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## 12-HETrE, a 12-LOX oxylipin of DGLA, inhibits thrombosis via $G\alpha_s$ signaling in platelets

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### Abstract

**Objective**—Dietary supplementation with polyunsaturated fatty acids (PUFAs) has been widely used for primary and secondary prevention of CVD in individuals at risk; however, the cardioprotective benefits of PUFAs remain controversial due to lack of mechanistic and *in vivo* evidence. We present direct evidence that an omega-6 PUFA, dihomo- $\gamma$ -linolenic acid (DGLA), exhibits *in vivo* cardioprotection through 12-lipoxygenase (12-LOX) oxidation of DGLA to its reduced oxidized lipid form, 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<sup>-/-</sup>). Similarly, wild-type mice treated with DGLA were able to reduce thrombus growth (platelet and fibrin accumulation) following laser-induced injury of the arteriole of the cremaster muscle, but not 12-LOX<sup>-/-</sup> 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, two hemostatic models, tail bleeding and arteriole rupture of the cremaster muscle, showed no alteration in hemostasis following 12(S)-HETrE treatment. Finally, the mechanism for 12(S)-HETrE protection was shown to be mediated via a  $G\alpha_s$ -linked GPCR pathway in human platelets.

**Conclusions**—This study provides the first direct evidence that an omega-6 PUFA, 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  $G\alpha_s$ -linked GPCR-dependent manner.

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**Disclosure:** None

## Keywords

thrombosis; platelets; lipoxygenase; 12-HETrE

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## Introduction

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 P2Y<sub>12</sub> receptor antagonists has significantly decreased morbidity and mortality associated with these debilitating conditions<sup>1, 2</sup>. Nonetheless, the fact that the rate of ischemic events still remains high in individuals on antiplatelet agents<sup>3</sup> 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- $\gamma$ -linolenic acid (DGLA), an  $\omega$ -6 polyunsaturated fatty acid (PUFA), has been suggested to play a role in inhibiting platelet aggregation *ex vivo*<sup>4-6</sup>. While 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<sup>7</sup>. In platelets, DGLA can be oxidized by cyclooxygenase-1 (COX-1) or platelet 12-lipoxygenase (12-LOX)<sup>8</sup> following its release from the phospholipid bilayer predominately through the actions of cytoplasmic phospholipase A<sub>2</sub><sup>9, 10</sup>. While both COX-1 and 12-LOX are able to oxidize DGLA to their respective metabolites, the relative contributions of these oxylipin products to the inhibitory effects of DGLA on platelet function remain unclear. Further, the antiplatelet effects of DGLA have been primarily attributed to the COX-1-derived metabolites that have been shown to inhibit platelet activation<sup>4-6, 11</sup>. However, the DGLA derived products of COX-1 (TXA<sub>1</sub> and PGE<sub>1</sub>) are liable and produced in low amounts in platelets<sup>12-15</sup>. Additionally, a recent study demonstrated for the first time that 12(S)-hydroxyicosatetraenoic acid (12-HETrE), the 12-LOX-derived oxylipin of DGLA, exhibits a potential antiplatelet effect *ex vivo*<sup>16</sup>. Hence, it is important to delineate if 12-LOX is required for DGLA-mediated inhibition of platelet function *in vivo* and whether DGLA and 12-HETrE play an essential role in regulation of thrombosis.

This study showed for the first time that an  $\omega$ -6 PUFA, DGLA, inhibited platelet thrombus formation *in vivo* following an insult to the vessel wall. Interestingly, DGLA was unable to inhibit thrombus formation in 12-LOX<sup>-/-</sup> mice suggesting the antithrombotic effects of DGLA were mediated by 12-LOX. The 12-LOX-derived oxylipin of DGLA, 12-HETrE, potently impaired thrombus formation following vessel injury irrespective of 12-LOX expression. Importantly, the antithrombotic effects of 12-HETrE did not disrupt primary hemostasis or result in increased bleeding. Finally, the antiplatelet effect of 12-HETrE was delineated here for the first time and shown to inhibit platelet function through activation of

the  $G_{\alpha_s}$  signaling pathway leading to formation of cAMP and PKA activation in the platelet. Hence, these findings are the first to demonstrate an antithrombotic role of DGLA 12-HETrE 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-HETrE, potently inhibited platelet aggregation<sup>16</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 to platelets from WT mice<sup>17</sup>, 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 to 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 due to the modification of the lipid membrane structure thus affecting platelet signaling or activation, other PUFAs including linoleic acid (LA) and AA were used as controls to rule out a lipid-membrane insulating effect in platelet activation<sup>18</sup>. Pretreatment of platelets with either LA or AA had no inhibitory effect on PAR4-AP or collagen-mediated platelet aggregation compared to vehicle alone (Figure 1A and 1B).

To determine if DGLA inhibited platelet aggregation by impinging on intracellular signaling, the activation of Rap1, a common signaling effector required for integrin  $\alpha_{IIb}\beta_3$  activation (Shattil et al., 2010; Shattil and Newman, 2004) 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). Since DGLA was unable to inhibit platelet aggregation in 12-LOX<sup>-/-</sup> mice, we assessed if 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 employed to examine thrombus formation (platelet and fibrin) in WT mice<sup>22</sup> (Figure 1D–F). Mice were intravenously injected with either vehicle control (DMSO) or 50 mg/kg of DGLA 10 minutes prior to the initiation of thrombosis by laser injury. Following 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; and Supplementary video 1). Simultaneously, fibrin formation can be seen at the base of the developing thrombus of vehicle control treated WT mice (Figure 1D and 1E; and Supplementary video 1). WT mice treated with DGLA showed a significant reduction in platelet, but not fibrin accumulation (Figure 1D and 1E; and Supplementary video 2).

Figure 1A–C suggests a requirement for 12-LOX in DGLA-mediated inhibition of platelet activation *ex vivo*. To determine if this observation translates to an attenuation of platelet reactivity *in vivo*, thrombus formation was measured in 12-LOX<sup>-/-</sup> mice following laser-induced injury of the cremaster arteriole (Figure 1D and 1F). As previously reported, platelets from 12-LOX<sup>-/-</sup> mice exhibited a bleeding diathesis compared to WT mice as determined by the tail-bleeding assay<sup>17</sup>. Therefore, it would be expected that the 12-LOX<sup>-/-</sup> mice show a significant attenuation of thrombus following injury compared to the WT (Figure 1D and 1F). Interestingly, the accumulation of platelet and fibrin in thrombi between DGLA-treated 12-LOX<sup>-/-</sup> and vehicle control did not differ (Figure 1D and 1F; and Supplementary videos 3 and 4) supporting the *ex vivo* observation that 12-LOX is required to mediate DGLA-dependent inhibition of platelet function as well as thrombosis.

### The 12-LOX oxylipin, 12-HETrE, inhibits platelet aggregation and thrombus growth

To confirm that 12-HETrE was the 12-LOX product of DGLA mediating the inhibitory effects observed in figure 1, washed platelets from either WT or 12-LOX<sup>-/-</sup> mice were treated with 12-HETrE followed by stimulation with either PAR4-AP or collagen. Notably, 12-HETrE (25  $\mu$ M) inhibited the aggregation of platelets from WT and 12-LOX<sup>-/-</sup> mice similarly in response to PAR4-AP or collagen (Figure 2A and 1B). As expected, no decrease in collagen- or PAR4-AP-mediated platelet aggregation was observed in either WT or 12-LOX<sup>-/-</sup> platelets pre-treated with 12-HETE, the pro-thrombotic 12-LOX-derived oxylipin of AA, compared to vehicle control. Additionally, incubation of platelets with 12-HEPE, a 12-LOX-derived oxylipin of eicosapentaenoic acid (EPA) with no known effects on aggregation<sup>16, 23, 24</sup>, did not inhibit collagen- or PAR4-AP-induced aggregation in platelets from either WT or 12-LOX<sup>-/-</sup> mice.

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

Although 12-HETrE significantly attenuated platelet activation, it remained unclear if 12-HETrE could directly inhibit platelet thrombus formation *in vivo*. To evaluate the effects of

12-HETrE on thrombus formation, the size and kinetics of the growing arterial thrombus were assessed following laser-induced injury of the cremaster muscle arterioles in WT and 12-LOX<sup>-/-</sup> mice treated with vehicle control or 6 mg/kg of 12-HETrE (Figure 1D and 2D). Following injury, platelets and fibrin were observed to rapidly accumulate at the injured arteriole wall in WT control mice (Figure 2D and Supplementary video 1). In contrast, WT mice treated with 12-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; Supplementary video 5). 12-LOX<sup>-/-</sup> mice treated with vehicle control exhibited a significant decrease in thrombus formation (platelet and fibrin accumulation) (Figure 2D and 2F; Supplementary video 3) compared to WT control mice following injury (Figure 2D and 2E; Supplementary video 1). Additionally, 12-LOX<sup>-/-</sup> mice treated with 12-HETrE exhibited significant inhibition of platelet accumulation compared to 12-LOX<sup>-/-</sup> alone (Figure 2F; Supplementary video 6). However, no difference in fibrin accumulation was observed between vehicle control and 12-HETrE treatment of 12-LOX<sup>-/-</sup>.

### DGLA-induced oxylipin production

Endogenously, only minute amounts of DGLA metabolites are produced by COX-1 (PGE<sub>1</sub> and TxB<sub>1</sub>) or 12-LOX (12-HETrE) due to the low abundance of DGLA in the platelet plasma membrane<sup>25</sup>. To determine if the exogenous addition of DGLA (10 μM) 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 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 to the DMSO control group (Figure 3A and 3B). The amount of AA-dependent metabolites from either 12-LOX (12-HETE) or COX-1 (TxB<sub>2</sub> or PGE<sub>2</sub>) 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<sup>26</sup>.

### 12-HETrE does not disrupt hemostasis

Since 12-HETrE potentially attenuated platelet accumulation in the laser-induced cremaster arteriole injury model of thrombosis, it is possible 12-HETrE also alters hemostasis resulting in increased bleeding. To determine if 12-HETrE treatment resulted in an increased bleeding diathesis, two hemostatic models were used to assess the impact of 12-HETrE on bleeding. First, the tail-bleeding time assay was utilized to determine the effects of 12-HETrE on primary hemostasis. 12-HETrE-treated mice showed no significant difference in tail bleeding time compared to the control mice following 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 following treatment with 12-HETrE. This model involved arteriole puncture of the cremaster muscle induced by severe laser injury<sup>27</sup> in order to monitor the cessation time of RBC leakage from the punctured arteriole wall (Figure 4B; Supplementary videos 7 and 8). No significant difference in the duration of RBC leakage

was observed between 12-HETrE and control treated mice. In both the control and 12-HETrE-treated mice, a stable, non-occlusive clot formed in response to laser puncture of the vessel wall, resulting in cessation of RBC leakage from the vessel. Both distinct hemostatic models suggested 12-HETrE did not disrupt hemostasis.

### 12-HETrE inhibits platelets in a $G\alpha_s$ -linked GPCR-dependent manner

COX-derived oxylipins that inhibit platelet function primarily exert their inhibition through the activation of a GPCR coupled to  $G\alpha_s$  resulting in adenylyl cyclase (AC) activation<sup>28, 29</sup> and the generation of cAMP<sup>30-34</sup>. To determine if the DGLA-derived 12-LOX oxylipin 12-HETrE could be regulating platelet reactivity in a similar manner, cAMP formation was measured in washed human platelets stimulated with 12-HETrE or 12-HpETrE, a peroxidated, labile precursor of 12-HETrE. Following a 1 minute stimulation with 12-HETrE or 12-HpETrE<sup>16</sup>, human platelets exhibited a significant increase in the level of intracellular cAMP compared to 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-HETrE-induced cAMP production was supportive of 12-HETrE inhibiting platelets through the activation of AC. To assess if 12-HETrE inhibited platelet aggregation in an AC-dependent manner, platelets were pre-treated with SQ 22536, an AC inhibitor<sup>35</sup> prior to incubation with 12-HETrE or iloprost, a prostacyclin receptor agonist known to signal through AC<sup>36, 37</sup>. Iloprost and 12-HETrE were unable to inhibit PAR4-AP-mediated platelet aggregation in platelets pre-treated with SQ 22536 (Figure 5B), supporting an AC-dependent mechanism of platelet inhibition by 12-HETrE.

The cAMP activated kinase, protein kinase A (PKA), phosphorylates multiple proteins in platelets including vasodilator-stimulated phosphoprotein (VASP). Since serine 157 (S157) in VASP is a known PKA substrate<sup>38</sup>, VASP phosphorylation was used as a surrogate readout for PKA activation. Washed human platelets treated with DGLA or its 12-LOX metabolites (12-HETrE, or 12-HpETrE) for 1 minute had enhanced VASP phosphorylation compared to DMSO treated platelets (Figure 5C). As expected, forskolin treated platelets also had an increase in VASP phosphorylation. This data demonstrates that the cAMP produced in platelets following exposure to 12-HETrE is capable of eliciting physiological effects.

The activation of a GPCR coupled to  $G\alpha_s$  leads to the dissociation of GDP and the subsequent binding of GTP to  $G\alpha_s$  initiating a well-established signaling cascade resulting in increases in cAMP levels through the activation of AC<sup>39, 40</sup>. Since 12-HETrE was shown to induce cAMP formation and inhibit platelet activation in an AC-dependent manner, we sought to determine if 12-HETrE could activate  $G\alpha_s$ . Activation of  $G\alpha_s$  was assessed by measuring the incorporation of the radiolabeled, non-hydrolyzable analog, [<sup>35</sup>S]GTP $\gamma$ S, to  $G\alpha_s$ , immunoprecipitated from isolated human platelet membranes following treatment<sup>41</sup> with vehicle control (DMSO), 12-HETrE, 12-HpETrE, PAR4-AP, or iloprost. Treatment of human platelet membranes with 12-HETrE, 12-HpETrE, and iloprost elicited a significant increase in [<sup>35</sup>S]GTP $\gamma$ S binding to immunoprecipitated  $G\alpha_s$  compared to platelet membranes incubated with DMSO (Figure 5D). Activation of PAR4, a receptor that is

known to selectively activate  $G_q$  and  $G_{12/13}$ , showed no [ $^{35}\text{S}$ ]GTP $\gamma$ S binding confirming the selectivity for  $G_{\alpha_s}$  activation in the assay.

## Discussion

Advances in antiplatelet therapy have significantly decreased the risk for morbidity and mortality due to thrombosis. However, even with the current standard-of-care antiplatelet therapies available, myocardial infarction and stroke due to occlusive thrombotic events remains 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  $\omega$ -6 PUFA, which has been shown to attenuate platelet aggregation *ex vivo*<sup>4-6</sup>. However, to date the mechanism by which this inhibition is regulated and the ability of DGLA to inhibit thrombus formation *in vivo* have not been elucidated. Recently, our lab identified a 12-LOX-derived oxylin of DGLA, 12-HETrE, which inhibits human platelet activation<sup>16</sup>. 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<sup>4-6, 11</sup>, we show here that DGLA, but not 12-HETrE, was unable to inhibit platelet aggregation in 12-LOX<sup>-/-</sup> 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-HETrE (Figure 5C), indicating other DGLA metabolites, such as COX-1 derived oxylin (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<sup>16</sup>.

The proposed inhibitory effect mediated through 12-LOX appears paradoxical based on previous work in our lab and others showing that 12-LOX is a positive mediator of platelet function<sup>17, 21, 42, 43</sup>. However, due to the fact that 12-LOX is an enzyme whose function is to add an oxygen to a free fatty acid in order to produce a bioactive oxylin, it is reasonable to conclude from the data presented here and elsewhere<sup>5, 8</sup> 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 which shows that oxidation of AA results in a pro-thrombotic milieu of oxylin<sup>44, 45</sup> while other substrates such as DGLA can result in production of anti-thrombotic oxylin<sup>4-6, 8, 46</sup>.

The potent inhibition of thrombus formation by both DGLA and 12-HETrE, raises the potential that 12-HETrE will cause excessive bleeding similar to other antiplatelet agents<sup>47-49</sup>. 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 if the DGLA metabolite 12-HETrE prolonged bleeding following



vascular injury. The mouse tail-bleeding assay for hemostasis is a physiological model, involving the measurement of cessation of bleeding following the excision of 5 mm of the distal tail, showed no prolonged bleed times in mice treated with 12-HETrE compared to the control. However, prolonged bleeding was observed in tail-bleeding following 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-HETrE-treated mice was observed, supporting the hypothesis that 12-HETrE exerts an antithrombotic effect while at the same time maintaining primary hemostasis. These data support 12-HETrE either given directly or formed through  $\omega$ -6 DGLA supplementation as a viable approach for prevention of thrombosis without creating a bleeding diathesis.

12-HETrE was shown here to directly activate a yet to be determined  $G\alpha_s$ -coupled GPCR. Direct addition of 12-HETrE to purified platelet membranes was shown to increase the binding of [ $^{35}$ S]GTP $\gamma$ S, the hydrolysis-resistant GTP analog, to the  $G\alpha_s$ -subunit resulting in cAMP formation, activation of PKA, and phosphorylation of VASP (Figure 6). Further studies are required to identify if 12-HETrE is binding to a novel receptor or a previously characterized  $G\alpha_s$ -coupled GPCR on the human and mouse platelet. Identification of this receptor will be essential to determine if the mechanism of action elicited by 12-HETrE is mediated through binding the GPCR in an allosteric or orthosteric manner and how this binding compares to other previously identified oxylipins shown to signal the platelet in a  $G\alpha_s$ -dependent manner.

The discovery of 12-HETrE regulation of platelet function at both the *ex vivo* and *in vivo* levels and the delineation of the mechanism of action through the  $G\alpha_s$ -coupled GPCR establishes this oxylipin as an important eicosanoid in platelet biology. Beyond the platelet, it is also possible that 12-HETrE 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, PGE, PGD, and thromboxane. Further, this study describes for the first time how an omega-6 essential PUFA such as DGLA, can be used to alter the platelet signalosome in order to attenuate unwanted platelet activation and occlusive thrombus formation common in atherothrombotic diseases often leading to myocardial infarction and stroke. Future 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-HETrE pathway through the identification of the 12-HETrE receptor as a first-in-class antiplatelet therapeutic with minimal risk of bleeding.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## Nonstandard Abbreviations and Acronyms

<b>12-LOX</b>	12-Lipoxygenase
<b>COX-1</b>	cyclooxygenase-1
<b>PUFA</b>	polyunsaturated fatty acid
<b>DGLA</b>	dihomo- $\gamma$ -linolenic acid
<b>AA</b>	arachidonic acid
<b>LA</b>	linoleic acid
<b>EPA</b>	eicosapentaenoic acid
<b>12-HETrE</b>	12(S)-hydroxy-8Z,10E,14Z-eicosatetrienoic acid
<b>12-HpETrE</b>	12(S)-hydroperoxy-8Z,10E,14Z-eicosatetrienoic acid
<b>12-HETE</b>	12(S)-hydroxy-5Z,8Z,10E,14Z-eicosatetraenoic acid
<b>12-HEPE</b>	12(S)-hydroxy-5Z,8Z,10E,14Z,17Z-eicosapentaenoic acid
<b>TxB<sub>1</sub></b>	thromboxane B <sub>1</sub>
<b>TxB<sub>2</sub></b>	thromboxane B <sub>2</sub>
<b>PGE<sub>1</sub></b>	prostaglandin E <sub>1</sub>
<b>PGE<sub>2</sub></b>	prostaglandin E <sub>2</sub>
<b>PAR4-AP</b>	protease-activated receptor-4-activating peptide
<b>RBC</b>	red blood cell
<b>GPCR</b>	G-protein-coupled receptor
<b>AC</b>	adenylyl cyclase
<b>PKA</b>	protein kinase A
<b>G<math>\alpha</math><sub>s</sub></b>	Gs $\alpha$ subunit
<b>cAMP</b>	cyclic adenosine monophosphate
<b>VASP</b>	vasodilator-stimulated phosphoprotein

[<sup>35</sup>S]GTPγS[<sup>35</sup>S]guanosine 5′O-[γ-thio]triphosphate

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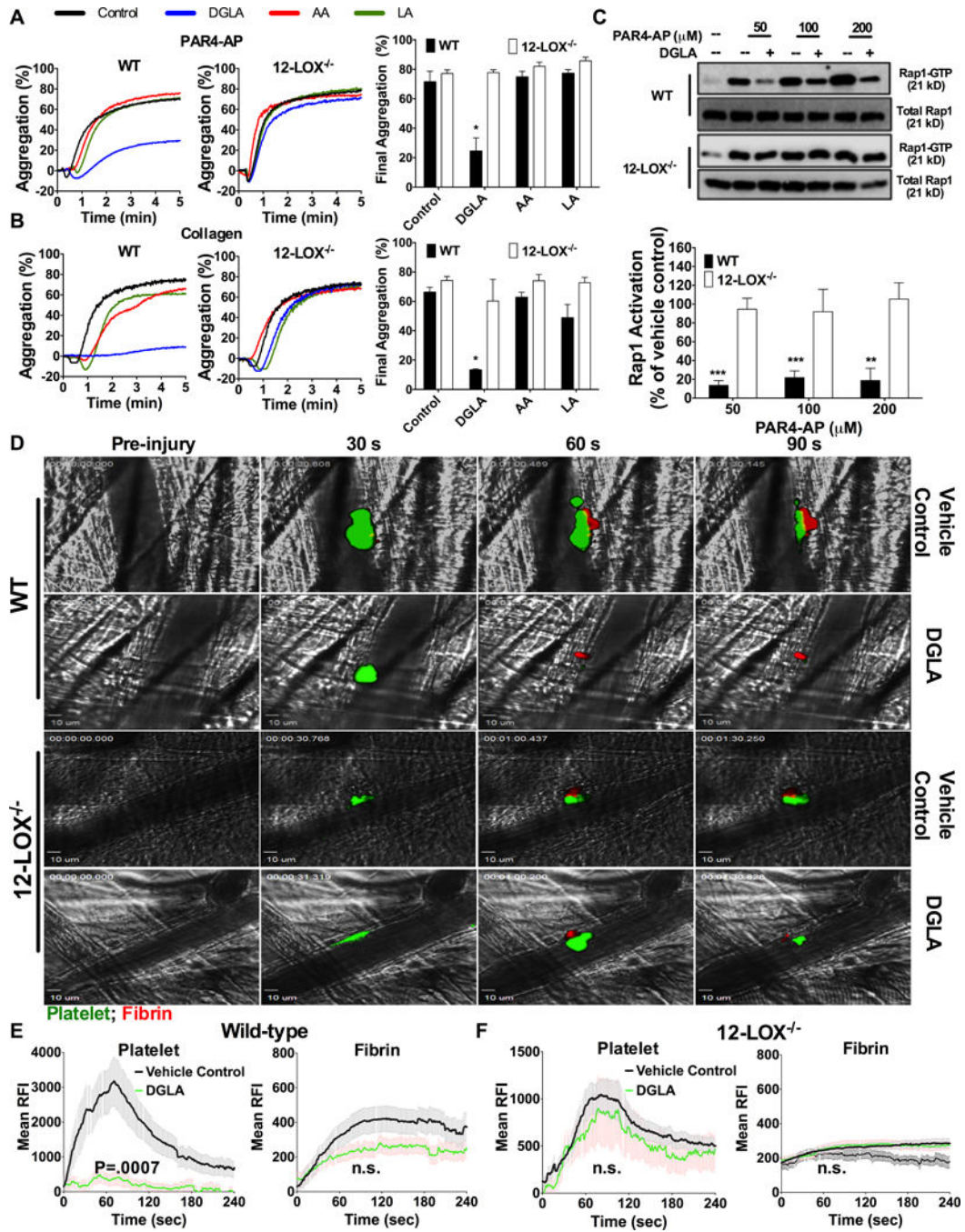
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### Highlights

- Dihomo- $\gamma$ -linolenic (DGLA), an  $\omega$ -6 polyunsaturated fatty acid inhibits platelet function and thrombosis in a 12-lipoxygenase (12-LOX) dependent manner
- 12-LOX-derived metabolite of DGLA, 12-HETrE, inhibits platelet activation and thrombosis independent of 12-LOX
- 12-HETrE does not alter hemostasis
- 12-HETrE inhibits platelet activation through a  $G\alpha_s$  signaling pathway

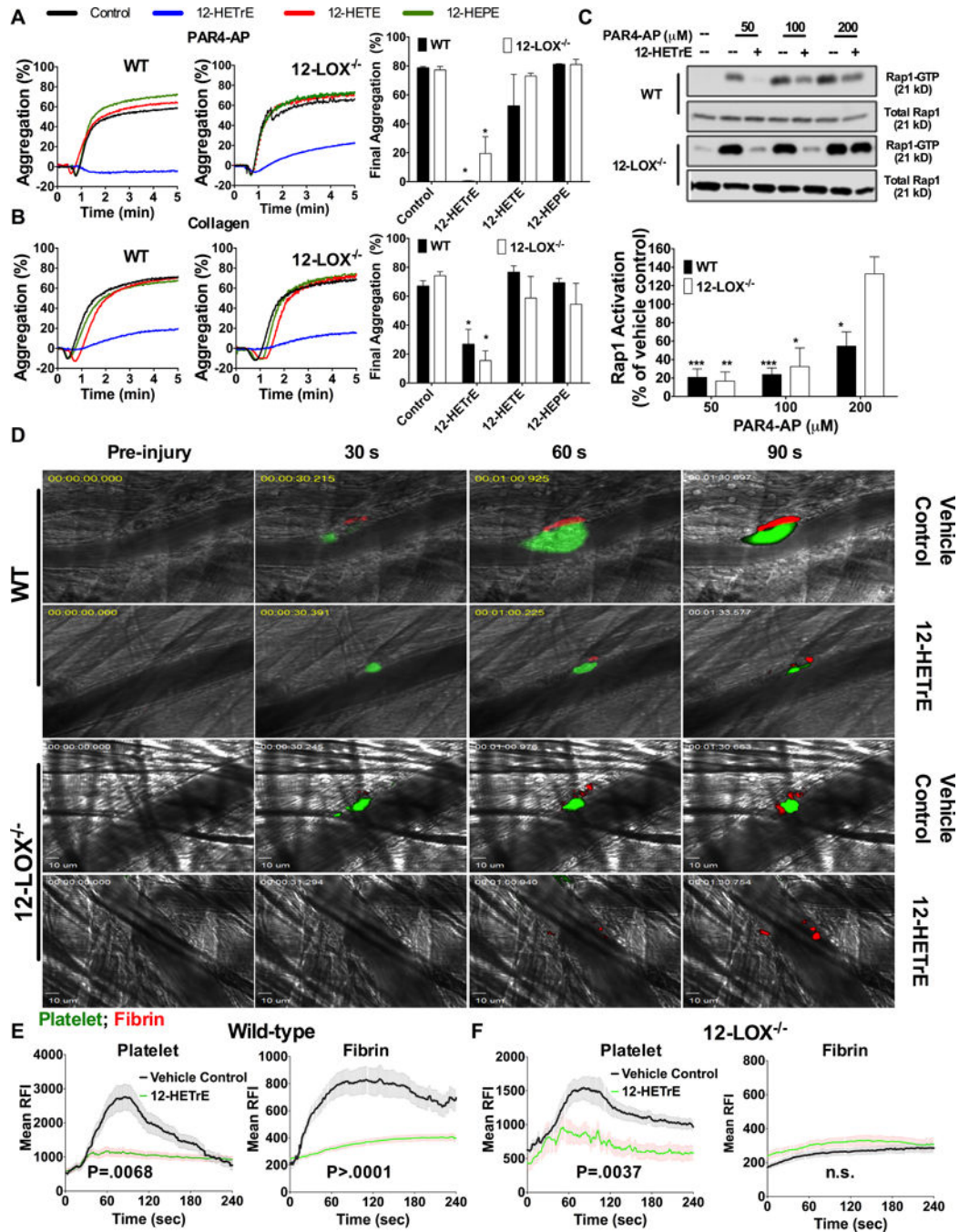


**Figure 1. 12-LOX is required for DGLA inhibition of platelet aggregation and thrombus formation**

Representative tracings and combined aggregation data of (A) WT ( $n=4$ ) or (B) 12-LOX<sup>-/-</sup> ( $n=4$ ) platelets stimulated with EC<sub>80</sub> concentration of PAR4-AP (WT 100 μM; 12-LOX<sup>-/-</sup> 200 μM) or collagen (WT 5 μg/mL; 12-LOX<sup>-/-</sup> 2 or 5 μg/mL) in the presence or absence of 10 μM of PUFAs (DGLA, AA, or LA). Aggregation was monitored for 10 minutes. Data represents mean ± SEM. \* $P < .05$  two-tailed unpaired t-test. (C) Active Rap1 (Rap1-GTP) was selectively precipitated from the lysates of platelets isolated from WT or 12-LOX<sup>-/-</sup> mice incubated with vehicle control or 10 μM DGLA ( $n=3$  to 4 mice) prior to stimulation

with increasing concentrations of PAR4-AP (50, 100, and 200  $\mu\text{M}$ ). 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  $\pm$  SEM.  $**P < .01$ ,  $***P < .001$  two-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<sup>-/-</sup> vehicle control ( $n=3$  mice, 10–15 thrombi per mouse), and 12-LOX<sup>-/-</sup> treated with DGLA ( $n=3$  mice, 10–15 thrombi per mouse). Scale bar: 40  $\mu\text{m}$ . Mean fluorescence intensity (MFI) of platelet and fibrin accumulation at the site of injury were recorded over time in (E) WT and (F) 12-LOX<sup>-/-</sup> mice. Data represents mean  $\pm$  SEM; two-way ANOVA.

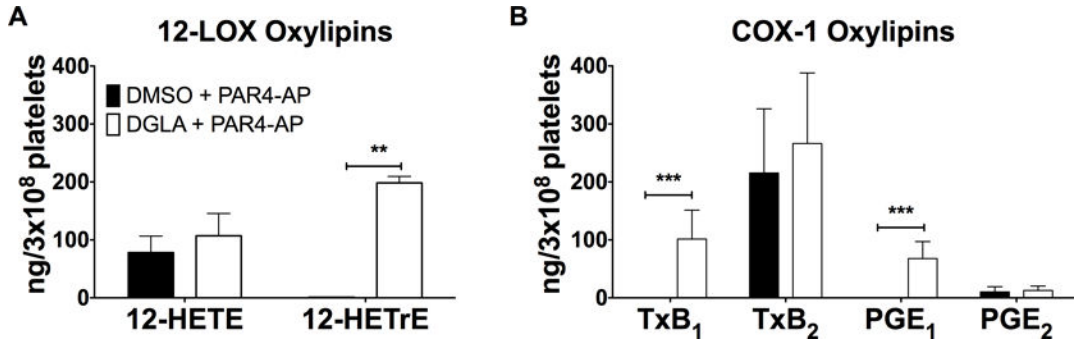




**Figure 2. 12-HETrE inhibits platelet aggregation and thrombus formation**

Representative tracings and combined aggregation data of washed platelets from (A) WT ( $n=4$ ) or (B) 12-LOX<sup>-/-</sup> ( $n=4$ ) mice pre-treated with 25 μM 12-LOX oxylipins (12-HETrE, 12-HETE, or 12-HEPE) for 10 minutes prior to stimulation with an EC<sub>80</sub> concentration of PAR4-AP (WT 100 μM; 12-LOX<sup>-/-</sup> 200 μM) or collagen (WT 5 μg/mL; 12-LOX<sup>-/-</sup> 2 or 5 μg/mL) in an aggregometer. Data represents mean ± SEM. \* $P < .05$  two-tailed unpaired t-test. (C) Active Rap1 (Rap1-GTP) was selectively precipitated from the lysates of platelets isolated from WT or 12-LOX<sup>-/-</sup> mice incubated with vehicle control or 25 μM 12-HETrE

( $n=3$  to 4 mice) prior to stimulation with increasing concentrations of PAR4-AP (50, 100, and 200  $\mu\text{M}$ ). 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  $\pm$  SEM.  $*P<.05$ ,  $**P<.01$ ,  $***P<.001$  two-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-HETrE treated group ( $n=3$  mice, 10–15 thrombi per mouse, 10–15 thrombi per mouse), 12-LOX<sup>-/-</sup> vehicle control ( $n=3$ –4 mice, 10–15 thrombi per mouse), and 12-LOX<sup>-/-</sup> treated with 12-HETrE ( $n=3$  mice, 10–15 thrombi per mouse). 12-LOX<sup>-/-</sup> vehicle control data is the same set as 12-LOX<sup>-/-</sup> vehicle control used for 12-LOX<sup>-/-</sup> DGLA treated comparison in figure 1F. Scale bar: 40  $\mu\text{m}$ . Mean fluorescence intensity (MFI) platelet and fibrin accumulation at the site of injury were recorded over time in (E) WT and (F) 12-LOX<sup>-/-</sup> mice. Data represents mean  $\pm$  SEM; two-way ANOVA.



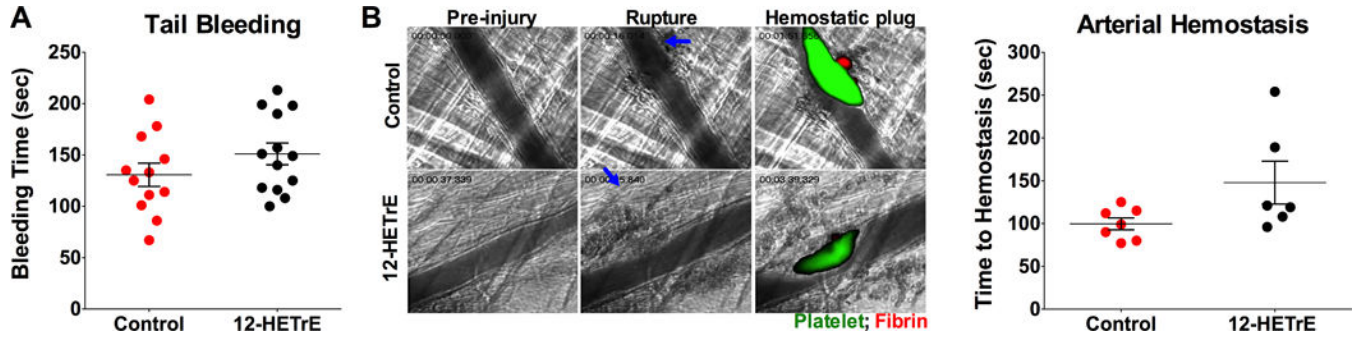
**Figure 3. Exogenous DGLA enhances platelet production of metabolites**  
12-LOX and COX-1 metabolites from washed human platelets ( $n=7$ ) treated with DGLA (10  $\mu$ M) or DMSO for 10 minutes prior to stimulation with PAR4-AP (200  $\mu$ M) were detected using mass spectrometry. Data represents mean  $\pm$  SEM; \*\* $P<.01$ , \*\*\* $P<.001$  two-tailed unpaired t-test.

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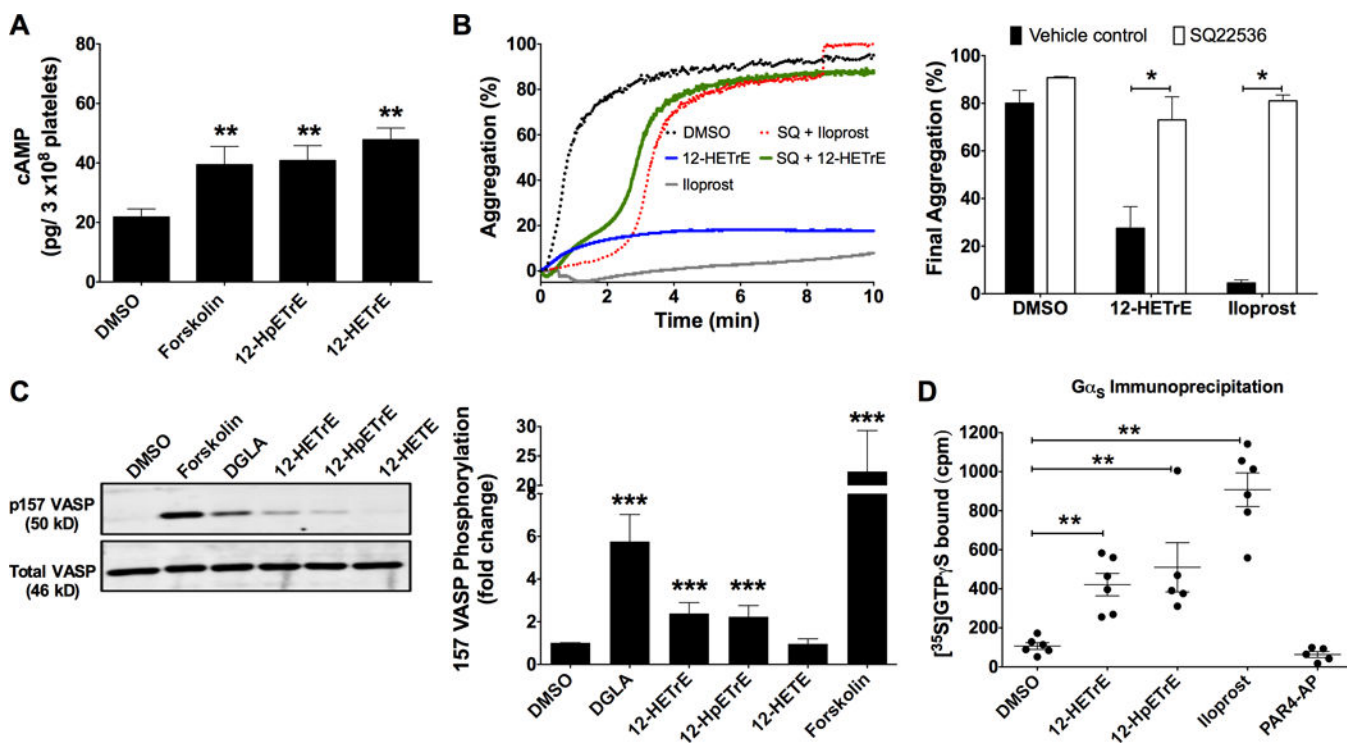
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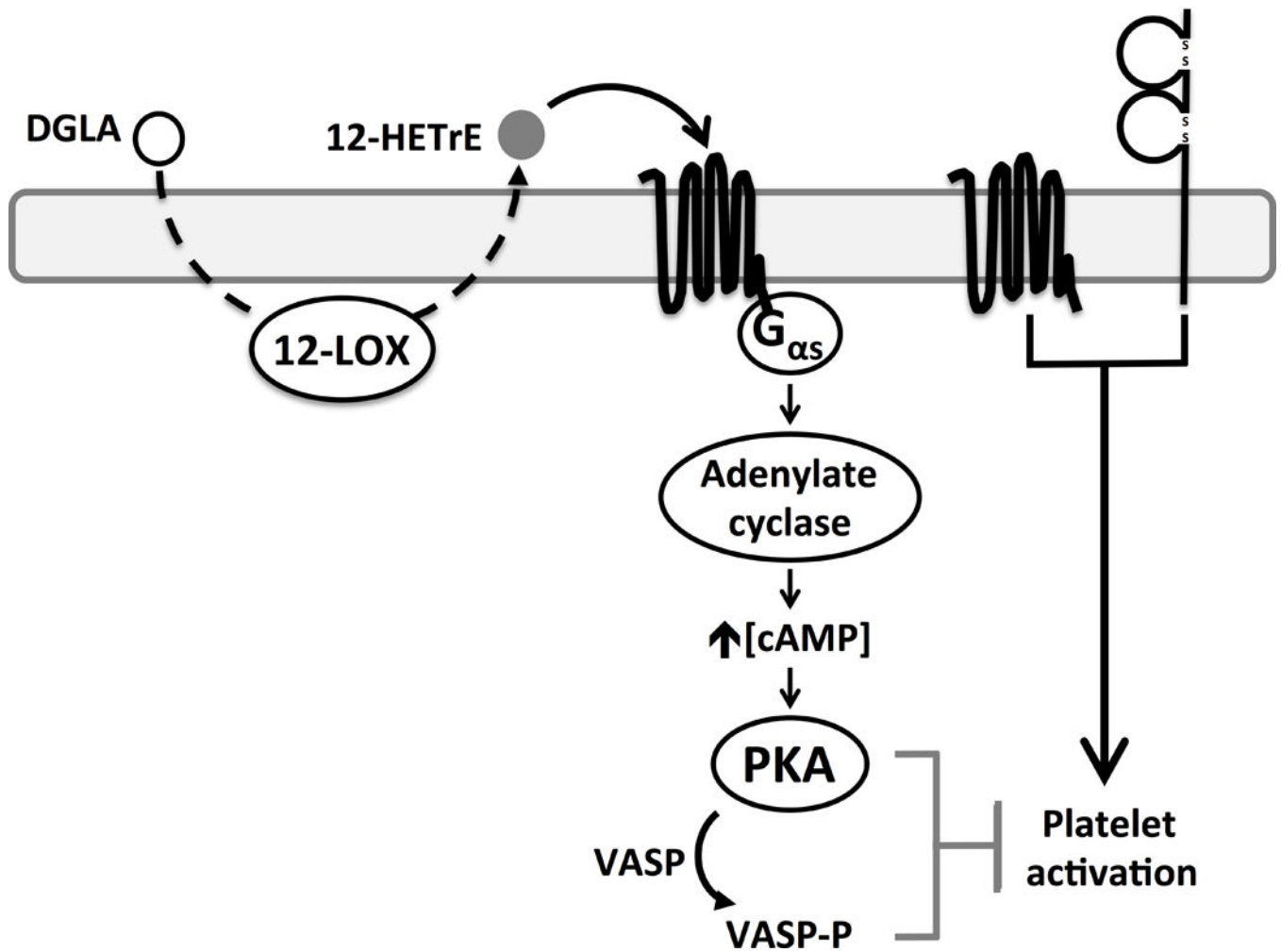


**Figure 4. Hemostasis is not affected by 12-HETrE**  
 Mice were retro-orbitally injected with DMSO or 12-HETrE dissolved in saline prior to tail-bleeding. (A) Mean tail-bleeding time of control ( $n=12$ ) or 12-HETrE ( $n=13$ ) treated mice is denoted by the horizontal line. Arterial hemostasis induced 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 plug (green) and fibrin (red) were acquired over time. Blue arrows denote the site of vessel rupture and leakage of RBCs. (C) Time to form hemostatic plug in control ( $n=7$ ) and 12-HETrE ( $n=6$ ) mice as assessed by RBC leakage. Data represent mean  $\pm$  SEM; two-tailed unpaired t-test.



**Figure 5. 12-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-HETrE (25  $\mu$ M), 12-HpETrE (25  $\mu$ M), or forskolin (.5  $\mu$ M) for 1 minute. (B) Washed human platelets ( $n=4$ ) were pre-treated with an adenylyl cyclase inhibitor, SQ22536 (25  $\mu$ M), or DMSO for 20 minutes and then incubated with 12-HETrE (7.5 to 25  $\mu$ M) or iloprost (.2 to .4 nM) for 1 minute prior to stimulation. Platelet aggregation induced by an EC<sub>80</sub> concentration of PAR4-AP (35 to 50  $\mu$ M) was measured for 10 minutes. 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) VASP phosphorylation was measured by western blot analysis on lysates from washed human platelets ( $n=8$ ) incubated with DMSO, DGLA (10  $\mu$ M), 12-HETrE (25  $\mu$ M), 12-HpETrE (25  $\mu$ M), or forskolin (.5  $\mu$ M) for 1 minute using antibodies specific for phospho-VASP (p157 VASP) or total VASP. Phosphorylated VASP was normalized to total VASP and DMSO for fold change in 157 VASP phosphorylation. (D) G $\alpha_s$  was immunoprecipitated following incubation of human platelet membranes with DMSO, 12-HETrE (25  $\mu$ M), 12-HpETrE (25  $\mu$ M), Iloprost (10  $\mu$ M) or PAR4-AP in the presence of [<sup>35</sup>S]GTP $\gamma$ S. The immunoprecipitates ( $n=6$ ) were then counted and background counts from normal IgG controls were subtracted. Data represent mean  $\pm$  SEM. \*  $P < .05$ , \*\*  $P < .01$ , \*\*\*  $P < .001$  two-tailed unpaired  $t$ -test (A–D).



**Figure 6. Proposed model of 12-HETrE inhibitory signaling in platelets**

Within platelets, 12-lipoxygenase (12-LOX) metabolizes free DGLA into the bioactive lipid, 12-HETrE. 12-HETrE can passively diffuse through the plasma membrane and presumably bind to an unidentified G $\alpha_s$ -coupled receptor in a paracrine or autocrine manner. G $\alpha_s$  activates adenylyl cyclase, which increases the intracellular level of cyclic AMP (cAMP). Elevated cAMP activates protein kinase A (PKA), which phosphorylates a number of proteins, including vasodilator-stimulated phosphoprotein (VASP), leading to platelet inhibition in response to either GPCR- or ITAM-mediated platelet activation.