platelet function and thrombosis in a 12-LOX-dependent manner.
- 12-LOX-derived metabolite of DGLA, 12(S)-HETrE, inhibits platelet activation and thrombosis independent of 12-LOX.
- 12(S)-HETrE does not alter hemostasis.
- 12(S)-HETrE inhibits platelet activation through a Gαs signaling pathway.

References:

12(S)-HETE, a 12-Lipoxygenase Oxylin of Dihomo-\(\gamma\)-Linolenic Acid, Inhibits Thrombosis via G\(\alpha_s\) Signaling in Platelets

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Materials and Methods

Preparation of washed murine platelets. The 12-LOX null C57BL/6 (12-LOX<sup>−/−</sup>) mice, generated using homozygous breeding pairs, and wild-type (WT) C57BL/6 (12-LOX<sup>+/+</sup>) mice were purchased from Jackson Laboratory. The Institutional Animal Care and Use Committees (IACUC) at the University of Michigan and Thomas Jefferson University approved all experimental procedures in this study involving mice. Blood was drawn from the inferior vena cava of anesthetized 8-12 week old mice with a 21-gauge needle attached to a 1 mL syringe containing 100 µl of 3.8% sodium citrate. Mouse blood was diluted with equal volumes of Tyrode’s buffer and centrifuged at 200 g. Platelet-rich plasma (PRP) was transferred to a tube containing 10x acid citrate dextrose solution (ACD) and apyrase (.02 U/mL) and centrifuged at 2000 g. Platelet count was adjusted to 3x10<sup>8</sup> platelets/mL with Tyrode’s buffer for all studies.

Preparation of washed human platelets. University of Michigan and Thomas Jefferson University Institutional Review Boards approved all protocols involving human subjects. In accordance with the Declaration of Helsinki, written informed consent was obtained from healthy donors prior to each blood draw. Blood was collected as previously described<sup>1</sup> and washed platelets were resuspended in Tyrode’s buffer at 3x10<sup>8</sup> platelets/mL, unless otherwise specified.

Extraction, liquid chromatography, and mass spectrometry (LC/MS) quantification of oxylipins. Washed human platelets were treated with agonists in the presence of vehicle control or specific fatty acids and acidified with 40 µL of 1M hydrochloric acid to pH of 3. Extraction standards were then added to the samples: 20ng each of PGE<sub>1</sub>-d<sub>4</sub> and 13(S)HODE-d<sub>4</sub>. Oxylipids were extracted with dichloromethane, reduced with trimethylphosphite, and dried under nitrogen gas. The samples were reconstituted in methanol containing 10 ng of each of the internal standards and transferred to MS vials with inserts. Chromatographic separation was performed on a Dionex UltiMate 3000 UHPLC with a C<sub>18</sub> column (1.7 µm, 150 mm x 2.1mm). The autosampler was held at 4°C and injection volume was 30 µL. Mobile phase A consisted of water with 0.1% (v/v) formic acid and mobile phase B was acetonitrile with 0.1% formic acid. Flow rate was 0.400mL/min. The initial condition (30% B) was maintained for 2.33 minutes. Mobile phase B was then ramped to 65% over 28.67 minutes, held at 65% for 1 minute, ramped to 100% over 0.1min, held at 100% for 7 minutes, and finally returned to 30% to equilibrate for 7 minutes. The chromatography system was coupled to a Velos Pro linear ion trap for mass analysis. Analytes were ionized via heated electrospray ionization with -4.0kV spray voltage, 60, 10, and 0 arbitrary units for sheath, auxiliary, and sweep gas, respectively. All analyses were performed in negative ionization mode at normal resolution setting. MS<sup>2</sup> was performed in both a data dependent and targeted manner, simultaneously from ions detected in a full scan with a mass-to-charge ratio (m/z) range of 200-400. The targeted MS<sup>2</sup> were selected for the parent ions of the analytes that were contained in a mass list -TxB<sub>1</sub>(m/z = 371.2), TxB<sub>2</sub>(m/z = 369.2), TxB<sub>2</sub>-d<sub>4</sub>(m/z= 373.3), PGE<sub>1</sub>(m/z = 353.2), PGE<sub>1</sub>-d<sub>4</sub>(m/z = 357.2), PGE<sub>2</sub>-d<sub>4</sub>(m/z = 355.2), 12HETE(m/z = 319.2) , 12HETE-d<sub>4</sub>(m/z = 321.2), 12(S)HETE-d<sub>8</sub>(m/z = 327.3), 13(S)HODE-d<sub>4</sub>(m/z = 299.3).

Mass spectrometry analysis, normalization, and relative quantitation of oxylipins. Retention times and fragmentation patterns of all analytes were determined with lipid standards prior to sample analyses. Total ion counts (TIC) of the m/z transitions of each analyte peak were used for relative quantitation. The m/z transitions of all analytes and standards were as follows: TxB<sub>1</sub>(m/z = 371.2→197), TxB<sub>2</sub>(m/z = 369.2→195), TxB<sub>2</sub>-d<sub>4</sub>(m/z= 373.3→199), PGE<sub>1</sub>(m/z = 353.2→317), PGE<sub>2</sub>(m/z = 351.2→315), PGE<sub>1</sub>-d<sub>4</sub>(m/z = 357.2→321), PGE<sub>2</sub>-d<sub>4</sub>(m/z = 355.2→319), 12HETE(m/z = 319.2→179) , 12HETE-d<sub>4</sub>(m/z =321.2→181), 12(S)HETE-d<sub>8</sub>
Platelet aggregation. A lumi-aggregometer (Chrono-log model 700D) was used to measure platelet aggregation under stirring conditions (1100 rpm) at 37°C. Prior to agonist stimulation platelets were incubated with a preselected PUFA or oxylipin for 10 minutes.

Rap1 activation. Washed platelets were incubated with PUFAs or oxylipins for 10 minutes prior to stimulation with PAR4-AP. Following 1 minute of stimulation, platelets were lysed and Rap1 activity was assessed by western blot analysis as previously described1.

Laser-induced cremaster arteriole thrombosis model. WT or 12-LOX−/− mice (12 weeks of age) were anesthetized by intraperitoneal injection of ketamine/xylazine (100 mg/kg) prior to the exposure of the cremaster muscle arterioles under a dissecting microscope with constant perfusion of preheated bicarbonate-buffered saline2-4. Anti-platelet (DyLight 488 anti-GPIb, 1 µg/g) and anti-fibrin (Alexa Fluor 647, 0.3 µg/g) antibodies were administered via jugular vein catheter prior to intravital microscopy. DGLA or DMSO were dissolved in a formulation of 5% DMSO and 45% PEG300 in sterile 1X PBS and intravenously injected into mice 10 minutes prior to induction of thrombosis. 12-HETRe or equal volume of DMSO was intravenously injected into mice 10 minutes prior to induction of thrombosis. Multiple thrombi were induced in the arterioles (30-50 µm diameter) in each mouse by a laser ablation system (Ablate! photo-ablation system; 3I). Images of thrombus formation were acquired in real-time under 63X water-immersion objective with a Zeiss Axio Examiner Z1 fluorescent microscope equipped with solid laser launch system and high-speed sCMOS camera. All captured images were analyzed on Slidebook.

Tail bleeding assay. Mice were anesthetized with ketamine/xyzaline and placed on a heating pad in prone position, the tip of the tail (5 mm) was excised with a sterile scalpel, and the tails were immediately immersed into isotonic saline solution (.9%) warmed to 37°C. Bleeding time was assessed until cessation of blood flow from the tail for 1 minute.

Cremaster muscle arterial puncture model of hemostasis. Mice were anesthetized, tail vein injected with anti-platelet and anti-fibrin antibodies and their cremaster muscle arterioles were prepared as described above. A high intensity laser pulse from the laser ablation system was used to puncture a hole in the cremaster muscle arteriole wall as visualized by red blood cell (RBC) leakage from the vessel. Images of RBCs leakage and hemostatic plug formation were acquired in real-time with a fluorescent microscope as described above. Arterial bleeding time was defined as the time from laser pulse injury until cessation of RBC leakage from the vessel.

Liquid chromatography extraction and mass spectrometry (LC/MS) quantification of cAMP. Washed human platelets were treated with the specific ligand or vehicle control for 1 min at room temperature, and quenched with an equal volume of ice-cold 2x platelet lysis buffer containing protease and phosphatase inhibitors. Lysed platelet samples were centrifuged to pellet cytoskeleton and the supernatant was stored in -80°C.

To prepare the sample for LC/MS injection, 200 µL of the supernatant were spiked with 400 pg of adenosine-3’;5’-cyclic-13C5 monophosphate (13C5-cAMP) and 600 µL of LC/MS grade
acetonitrile. The sample was vortexed and centrifuged for 10 min. The supernatants were dried and reconstituted in 200 µL of LC/MS grade water, centrifuged for 5 min at 4°C, and used for LC/MS injection.

The separation and detection of cAMP was performed on a Waters ACQUITY UPLC system equipped with a Xevo Triple Quadrupole Mass Spectrometer (TQ-S MS/MS). The extraction/purification of cAMP from the sample was carried out using a Waters HSS C18 column (1.8 µm, 2.1*100mm). The sample (10 µL) was then injected.

The mass spectrometer was operated with an ESI source in positive mode. The electrospray voltage was 3.9kv. The source temperature was maintained at 150°C, and the desolvation temperature was 525 °C with a nitrogen desolvation gas flow of 1000L/h. cAMP was quantitated using ^13C5-cAMP as the internal standard. For cAMP monitoring in the MRM mode with a collision energy of 22 volts, 330/136 mass transition was used, and for ^13C5-cAMP, 335/136 was used.

**VASP phosphorylation in human platelets.** Washed platelets were treated with PUFA or PUFA metabolite for one minute then directly lysed in 5x Laemmeli sample buffer. The samples were boiled for five minutes and assayed by western blot for total and phospho-VASP (serine 157).

**Membrane preparation and[^35S]GTPγS binding.** PRP was isolated from whole blood as described above and incubated with 1 mM aspirin for 1 hour at 37°C. Washed platelets were prepared as previously described. The platelets were flash frozen with liquid nitrogen and resuspended in cold detergent-free TME buffer (50 mM Tris-HCl, pH 7.5, 20 mM MgCl₂, 2 mM EDTA, and 100 mM NaCl) with 1 µM GDP and protease and phosphatase inhibitors to lyse the platelets. The lysed platelets were centrifuged at 1500 g at 4°C for 5 min. The supernatant was collected and centrifuged at 100,000 g for 30 min at 4°C. The pellet membranes were resuspended in TME buffer with 1 µM GDP and stored at -80°C prior to use.

Fatty acid metabolites, agonists, or DMSO and[^35S]GTPγS (10 nM) were added to platelet membranes and the tubes were immediately transferred to a 30°C water bath shaker for 20 min. The reaction was terminated by the addition of ice-cold IP buffer (50 mM Tris-HCl, pH 7.5, 20 mM MgCl₂, 150 mM NaCl, .5% Nonidet P-40, .33% aprotinin, .1 mM GDP, and .1 mM GTP). The samples were pre-cleared with Protein A agarose beads and normal rabbit IgG and aliquoted equally into two tubes containing either normal rabbit IgG or a Gas antibody that had been conjugated to Protein A agarose beads. The samples were incubated on a nutator for 1 hr at 4°C and washed 4 times with IP buffer and 1 time in TME buffer. The samples were boiled in .5% SDS for 30 sec and the supernatants were collected following brief centrifugation. The supernatants were analyzed in 8 mL of scintillation fluid. The background counts for the normal rabbit IgG (50-200 cpm) for each sample were subtracted from the anti-Gα immunoprecipitated samples prior to analyzing the data.

**Statistics.** Unpaired, paired two-tailed student t-tests, and two-way analysis of variance (ANOVA) were used to compare between experimental groups with Prism 6.0 software (GraphPad). Where appropriate the statistical test used is contained in the figure legend. Data represents mean values +/- SEM.

**Online supplemental material.** Supplementary videos 1-7 show representative real-time recording of laser injury model of thrombosis in WT and 12-LOX⁻/⁻ mice treated with vehicle control, DGLA, or 12-HETE. Supplementary videos 8 and 9 show representative hemostatic model of cremaster muscle arterial rupture in WT mice treated with vehicle control or 12-HETE.
References


Supplemental Data

Supplemental Video 1. Laser-induced thrombus formation in cremaster muscle arteriole in WT mouse treated with DMSO vehicle control. Visual representation of platelet (green) and fibrin (red) accumulation following laser injury to the cremaster arteriole of WT mice treated with DMSO.

Supplemental Video 2. Laser-induced thrombus formation in cremaster muscle arteriole in WT mouse treated with DGLA. Visual representation of platelet (green) and fibrin (red) accumulation following laser injury to the cremaster arteriole of WT mice treated with DGLA (50 mg/kg).

Supplemental Video 3. Laser-induced thrombus formation in cremaster muscle arteriole in 12-LOX−/− mouse treated with DMSO vehicle control. Visual representation of platelet (green) and fibrin (red) accumulation following laser injury to the cremaster arteriole of 12-LOX−/− mice treated with vehicle control.

Supplemental Video 4. Laser-induced thrombus formation in cremaster muscle arteriole in 12-LOX−/− mouse treated with DGLA. Visual representation of platelet (green) and fibrin (red) accumulation following laser injury to the cremaster arteriole of 12-LOX−/− mice treated with DGLA.

Supplemental Video 5. 12-HETrE attenuation of the cremaster muscle arteriole thrombus formation in WT mouse following laser injury. Visual representation of platelet (green) and fibrin (red) accumulation following laser injury to the cremaster arteriole of WT mice treated with 12-HETrE.

Supplemental Video 6. 12-HETrE attenuation of the cremaster muscle arteriole thrombus formation in 12-LOX−/− mouse following laser injury. Visual representation of platelet (green) and fibrin (red) accumulation following laser injury to the cremaster arteriole of 12-LOX−/− mice treated with 12-HETrE.

Supplemental Video 7. Formation of a hemostatic plug at the site of cremaster arteriole vessel wall rupture by laser in WT control mice treated with DMSO. DyLight488 anti-GPlb and Alexa Fluor 647 anti-fibrin were intravenously administered into WT mice. High intensity laser pulse was induced on the cremaster arteriole to rupture the vessel wall that resulted in bleeding as visualized by red blood cell leakage. A visual representation of hemostatic plug formation composed of platelets (green) and fibrin (red) resulted in cessation of red blood cell leakage in WT mouse treated with vehicle control. Images were recorded at 63X water immersion objective.

Supplemental Video 8. Formation of hemostatic plug at the site of cremaster arteriole vessel wall rupture by laser in WT mouse treated with 12-HETrE. Visual representation of hemostatic plug formation composed of platelets (green) and fibrin (red) resulting in cessation of red blood cell leakage in WT mouse treated with 12-HETrE (6 mg/kg).