Networks of enzymatically oxidized membrane lipids support calcium-dependent coagulation factor binding to maintain hemostasis

Sarah N. Lauder,1,2 Keith Allen-Redpath,1,2* David A. Slatter,1,2* Maceler Aldrovandi,1,2 Anne O’Connor,1,2 Daniel Farewell,3 Charles L. Percy,1,2 Jessica E. Molhoek,4 Sirpa Rannikko,5,6 Victoria J. Tyrrell,1,2 Salvatore Ferla,7 Ginger L. Milne,8 Alastair W. Poole,9 Christopher P. Thomas,1,2,7 Samya Obaji,1,2 Philip R. Taylor,1,2 Simon A. Jones,1,2 Phillip G. de Groot,4 Rolf T. Urbanus,4 Solvi Hörkkö,5,6 Stefan Uderhardt,10 Jochen Ackermann,10 P. Vince Jenkins,11 Andrea Brancale,7 Gerhard Krönke,10 Peter W. Collins,1,2† Valerie B. O’Donnell1,2†

Blood coagulation functions as part of the innate immune system by preventing bacterial invasion, and it is critical to stopping blood loss (hemostasis). Coagulation involves the external membrane surface of activated platelets and leukocytes. Using lipidomic, genetic, biochemical, and mathematical modeling approaches, we found that enzymatically oxidized phospholipids (eoxPLs) generated by the activity of leukocyte or platelet lipoxigenases (LOXs) were required for normal hemostasis and promoted coagulation factor activities in a Ca2+- and phosphatidylserine (PS)-dependent manner. In wild-type mice, hydroxyeicosatetraenoic acid–phospholipids (HETE-PLs) enhanced coagulation and restored normal hemostasis in clotting-deficient animals genetically lacking p12-LOX or 12/15-LOX activity. Murine platelets generated 22 eoxPL species, all of which were missing in the absence of p12-LOX. Humans with the thrombotic disorder antiphospholipid syndrome (APS) had statistically significantly increased HETE-PLs in platelets and leukocytes, as well as greater HETE-PL immunoreactivity, than healthy controls. HETE-PLs enhanced membrane binding of the serum protein β2GPI (β2-glycoprotein 1), an event considered central to the autoimmune reactivity responsible for APS symptoms. Correlation network analysis of 47 platelet eoxPL species in platelets from APS and control subjects identified their enzymatic origin and revealed a complex network of regulation, with the abundance of 31 p12-LOX–derived eoxPL molecules substantially increased in APS. In summary, circulating blood cells generate networks of eoxPL molecules, including HETE-PLs, which change membrane properties to enhance blood coagulation and contribute to the excessive clotting and immunoreactivity of patients with APS.

INTRODUCTION

Blood clotting is an essential first step in innate immunity; it is required to prevent bacterial invasion and ensure effective cessation of blood flow (hemostasis) after injury. Excess clotting in the vasculature underlies vascular inflammatory conditions, including myocardial infarction, stroke, pulmonary embolism, and deep vein thrombosis, whereas impaired coagulation contributes to excessive blood loss during surgery and childbirth, a common cause of mortality during childbirth. Thus, a better understanding of the molecular processes underlying coagulation and hemostasis could drive the development of effective new treatments and inform prevention strategies for several major human disorders.

Hemostasis depends on the coagulation cascade, a series of serine proteases and cofactors in plasma. The coagulation cascade is initiated by tissue factor (TF). For clotting to occur, aminophospholipid (aPL) externalization on the surface of activated platelets is required. At the platelet membrane, scramblase leads to the translocation of phosphatidylethanolamine (PE) and phosphatidylserine (PS), providing a negative charge to facilitate calcium binding and factor association (1, 2). Externalization of aPLs alone is only part of the physiological coagulation process, because the rare disorder Scott syndrome presents with an inability to externalize PS and PE, but only a relatively minor bleeding phenotype, unless the patients are experiencing a severe hemo- static challenge (3). This suggests that additional PLs are involved. Activated platelets and leukocytes rapidly generate oxidized PEs and phosphatidylcholines (PCs) through the action of lipoxigenase (LOX) enzymes. LOX-generated PLs are termed hydroxyeicosatetraenoic acid–PLs (HETE-PLs), and these lipids are present at or within the plasma membrane (4–10). HETE-PL positional isomers (in which the oxidized moiety can be located at one of up to six different positions on the fatty acid side chain) are cell type–specific and LOX isoform–dependent: 5-HETE-PLs are generated by human neutrophils, 12-HETE-PLs by platelets, and 15-HETE-PLs by monocytes/ eosinophils. In mice, leukocytes express not only a 12/15-LOX homolog, which generates mainly 12-HETE-PLs, but also small amounts of 15-HETE-PLs (7). We previously identified cyclooxygenase 1 (COX-1)–derived, enzymatically oxidized PLs (eoxPLs) that are generated by platelets that have either prostaglandin E2 (PGE2), prostaglandin D2 (PGD2), or dioxolane A3 (DXA3) (11, 12). Using global lipidomic mass spectrometry (MS), we identified...
103 eoxPL molecular species in thrombin-activated human platelets (13). These data indicated that eoxPL generation is a broader phenomenon than was previously thought. However, the enzymatic origin of most members of this large group remains unknown.

Antiphospholipid syndrome (APS) is an acquired prothrombotic disorder caused by a diverse family of circulating “antiphospholipid” antibodies. These can be directed against PLs, including PE or cardiolipin, or against proteins, such as the PL-binding protein β2-glycoprotein 1 (β2GP1) or other PL-binding proteins. In APS, pathogenic antibodies contribute to thrombotic episodes or pregnancy complications (14–16). An interaction between β2GP1 and negatively charged PLs on the surface of cells is thought to be required for disease development (14, 16). The PLs that provide optimal binding of β2GP1 to membranes are unknown. Given that eoxPLs are generated by isolated blood cells and platelets, contain electronegative hydroxyl groups on their fatty acids, and remain cell-associated after their formation, we explored their generation in APS (4–10).

Here, we used biochemical, genetic, clinical, mathematical, and lipidomic approaches to reveal the procoagulant mechanisms of endogenously generated LOX-derived eoxPLs from platelets and leukocytes in vitro and in vivo. We found that LOX-derived eoxPLs increased the binding of β2GP1 to membranes. We performed correlation network analysis of the 47 most abundant eoxPL species in platelets from a human APS cohort, which revealed several levels of enzymatic regulation. We found that increased amounts of eoxPL species were generated by blood cells from APS patients and that these lipids enhanced immune recognition in disease. Overall, our studies support the idea that hemostasis requires the generation of multiple eoxPL species by platelets and leukocytes, and that their production is chronically increased in human venous thrombotic disease associated with APS.

RESULTS
HETE-PL species stimulate coagulation
Coagulation requires multiple complexes of enzymes and their cofactors. These are termed (i) TF/FVIIa (extrinsic tenase), (ii) FIXa/FVIIa (intrinsic tenase), and (iii) FXa/FVa (prothrombinase) and they are active on PL membranes (17–19).

To support this cascade, plasma membranes externalize aPLs, specifically PS and PE (18–21). PS is required to support coagulation, whereas PE enhances PS-mediated coagulation (1, 2, 22). PS and PE bind coagulation factors FII, FVII, FIX, and FX in a calcium-dependent manner, through the γ-carboxylated glutamic acid (Gla) domains on the coagulation factors (18, 23), whereas FVIII and FV bind PS and PE directly through their C domains (24, 25). To examine the effects of HETE-PLs on coagulation, we incorporated specific HETE-PLs into TF-containing liposomes and added the liposomes to plasma. We monitored coagulation by measuring the production of thrombin. Increasing concentrations of 5-, 12-, or 15-HETE-PE (Fig. 1A) or 5-, 12-, or 15-HETE-PC (Fig. 1B) enhanced coagulation in a concentration-dependent manner.

The accepted model of coagulation is that PS supports the formation of the prothrombinase and intrinsic tenase complexes, and PE enhances the effect of PS (2). PC is thought to have no role in supporting coagulation. To determine the procoagulant mechanisms of HETE-PLs, we monitored thrombin generation varying PS concentrations in the presence of PE (2, 26) or HETE-PE or HETE-PC. In the absence of PS, PE supported low thrombin generation, which increased upon substitution with 15-HETE-PE (Fig. 1C, left graphs). Increasing PS led to dose-dependent increases in thrombin generation, which were similarly increased by HETE-PE (Fig. 1C, left graphs). In contrast, neither PC nor HETE-PC supported thrombin generation without PE and PS (Fig. 1C, right graphs). Increasing PS led to a dose-dependent increase in thrombin generation, with HETE-PC having an enhancing effect (Fig. 1C, right graphs). The enhancing effect of HETE-PC was greater at higher PS concentrations than that for HETE-PE. The data indicated a mechanism by which HETE-PLs enhance PS-dependent coagulation, in a manner similar to, but more potent than, that of PE. The procoagulant action of HETE-PC is highlighted because PC was previously assumed not to support PS in coagulation reactions (2, 26).

Thrombin generation in platelet-poor plasma is also influenced by anticoagulant factors (27). Therefore, we repeated the thrombin generation experiments in a synthetic system using purified proteins (FII, FV, FVII, and FVIII) at physiological concentrations without inhibitors of coagulation. We stimulated thrombin generation with TF-containing liposomes containing specific HETE-PE and HETE-PC species. 15- and 12-HETE-PEs and 15-, 12-, and 5-HETE-PCs statistically significantly increased thrombin generation (Fig. 1D).

Molecular modeling shows bending of HETE with localization of –OH at the membrane surface and association with calcium molecules
A molecular dynamics (MD) simulation was performed to understand how the hydroxyl group of 12-HETE-PC would behave within the membrane. An available preequilibrated dioleoylphosphatidylcholine (DOPC) membrane with 128 molecules was modified to give a composition of 5% 1-stearoyl-2-arachidonoyl-PS (SAPS), 5% 1-stearoyl-2-arachidonoyl-PC (SAPC), 30% 1-stearoyl-2-arachidonoyl-PE (SAPE), 55% DOPC, and 5% 12-HETE-PC. This includes three molecules of PS and HETE-PC per leaflet. On one side, HETE-PCs were placed with HETEs buried in the membrane (in the yellow hydrophobic compartment), whereas on the other side, they were placed in a bent-up configuration, with the –OH close to the polar membrane surface. During the 300-ns simulation, the system remained stable. During the 300-ns simulation, all of the HETE-PCs with HETEs pointing downward changed conformation to place the –OH group at the charged surface of the membrane (blue), whereas those already in that configuration remained stable (Fig. 2, A to D, and movie S1). In this upward-facing position, the –OH could establish a hydrogen bond with the nearby lipid phosphates and, in some cases, with the carboxylic acid group of a neighboring PS (Fig. 2D). The OH– also appeared to interact with calcium ions (Fig. 2, B and C). As expected, calcium ions (red) strongly interacted with PS head groups during the simulation (Fig. 2, C and D). As a result, both the HETE-PE and the PS carboxylic acid appeared to favor a close interaction between the surface of the membrane and the calcium ions of the water phase. We also visualized the bilayer from above (Fig. 2E; blue, positive charge; brown, negative charge) and found that the –OH was visible for all three HETE-PCs (red), facilitating the formation of a more negatively charged space, pushing head groups apart, and making space for calcium to bind, in some cases near PS (pink) (Fig. 2E).

HETE-PE increases calcium membrane binding
To experimentally test for an interaction between calcium and HETE-PLs, we measured Fluo-FF fluorescence in the presence of liposomes, maintaining PC, PS, and total PE concentrations, but gradually
Fig. 1. HETE-PEs and HETE-PCs dose-dependently enhance TF-dependent thrombin generation through a PS-dependent mechanism. Thrombin generation was initiated by the addition of liposomes to pooled platelet-poor plasma (PPP) with a thrombinoscope, as described in Materials and Methods. (A) Hydroxyecosatetraenoic acid-phosphatidylethanolamines (HETE-PEs) enhance tissue factor (TF)-dependent thrombin generation in plasma. Liposomes contained 10 pM recombinant TF with 65% 1,2-di-stearoyl-phosphatidylcholine (DSPC), 5% 1-stearoyl-2-arachidonoyl-phosphatidylserine (SAPS), and 30% SAPE, with 0 to 10% SAPE replaced with 0 to 10% of the indicated HETE-PE species. (B) HETE-PCs enhance TF-dependent thrombin generation in plasma. Liposomes contained 10 pM TF, 55% DSPC, 5% SAPS, and 30% SAPE, with 0 to 10% SAPS replaced with <10% of the indicated HETE-PC species. Data in (A) and (B) are representative traces of experiments that were repeated at least three times. (C) 15-HETE-PE and 15-HETE-PC enhance PS-dependent thrombin generation. Pooled PPP was activated with liposomes as described earlier, where SAPE was replaced with 15-HETE-PE (left graphs) or 15-HETE-PC (right graphs), with or without SAPS replacing PC, as indicated. Representative traces and maximum thrombin at varying PS concentrations are shown. Data are means ± SEM of three runs. (D) HETE-PEs and HETE-PCs promote the activities of coagulation factors in a full reconstitution system. Thrombin generation was initiated by the addition of liposomes to purified factors II, V, VII, VIII, IX, and X at physiological concentrations (see Materials and Methods). Liposomes contained (left) 65% DSPC, 5% SAPS, and 30% SAPE, with 10% SAPE (control) or 10% HETE-PE, or (middle) 55% DSPC, 5% SAPS, and 30% SAPE, with 10% SAPC (control) or 10% HETE-PC. Right: Summary data presented as fold change for the maximum thrombin generation rate observed (slope of lines in left and middle graphs) for each isomer. Data are means ± SEM of three runs. **P < 0.01 by single-factor analysis of variance (ANOVA) and post hoc Tukey tests.
Fig. 2. MD simulation suggests the association of the HETE-PL hydroxyl group with the polar environment, serine, and Ca²⁺, whereas HETE-PL membranes bind more Ca²⁺. (A to E) Molecular dynamics (MD) simulation shows the HETE hydroxyl group altering membrane behavior. MD simulations were undertaken for 300 ns using 5% SAPS, 5% SAPC, 30% SAPE, 55% dioleoylphosphatidylcholine (DOPC), and 5% 12-HETE-PC, as described in Materials and Methods. (A to D) Side views showing hydrophobic region (yellow), charged phosphate groups (cyan), and PC head groups (gray). (E) Top view looking down on the membrane showing areas of positive charge (blue), negative charge (brown), PS head groups (pink), and HETE hydroxyl groups (red). (F) Binding of calcium to membranes is increased by 15-HETE-PE. Liposomes consisting of 65% DSPC, 30% SAPE, and 5% SAPS, with up to 10% SAPE replaced by 15-HETE PE, were tested for calcium binding by measuring Fluo-FF fluorescence (see Materials and Methods). *P < 0.05, when compared to no added 15-HETE-PE. Data are means ± SEM of three experiments and were analyzed with the Mann-Whitney U test.
replacing PE with 12-HETE-PE up to 10%. In this assay, lipid-dependent reduction of FluorFF fluorescence indicates elevated membrane calcium binding. In the presence of either 10 or 20 μM CaCl₂, control liposomes bound 1 or 2.5 μM calcium, respectively. This increased significantly with 3 to 10% HETE-PE (Fig. 2F). These data support our hypothesis that HETE-PLs facilitate calcium binding on the surface of membranes to enhance PS-dependent clotting factor binding and activity.

HETE-PLs enhance coagulation and promote hemostasis in vivo

We next tested the ability of HETE-PLs to promote coagulation and hemostasis in vivo. For this, liposomes containing two different concentrations of 12-HETE-PE or 12-HETE-PC were injected intradermally into the tails of wild-type mice, immediately proximal to a tail cut. Control liposomes were without effect, whereas either 78 or 19 ng of HETE-PL (per injection) significantly inhibited bleeding, in some cases leading to total cessation (Fig. 3A). Separately, intravenous injection of liposomes with 19 ng of 12-HETE-PE into wild-type mice significantly increased thrombin-antithrombin (TAT) complexes (Fig. 3B). These data suggest that HETE-PLs are procoagulant in healthy mice in vivo. We measured eoxPL generation in vitro by platelets isolated from mice genetically lacking p12-LOX (encoded by the ALOX12 gene).

Washed platelets from wild-type but not ALOX12−/− mice generated multiple isomers of 12-HETE-PE and 12-HETE-PC in response to thrombin activation (Fig. 3C and fig. S1). PLs with either plasmalogen or acyl bonds at the Sn1 position, as well as analogous eoxPLs from 22:4, 22:5, and 22:6 fatty acids, were found, with all requiring p12-LOX activity (Fig. 3C). Time courses showed that HETE-PLs were already increased in abundance at 5 min but continued to increase at least until 30 min after thrombin treatment (Fig. 3D).

Genetic deficiency of p12-LOX or 12/15-LOX leads to a bleeding defect that can be corrected with HETE-PE administration

To test the involvement of p12-LOX in regulating coagulation in vivo, we induced venous thrombosis with FeCl₃ in mice. In the FeCl₃ venous thrombosis model, a statistically significantly reduced thrombus weight was observed in ALOX12−/− mice (Fig. 4, A and B). Mice lacking either ALOX12 (Fig. 4C) or ALOX15 (Fig. 4D) also showed a significantly increased tail bleeding time, as well as increased blood loss, measured as hemoglobin (Hb) loss from the tail. We next tested whether administration of HETE-PLs restored hemostasis in the ALOX12 or ALOX15−/− mice by administering small doses immediately proximal to a tail cut. Control liposomes containing 65% PC, 35% PE, and 5% PS may be expected to have partial procoagulant activities depending on dose. Administration of 19 ng of 12-HETE-PE in liposomes restored hemostasis back to that observed with wild-type amounts and was statistically significantly more effective than control liposomes at reducing bleeding time (Fig. 4, C and D).

Leukocyte and platelet HETE-PEs are increased in abundance in APS

To characterize HETE-PL generation in a human disease associated with venous thrombosis, we quantified HETE-PEs in isolated platelets and leukocytes from patients with APS and healthy controls (HCs) (tables S1 and S2). We detected statistically significantly increased basal concentrations of 5-HETE-PE (from neutrophils and monocytes, via 5-LOX) and 15-HETE-PE (from eosinophils, via 15-LOX) in leukocytes from APS patients compared to the amounts detected in cells from healthy volunteers (Fig. 5, A and B). After the activation of leukocytes with a calcium ionophore, HETE-PEs increased in abundance in both groups to a similar extent (Fig. 5, A and B). This suggests that leukocyte LOXs generate HETE-PEs basally in APS. The lack of difference between HCs and APS after stimulation with ionophore could imply that 15- and 5-LOX abundances are similar in leukocytes from both groups, although HETE-PL generation also depends on arachidonic acid (AA) substrate availability and the rate of reesterification into lysophospholipids. Platelets from APS patients contained statistically significantly more 12-HETE-PE than did those from HCs, both basally and after thrombin activation (Fig. 5C).

Our methods are optimized so that spontaneous activation is minimized during platelet isolation, specifically by using a wide-bore needle, ensuring a constant room temperature, and minimizing the numbers of wash steps and manipulations performed. Despite these steps, platelets from APS patients tended to spontaneously aggregate during isolation. Specifically, no platelets from healthy subjects were aggregates until challenged with thrombin, whereas of the platelets from 12 APS patients, 9 appeared to at least slightly aggregate during the last washing step of platelet isolation. Of those, seven generated a platelet “clot” that we removed and analyzed separately. Thus, our data suggest that platelets from APS patients are inherently more “poised” to activate. The amount of 12-HETE-PE in these spontaneously aggregated platelets was similar to the amounts in thrombin-activated APS platelets (Fig. 5, C and D), suggesting that they also generated increased amounts. We confirmed increased platelet activation in vivo with APS by measuring 11-thromboxane B₂ (TXB₂), a urinary metabolite of thromboxane A₂ formed by platelet COX-1 (Fig. 5E). The increased TXB₂ abundance in urine is consistent with platelets from APS patients circulating in a heightened activation state in vivo.

Next, we analyzed leukocytes for platelet-derived 12-HETE-PEs and platelets for leukocyte-derived 5- and 15-HETE-PEs and found that these were increased basally in platelets from APS patients (Fig. 5, F and G). Unlike 12-HETE-PEs, these did not show a statistically significant increase in abundance in response to thrombin activation, indicating that they were not from platelets. Instead, 15- and 5-HETE-PEs on the platelets could represent microparticles from other cells that had attached to the platelets. Leukocytes from APS patients had increased amounts of platelet 12-HETE-PEs compared to those of controls, and this lipid increased further upon ionophore activation (Fig. 5H). Thus, platelets or platelet-derived microparticles may be associated with APS patient leukocytes during isolation and may remain responsive to ionophore activation in vitro. It is well known that platelet-derived microparticles are increased in number in APS (28), and we previously showed that 12-HETE-PE and 12-HETE-PC are present in vesicles generated by thrombin-activated platelets in vitro (10, 29). Most of the patients were taking anticoagulant medication; thus, an influence of this on eoxPLs cannot be excluded (table S1). Although sample sizes were small, gender, age, and concurrent arterial thrombosis did not appear to influence eoxPL amounts (fig. S2 and table S2).

APS patients have increased amounts of plasma IgG directed against HETE-PEs

We sought to determine whether HETE-PLs acted as an antigen for antibodies in APS by measuring the amount of immunoglobulin G
Fig. 3. HETE-PL liposomes prevent tail bleeding and increase TAT concentrations in vivo, whereas platelets from mice genetically lacking p12-LOX generate few eoxPLs. (A) 12-HETE-PE/TF intradermal administration prevents tail bleeding in adult mice. Eleven-week-old male C57BL/6J mice were injected with liposomes containing TF with or without 12-HETE-PE or 12-HETE-PC immediately proximal to a tail cut, and bleeding time and blood loss were recorded as described in Materials and Methods. Data are from 10 to 16 mice per group. *P ≤ 0.01, **P ≤ 0.05 by Mann-Whitney U test. (B) 12-HETE-PE increases thrombin-antithrombin (TAT) complexes in vivo. Control or HETE-PE-containing liposomes were injected intravenously into wild-type (WT) mice, and plasma was obtained after 1 hour. TAT concentrations were measured by enzyme-linked immunosorbent assay. Data are from six mice per group. *P ≤ 0.05. (C) Thrombin stimulates the production of enzymatically oxidized phospholipid (eoxPL) species from washed murine platelets in a p12-lipoxygenase (LOX)-dependent manner. Washed platelets from WT or ALOX12−/− mice were activated for 30 min with thrombin (0.2 U/ml), and then the lipids were extracted and analyzed to detect eoxPL species. Data are means of three experiments. Data in the heatmap are normalized to basal amounts of the indicated eoxPLs in WT mouse platelets. (D) Time course for the generation of 12-HETE-PE and 12-HETE-PCs. Platelets from WT or ALOX12−/− mice (2 × 10^9/ml) were activated with human thrombin (0.2 U/ml) for 0 to 30 min at 37°C. The lipids were then extracted and quantified. HETE-PE represents the sum of 16:0p/12-HETE-PE, 18:1p/12-HETE-PE, 18:0/12-HETE-PE, and 18:0a/HETE PE. HETE-PC represents the sum of 16:0a/12-HETE-PC and 18:0a/12-HETE-PC. Data are means ± SEM of eight mice per group. ns, not significant.
Fig. 4. Mice lacking either 12/15-LOX or p12-LOX have impaired venous coagulation, which is restored by the administration of 12-HETE-PE liposomes. (A and B) ALOX12-deficient mice show reduced thrombus formation in vivo after challenge. Venous thrombosis was induced in WT and ALOX12−/− mice. (A) Thrombus weights were measured. Data are means ± SEM of seven to nine mice per group. *P < 0.05 by Mann-Whitney U test. (B) A representative thrombus is shown for each genotype. (C) ALOX12-deficient mice have impaired hemostasis in vivo. Male WT C57BL/6J or ALOX12−/− mice (8 to 11 weeks old) were administered a tail cut, and bleeding time and blood loss were recorded. Liposomes containing 19 ng of 12-HETE-PE or control liposomes were administered 10 μl of phosphate-buffered saline into the tail tissue just ahead of the cut and immediately before the cut was made. Data are means ± SEM of 6 to 12 mice per group. (D) ALOX15-deficient mice have impaired hemostasis in vivo. Experiments were performed, and data were collected as described in (C) except that WT C57BL/6J mice were compared to ALOX15-deficient mice. Data are means ± SEM of 12 to 19 mice per group. Data in (C) and (D) were analyzed by Mann-Whitney U test. *P < 0.05, **P < 0.01, ***P < 0.005.
IgG specific for HETE-PE compared with the amount of IgG specific for the unoxidized analog, SAPE. APS serum had a substantial increase in the amount of IgG recognizing 5-, 12-, and 15-HETE-PEs (Fig. 6, A to C). IgG against SAPE was also increased, but this was not statistically significant. Total IgG was comparable between both groups (Fig. 6D). This suggests that chronic higher exposure to HETE-PEs leads to the enhanced immune recognition of these lipids in patients.
**HETE-PEs enhance APS-associated plasma protein binding to the cellular surface**

β2GP1 is a positively charged protein that binds to anionic PLs, such as cardiolipin, resulting in a conformational change that exposes cryptic epitopes recognized by pathogenic disease–associated antibodies (14). This interaction is believed to result in downstream signaling with a net procoagulant effect. Because HETE-PLs can enhance calcium binding (Fig. 2F), we tested whether they also increased β2GP1 interactions with the plasma membrane using PS- and PC-containing liposomes. Binding of human purified β2GP1 was enhanced by cardiolipin substitution of PE with 15-HETE-PE (Fig. 6E). In addition, 5- or 12-HETE-PE increased the cardiolipin-dependent binding of β2GP1 to the liposome in comparison to HETE-PEs or cardiolipin alone (Fig. 6F).

Thus, HETE-PEs enhance the binding of β2GP1 to membranes, a process that is required for the protein to become antigenic.

**Lipidomics defines the complexity of eoxPL enzymatic generation in human platelets, including previously uncharacterized control networks**

More than 100 eoxPLs are generated by thrombin-activated human platelets (11–13). The enzymatic origin of most of these lipids is not defined. Here, the 47 most abundant lipids were profiled in patients from HC and APS patients (both unstimulated and after thrombin activation) to examine their behavior in the context of a thrombotic disease (Fig. 7A and fig. S3). Where the enzyme responsible for synthesizing the lipid was known, lipids were labeled

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**Fig. 6. Circulating IgG against HETE-PEs is increased in abundance in APS, and β2BP1 binding to membranes is enhanced by HETE-PEs.** (A to C) Immunoglobulin G (IgG) against HETE-PEs is statistically significantly increased in abundance in plasma from APS patients. The amounts of IgG antibodies against HETE-PEs in HC and APS patient plasma were determined by diluting plasma 1:12 and testing binding to the indicated antigens. SAPE was used as an unoxidized lipid for comparison. All samples were analyzed in triplicate. Data are means ± SEM of 18 (HC) or 9 (APS) subjects. *P < 0.05, **P < 0.01 by Mann-Whitney U test. (D) IgG titers are comparable in plasma from HC and APS patients. Total IgG amounts in plasma from HC and APS patients were determined. Data are means ± SEM of 34 (HC) and 10 (APS) subjects. (E) HETE-PEs enhance the binding of β2-glycoprotein 1 (β2GP1) to lipid membranes. Binding of β2GP1 to liposomes in the presence of cardiolipin, 15-HETE-PE, 5-HETE-PE, or 12-HETE-PE was determined as described in Materials and Methods. (F) HETE-PEs enhance the cardiolipin-dependent binding of β2GP1 to lipids. Binding of β2GP1 to liposomes in the presence of cardiolipin with or without 15-HETE-PE, 5-HETE-PE, or 12-HETE-PE was determined. In (D) and (E), data are means ± SEM of three experiments, each performed in triplicate. Statistical significance was determined by one-way ANOVA and Tukey-Kramer test. *P < 0.05, **P < 0.01, ***P < 0.001. RLU, relative light units.

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Fig. 7. Lipidomic profiling of 47 eoxPL defines their enzymatic origin and regulatory networks. (A and B) Washed platelets were isolated from HC or APS patients and were left unstimulated or were stimulated with thrombin (0.2 U/ml, 30 min) before lipids were extracted and analyzed by liquid chromatography-MS/MS for 47 eoxPL species, as described in Materials and Methods. Data are from 16 (HC) and 10 (APS) subjects. (A) p12-LOX– and cyclooxygenase 1 (COX-1)–derived lipids cluster into distinct families based on Sn2 fatty acid composition. A heatmap of the effect of thrombin on the abundances of the indicated lipids is shown. In the heatmap, an increase in abundance is denoted in red, whereas a decrease in abundance is denoted in blue. Deeper tones indicate greater differences, with comparisons for individual lipids made across samples. (B) Plotting correlation between individual lipids illustrates additional relatedness between families of ions. Correlations between lipids across the whole cohort were plotted in a grid in order of decreasing correlation with 18:0a/12-HETE-PE (see fig. S4 and Materials and Methods). Red, p12-LOX; blue, COX-1; green, polyoxygenated PL; black, unknown origin. Lipids marked with red arrows indicating a relationship are listed. All lipid abundances were normalized to the mean of those of the control unstimulated values.
as being derived from either COX-1 (blue) or p12-LOX (red) (Fig. 3C), based on our observation of their absence in ALOX12−/− mouse platelets or having a known sensitivity to aspirin, which define PGE2- or DTX3- PEs that are produced by COX-1 (11, 12). Cluster analysis revealed two prominent groups of related lipids visible based on COX-1 or p12-LOX. Several polyhydroxylated lipids grouped with COX-1, suggesting that they may also originate from that pathway (green), and five monohydroxylipids (black) grouped with p12-LOX (Fig. 7A). Note that eoxPL clustered strongly based on the Sn2 fatty acid, with hydroxycosahexanoic acids (HDOHEs), HETEs, and monohydroxy C22 lipids forming associated groups. From visual inspection, 12-LOX–derived lipids appeared to increase in abundance to a greater extent than did other lipids in response to thrombin and are increased in abundance in the APS patients compared to the HCs (Fig. 7A).

Next, we plotted correlations between lipids (that is, how each lipid behaved in relation to others individually) to further examine their behavior within the APS cohort (Fig. 7B). Here, lipids were sorted by decreasing correlation with 18:0a/12-HETE-PE, an abundant eoxPL that is generated by p12-LOX. p12-LOX– and COX-1–derived lipids grouped together, with polyoxygenated lipids correlating more closely with COX-1. However, this visualization also revealed a family that correlated with 16:0e/22:5(O)-PE (red arrows and table) (Fig. 7B and fig. S4). Note that almost all were acyl-PEs and PCs, with exclusively either 22:5 or 22:4 monoxygenated fatty acids at Sn2, and several were p12-LOX–derived. This suggests that 12-LOX forms 22:4(O) or 22:5(O) PLs that are regulated as a group. Together with the hierarchical cluster analysis (Fig. 7A), this further supports the idea of differential behavior based on the composition of the Sn2 oxidized fatty acid.

For both the cluster analysis and correlation analyses, we normalized all lipids to the mean of the control unstimulated values. Thus, we could examine how each lipid behaved individually, relating to activation or health and disease. There are major differences in abundance between individual lipids (for example, HETE-containing PLs predominate), whereas lipids from less abundant fatty acids or with multiple oxygens are present in lower amounts (11–13). If data are not normalized, then correlations will be influenced by amounts rather than biological pathways. We also generated correlations in which we separated the APS and HC data sets (fig. S5). Whereas these appeared to be visually distinct, the same trends whereby 12-LOX–derived lipids or COX-1/polyoxygenated lipids correlated in groups based on biological pathway were maintained.

We then characterized the 47 lipids using a network analysis (Cytoscape 3.2.1), in which nodes represented individual lipids and node size was determined by the number of links to other lipids (Fig. 8A). Edge thickness represented the strength of correlation between individual nodes. Nodes with the highest degree clustered toward the center. The network diagram illustrates that the COX-1– and p12-LOX–derived lipids behaved as two separate groups, with only a small degree of relatedness between them. The analysis further showed that all remaining 20:4(3O) lipids located with COX-1, suggesting that they are PG-containing PLs. Four PEs correlated with p12-LOX, indicating this as their origin. When analyzed as a single group, the 31 p12-LOX–derived eoxPLs were statistically significantly increased in abundance in APS platelets both basally and after thrombin stimulation, whereas for COX-1–derived eoxPLs, a trend toward increased amounts in APS was noted (Fig. 8B). Last, a group of exclusively plasmalogen mono- or dioxygenated-PEs from 22:5 or 20:4 behaved as a separate family, suggesting that they have a different enzymatic origin than from either COX-1 or p12-LOX (Fig. 8A). Unlike COX-1– and p12-LOX– derived eoxPLs, these lipids did not exhibit an increase in abundance upon thrombin activation and were present basally (fig. S3). It is also noteworthy that COX-1–derived eoxPLs are almost exclusively plasmalogen PE species, whereas p12-LOX–derived eoxPLs include both acyl and plasmalogen forms, as well as PC and PE.

**DISCUSSION**

Here, we showed using in vitro coagulation studies, murine venous thrombosis models, and MD simulations that HETE-PLs increase the activities of calcium/PS-dependent factors in vitro and in vivo (Figs. 1 to 4). Coagulation factors associate with anionic PLs (classically, native PS and PE) through interactions with positively charged calcium ions. Positioning the HETE hydroxyl group near the membrane surface appears to enhance this and provides a mechanistic basis for our observations (Fig. 2). Mice lacking either ALOX15 (leukocyte-type 12/15-LOX or 15-LOX) or ALOX12 (platelet-type 12-LOX) were affected in venous bleeding challenge models, suggesting that both LOX isoforms likely act in concert to achieve effective hemostasis in vivo (Fig. 4).

Circulating blood cells from patients with the thrombotic disorder APS had substantially greater amounts of leukocyte- and platelet-derived eoxPLs basally, in concert with enhanced anti–HETE-PL immunoreactivity (Figs. 5 and 6). Furthermore, by undertaking a comprehensive lipidomic analysis of platelets from APS patients and controls, a complex network of independently regulated eoxPL families from COX-1 and p12-LOX was revealed (Figs. 7 and 8). A large group of platelet lipids, generated by p12-LOX, were substantially increased in abundance in APS (Fig. 8B). Together, these data are suggestive of a contribution of eoxPLs from this pathway to human thrombotic disease through their procoagulant or immunogenic activities, but this remains to be conclusively proven. The clear stratification of lipids into groups that behaved differently based on head group, Sn2 fatty acid, or Sn1 acyl/plasmalogen composition shown by the clustering and network analyses suggests additional enzymatic regulation mechanisms for eoxPL formation and metabolism in platelets, which remain to be characterized. These could include differential enzymatic control of acylation of the oxidized fatty acids into specific lysosphopholipid species. In this regard, little is yet known about fatty acyl–coenzyme A ligase or lysosphopholipid acyltransferase isoform preference for different oxidized fatty acids or eicosanoids as substrates for esterification, in platelets or any cell type.

Previous studies proposed a role for lipid oxidation in APS, because nonenzymatic oxidation products are increased in abundance in urine (30). We suggest that this may be initiated by leukocyte and platelet LOXs, with primary enzymatic oxidation products, such as peroxides and lipid radicals decomposing and mediating propagation reactions nonenzymatically. Although there are numerous antigenic targets for antiphospholipid antibodies, only some have been implicated in APS (31, 32). Further study is necessary to determine whether HETE-PE–specific antibodies are causally involved; however, increased amounts of IgG indicate that the lipids would likely be chronically increased in abundance in APS patients with thrombotic disease.

A mechanism proposed to promote thrombosis in APS involves increased binding of β2GP1 to PF4 (platelet factor 4), causing platelet activation that is indicated by the measurement of increased TXB2 amounts in vivo (33–35). In this regard, we found the HETE-PEs facilitated increased β2GP1 binding to membranes, both directly and through enhancing cardiolipin-dependent binding, and we also observed increased amounts of TXB2 in our cohort of APS patients compared to HC.
to those in controls (Figs. 5E and 6, E and F). In addition, APS monocytes can promote both coagulation and inflammation through the production of increased amounts of TF, tumor necrosis factor–α (TNF–α), and interleukin-1 (IL-1) (36, 37). The binding of β2GP1 to monocytes in complex with anti–β2GP1 IgG increases the production of TF (38). Thus, our studies suggest two potential mechanisms for increased thrombosis linked to eoxPLs: (i) direct HETE-PL enhancement of PS-dependent coagulation on the platelet or leukocyte surface by the mechanisms characterized here, and (ii) increased β2GP1 binding to oxidized PLs on the surface of circulating immune cells, which in turn promotes inflammation and TF production. Further work is required to determine the relative contributions of these mechanisms to
membranes. Cardiolipin is negatively charged at pH 7.4. Although it
interacts with calcium through its phosphate, suggesting that they act
similarly to native PE through calcium bind-

ing (Fig. 1, G to J). Our MD simulation supports this (Fig. 2). The HETE-
PLs enhanced coagulation in vitro and in vivo (Figs. 1, 3, and 4). Native PE alone does not support coagula-
tion, because its bulky head group inhibits GLA domain interac-
tions with its phosphate (2). However, in multiple experiments in vitro and in vivo, we found that HETE-PE promoted coagulation (Figs. 1, D to F, and 3A). Most previous studies have focused on the head group; how-
ever, PLs also differ with respect to fatty acid composition (3, 26). Thus,
the action of an eoxPL is entirely dependent on the oxidized epite-
This represents a paradigm shift in our understanding of hemostasis,
whereby certain forms of PE rapidly generated by activated leukocytes
and platelets promote clotting. Determining which specific factors
and cofactors are sensitive to HETE-PLs will be undertaken using recom-
binant factors and surface plasmon resonance in a follow-up study.

HETE-PLs directly enhanced PS-dependent thrombin generation,
suggesting that they act similarly to native PE through calcium bind-
ing (Fig. 1, G to J). Our MD simulation supports this (Fig. 2). The HETE-
hydroxyl group quickly moved near the surface of the membrane,
where it interacted with calcium. It also increased the distance between
the head groups, enabling greater accessibility to the phosphate group.
To some extent, the effect of HETE-PL mimics the role of the carbox-

HETE-PE and HETE-PC are generated and purified as previously de-
scribed (23, 24). The cofactors and platelets promote clotting. Determining which specific factors
and cofactors are sensitive to HETE-PLs will be undertaken using recom-
binant factors and surface plasmon resonance in a follow-up study.

The concentrations of HETE-PLs in thrombinscope assays ranged
from 10 to 100 ng/ml. We calculated the amounts of HETE-PL gen-
drated by human and mouse platelets to be 23 ng/4 × 10^7 cells and
35 ng/2 × 10^8 platelets, respectively (Fig. 3D) (9). Given typical blood
platelet concentrations (2 × 10^9/ml or 4 × 10^9/ml for human or mouse,
respectively), this would be expected to equate to around 120 or 70 ng/ml
of HETE-PLs for mouse or human blood. HETE-PE and HETE-PC
isomers represent only a proportion of the total PL oxidation products
that form during platelet activation, with more than 100 individual mo-
lecular species being formed acutely (13). Thus, the actual concentra-
tion of potential procoagulant eoxPLs in the platelet membrane is likely
to be considerably greater than our estimates. Furthermore, studies
showed that annexin V binding is clustered to discrete domains of the
activated platelet membrane, where procoagulant PLs concentrate, thus
achieving even greater local amounts (41).

**MATERIALS AND METHODS**

**Reagents**

Corn trypsin inhibitor (recombinant) and full-length TF (human re-
combinant) were from Haematologic Technologies Inc. FITC fluorogenic
substrate (Z-Gly-Gly-Arg-AMC) was from Bachem. T-cal Thrombin-
scope calibrator was from Stago. SAPE, SAPC, SAPS, and 1,2-di-stearoyl-
9-stearoyl-PC (DSPC) were from Avanti Polar Lipids. LiposoFast membranes were from Sigma-Aldrich, except for N-methyl
benzoxhydroxamic acid, which was synthesized in-house as previously
described (45). Solvents were from Thermo Fisher Scientific. HETE-
PEs and HETE-PCs were generated and purified as previously de-
scribed (46).
Study design
All studies were carried out in accordance with the principles of the Declaration of Helsinki and with informed consent and full ethical approval. APS: The study was approved by the South West Wales Research Ethics Committee (12/WA/0229). Patients (total, 18) with APS associated with venous thrombosis were recruited from hematology clinics. All had at least one incidence of venous and/or arterial thrombosis and tested positive for at least one laboratory criterion (lupus anticoagulant and/or high anticardiolipin IgG titers and/or high anti-β2GP1 IgG titers measured on two occasions at least 12 weeks apart). Patients were not taking aspirin or other nonsteroidal anti-inflammatory drugs at the time of venipuncture. Most were taking anticoagulants (for example, warfarin or rivaroxaban), none had diabetes, and only one had high cholesterol at the time of sampling. Full details on all patients are shown in table S1. Blood samples were collected from patients following a routine clinical visit, in a quiescent state with no clinical thrombotic episode at the time of sampling. HCs (total, 34) were recruited from the local population and were excluded if they had a history of arterial or venous thrombosis, recurrent fetal loss, cardiac disease, or any other chronic inflammatory diseases such as rheumatoid arthritis, systemic lupus erythematosus, diabetes, high cholesterol, abnormal renal or liver function, or other diseases that may conflict with the study parameters. Individuals within the HC group had not taken aspirin, non-steroidal anti-inflammatory drugs, or any other medication in the preceding 14 days. Informed consent was obtained from all participants. Age and gender demographics are given in table S2. Blood samples were collected from APS patients and HCs via venipuncture from the median cubital vein. Full blood counts (FBCs) were collected, and any patients or HCs with an abnormal FBC at the time of sample collection were retrospectively omitted from the study. Washed platelets and leukocytes were then isolated and activated. Serum, plasma, and urine were also collected. Blood donations from healthy volunteers were approved by the Cardiff University School of Medicine Ethics Committee and were with informed consent (SMREC 12/37, SMREC 12/10). A power calculation was not undertaken because preliminary data were not available for our studies. Rules for stopping data collection were not defined. Outliers were not removed, and end points were not prospectively selected. Replicates for all experiments are included in figure legends. The objectives of the research were to define whether eoxPLs could modulate coagulation reactions, determine the mechanisms involved, and examine whether eoxPLs were increased in human thrombotic disease. Research subjects included patients with APS (total number, 18) and HCs (total number, 34), mice (wild-type and genetically modified), and liposomes of defined composition. There were several different studies in our design, including controlled laboratory experiments and observational studies. Randomization is not relevant because we did not conduct a clinical trial. Blinding was used during analysis of lipids from patients and controls, but not in animal, cellular, or liposome experiments.

Generation of liposomes
Liposomes were made by freeze-thawing followed by extrusion in Heps (20 mM), NaCl (100 mM), and pH 7.35 (buffer A). Where HETE-PE was substituted for unoxidized PE, the liposomes used were as follows: 5% SAPS, 20 to 30% SAPE, 0 to 10% HETE-PE, and 65% DSPC (mole percent). Where unoxidized PC was substituted for HETE-PC, the liposomes used were as follows: 5% SAPS, 30% SAPE, 55% DSPC, 0 to 10% SAPC, and 0 to 10% HETE-PC. Liposomes were made in the presence of full-length recombinant TF (10 pM) and used at a final concentration of 4 μM total lipid unless otherwise stated. Binding of β2GP1 to liposomes was also determined.

Thrombin generation assays
Thrombin generation assays were performed using a Fluoroskan Ascent plate reader (Thermo LabSystems). Cleavage of prothrombin to thrombin was measured using the fluorogenic substrate Z-Gly-Gly-Arg-AMC (0.5 mM), and thrombin activity was compared to the thrombin calibrator (Stago). Thrombin generation was calculated from raw fluorescence data, as described previously (47). Cleavage of prothrombin to thrombin in simplified coagulation cascade experiments was performed according to the same methods but using a mixture of FII (1.4 μM), FV (26 nM), FVII (10 nM), FVIII (300 pM), FIX (80 nM), and FX (136 nM) in buffer A with 1% bovine serum albumin (buffer B). The reaction was initiated by the addition of CaCl₂ (20 mM) and fluorogetic substrate (0.5 mM) in buffer B.

Lipid extraction and analysis
1,2-Dimyristoyl-PE or 1,2-dimyristoyl-PC (10 ng) was added to each sample before extraction as an internal standard. Lipids were extracted by adding a solvent mixture [1 M acetic acid, 2-propanol, hexane (2:20:30)] to the sample at a ratio of 2.5 ml of solvent mixture to 1 ml of sample, vortexing, and then adding 2.5 ml of hexane. After vortexing and centrifugation (400g, 5 min), lipids were recovered in the upper hexane layer. The samples were then reextracted by the addition of an equal volume of hexane followed by further vortexing and centrifugation. The combined hexane layers were then dried under vacuum and analyzed for HETE-PE using liquid chromatography (LC)–MS/MS. Samples were separated on a C₁₈ Luna, 3 μm, 150 mm × 2 mm column (Phenomenex) gradient of 50 to 100% solvent B for 10 min followed by 30 min at 100% B (solvent A: methanol/acetonitrile/water, 1 mM ammonium acetate, 60:20:20; solvent B: methanol, 1 mM ammonium acetate) with a flow rate of 200 μl/min. Electrospray mass spectra were obtained on a Q-Trap instrument (Applied Biosystems 4000 Q-Trap) operating in the negative mode. Products were analyzed in the multiple reaction monitoring (MRM) transitions from the parent ion to the daughter ion of 179.1 (12 HETE [M-H]−) every 200 ms with a collision energy of −45 to −42 V. The area under the curve for the parent ion to the 179.1 ion was integrated and normalized to the internal standard for 16:0, 18:1p/18:0p, and 18:0a/12-HETE-PEs. For quantification of these lipids, standard curves were generated with purified 18:0a/12-HETE-PE/PC. Where large numbers of HETE-PLs were measured in lipidomic assays, fold changes relative to PE or PC internal standards were determined using MRM transitions from a previous study (13).

Liposome composition for β2GP1 binding experiments
All liposomes were as described earlier except, but with the following lipid composition: 55% DSPC, 15% SAPS, 10% SAPC, 1% SAPE–biotin (generated by reacting N-hydroxysuccinimide–biotin with PE and purification using HPLC), and 19% SAPE. In some experiments, an equivalent amount of SAPE was replaced with 1% 15-, 12-, or 5-HETE-PE and/or 1% cardiolipin. Liposomes were immobilized onto Polysorp plates (Nunc) coated with neutravidin (5 μg/ml). β2GP1 (1 μg/ml) was added for 1 hour at room temperature. Bound β2GP1 was determined using an anti-β2GP1 horseradish peroxidase antibody (1:20,000) (Cedarlane Laboratories) and ECL detection (Pierce). Data are expressed as the relative light units (RLU) per 100 ms.
Determination of circulating antibodies to HETE-PEs
Specific antibody titers to individual HETE-PEs were determined by chemiluminescent enzyme-linked immunosorbent assay (ELISA), as previously described (48). Lipids were coated onto Microfluor plates at 20 μg/ml and subsequently blocked with 0.5% fish gelatin in 0.27 mM phosphate-buffered saline (PBS)–0.27 mM EDTA. EDTA plasma samples (1:12) were diluted in PBS–0.27 mM EDTA and incubated for 1 hour at room temperature. The bound IgG was measured using an anti-human IgG alkaline phosphatase–conjugated secondary antibody (Sigma-Aldrich) and LumiPhos 530 (Lumigen Inc.). Data are expressed as RLU/100 ms.

Isolation of human washed platelets
Washed platelets were prepared from whole blood drawn from a central venous catheter into syringes containing acidified citrate dextrose (ACD; 85 mM trisodium citrate, 65 mM citric acid, and 100 mM glucose) at a ratio of 8.1 parts whole blood to 1.9 parts ACD, as described previously (46), and resuspended in modified Tyrode’s buffer (134 mM NaCl, 12 mM NaHCO₃, 2.9 mM KCl, 0.34 mM Na₂HPO₄, 1.0 mM MgCl₂, 10 mM Heps, and 5 mM glucose, pH 7.4) at 2 × 10⁸/ml. Platelets (1 ml) were incubated at 37°C for 30 min with 1 mM CaCl₂, human thrombin (0.2 U/ml; Sigma-Aldrich), or modified Tyrode’s buffer.

Isolation of human leukocytes
Leukocytes were isolated from 20 ml of citrate anticoagulated whole blood. Briefly, 20 ml of whole blood was mixed with 4 ml of 2% citrate and 4 ml of Hetasep (STEMCELL Technologies) and allowed to sediment for at least 45 min. The upper plasma layer was recovered and centrifuged at 250g for 10 min at room temperature. The pellet was resuspended in an ice-cold 0.4% trisodium citrate/PBS and centrifuged at 250g for 5 min at 4°C. Erythrocytes were removed by hypotonic lysis. Leukocytes were resuspended in Krebs buffer at 4 × 10⁹/ml. Leukocytes were activated at 37°C with 10 μM A23187 in the presence of 1 mM CaCl₂ for 30 min before lipid extraction.

Isolation of serum, plasma, and urine
Blood was collected into EDTA, lithium heparin, or citrate vacutainers before centrifugation at 900g. The plasma layer was collected and centrifuged again at 900g to remove any residual platelets. Plasma was stored at −80°C for use in vitro assays. Whole blood was collected into a clot-activating vacutainer and centrifuged at 900g. The serum was collected and centrifuged again at 900g to remove any residual cells. Serum was stored at −80°C for use in vitro assays. Urine was collected into universal containers, and samples were aliquoted and stored at −80°C until use. 11-Dehydro-TXB₂ was measured using gas chromatography–MS, as previously described (49).

Isolation and activation of mouse washed platelets
Mouse blood was obtained by cardiac puncture directly into a syringe containing 150 μl of ACD [2.5% (w/v) trisodium citrate, 1.5% (w/v) citric acid, and 100 mM glucose]. The syringe was emptied into an Eppendorf tube containing 150 μl of 3.8% (w/v) sodium citrate, and 300 μl of modified Tyrode’s buffer was then added (145 mM NaCl, 12 mM NaHCO₃, 2.95 mM KCl, 1 mM MgCl₂, 10 mM Heps, and 5 mM glucose). The blood was spun for 5 min at 150g at 25°C, and platelet-rich plasma (PRP) was removed. Another 400 μl of Tyrode’s buffer was added and carefully mixed into the blood without inverting the tube, and more PRP was removed after a second identical spin. A third spin at 530g for 5 min on the pooled PRP pelleted the platelets, plasma was removed, and the platelets were resuspended in Tyrode’s buffer at 2 × 10⁹/ml. Half of the platelets were used as unstimulated controls, and the rest were activated with thrombin (0.2 U/ml) and 1 mM CaCl₂ followed by gentle mixing every 2 to 3 min for 30 min at 37°C.

TAT complex measurement
Whole blood was collected via a cardiac puncture into 1/10 volume of 3.8% sodium citrate as an anticoagulant and centrifuged at 3000g for 10 min. Plasma TAT amounts were determined using a commercially available ELISA kit (Mouse Thrombin-Antithrombin Complexes ELISA Kit; ab137994, Abcam).

Quantification of blood loss from tail bleeding assays
C57/BL6 wild-type (Charles River Laboratories), ALOX15<sup>−/−</sup>, and ALOX12<sup>−/−</sup> mice bred in-house were kept in constant temperature cages (20°C to 22°C) and given free access to water and standard chow. Tail bleeding assays and breeding of mice were performed under Home Office Licence PPL/3150. Male mice (11 weeks old) were anesthetized using 5% isoflurane and maintained with 2% isoflurane. Where administered, liposomes [10 μl; generated as described earlier, using either (i) 30% SAPE, 65% DSPC, 5% SAPS, and 2.5 mM TF or (ii) 20% SAPE, 65% DSPC, 5% SAPS, 10% 12-HETE-PE, and 2.5 mM TF] were injected immediately proximal to the cut site directly before transection of 2 to 5 mm from the distal end and immediate immersion in 37°C physiological saline. Bleeding was observed as blood loss, and time to the beginning of stable (1 min) cessation of blood flow was determined before euthanasia via cervical dislocation. Blood loss was quantified by measuring the Hb content of the saline as follows: Hb quantitation was achieved via centrifugation of the tube at 250g for 15 min and resuspending the red cells in 5 ml of erythrocyte lysis buffer [NH₄Cl (8.3 g/liter), KHCO₃ (1 g/liter), and EDTA (0.037 g/liter) in distilled H₂O]. The concentration of Hb was measured as optical density at 575 nm using a UVIKON 923 double beam UV/VIS spectrophotometer (BioTek Kontron Instruments) and expressed as absorbance units (AU).

Injury-related venous thrombosis
Thrombosis was induced as described previously with minor modifications (50). In brief, mice (20 to 25 g body weight) were anesthetized with ketamine (100 mg/kg body weight) and xylazine (20 mg/kg body weight) and placed under a heating lamp to maintain a constant body temperature of 37°C. A ventral midline incision was performed, and the intestines were gently put aside. The inferior vena cava was laid free carefully, and a filter paper (1 mm × 2 mm × 4 mm) soaked with 4% of aqueous ferric chloride solution was placed on top of the vessel. After 3 min, the filter paper was removed and the peritoneal cavity was thoracically inflated with ketamine (100 mg/kg body weight) and xylazine (20 mg/kg body weight) and placed under a heating lamp to maintain a constant body temperature of 37°C. A venous midline incision was performed, and the intestines were laid aside. The vena cava was washed with saline, and the clot was dissected free from the vessel and prepared under a microscope for further analysis. Wet thrombus weight was measured using a precision balance (Sartorius MS, Germany) after removal of excess water.

Lipid bilayer model preparation and membrane energy minimization and simulation protocol
A equilibrated hydrated lipid bilayer consisting of 128 molecules of DOPC, based on a previously reported study, was downloaded from the ATM database (Box ID: 30) (51, 52). The lipid bilayer was structurally

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**Further Reading**


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**Citation**

modified by removing DOPC using the MOE (Molecular Operating Environment) (53) builder tools according to the experimental composition of the tested liposomes to give the following composition: 5% SAPS, 5% SAPC, 30% SAPE, 55% DOPC, and 5% 12-HETE-PC. Initially, a conformational analysis of 12-HETE-PC was performed with two different low-energy conformations included in the membrane. The first conformation presented the oxidized lipid chain in an elongated structure and was used for the three 12-HETE-PCs in one monolayer. The second presented the oxidized chain in a bent conformation and was used in the other monolayer. As a result, the 12-HETE-PC presented the hydroxyl groups differently on the two different membrane layers: On one side, the hydroxyl groups were placed deep in the membrane, whereas on the other side, they were close to the membrane surface. All the modified lateral chains were minimized in MOE using an optimized potentials for liquid simulations (OPLS)–AA force field with a gradient of 0.1 kcal mol⁻¹ Å⁻¹. Hydrate membrane energy minimization and simulation were performed by using Desmond (Maestro Interface) with OPLS_2005 set of force field parameters (54, 55). The TIP3P water model was used, and the assembled system consisted of 35,426 atoms enclosed in a 69 × 70 × 80 Å³ triclinic box. Fourteen trajectory was recorded every 480 ps. Energy of the simulation was recorded every 120 ps, whereas the time step was set to 2 fs. Energy of the simulation was recorded every 120 ps, whereas the trajectory was recorded every 480 ps.

**Measurement of calcium binding to membranes**

Glass tubes were washed using 1 M hydrochloric acid and methanol. All buffers were treated with prewashed Chelex-100 (10 mg/ml) and kept in plasticware to reduce calcium contamination to 1 to 2 ppm. All buffers were treated with prewashed Chelex-100 (10 mg/ml) and methanol. All buffers were treated with prewashed Chelex-100 (10 mg/ml) and kept in plasticware to reduce calcium contamination to 1 to 2 ppm.

**Heatmap and Cytoscape correlation**

Heatmaps were generated using the pheatmap package in R using hierarchical clustering (complete linkage method) to group similar lipids (version 3.3.1). Data were first normalized to the mean of the unstimulated control values for each lipid. Network analysis was performed with Cytoscape (version 3.4.0) using pairwise correlations between lipids generated with R. The network diagram shows only correlations with a Pearson product-moment correlation coefficient value (r) of >0.8 because of the high number of interactions.

**Statistical analysis**

Statistical significance was calculated with the Mann-Whitney U test, unless otherwise stated. The statistical differences between β2GP1 binding to HETE-PL and cardiolipin liposomes were calculated by one-way analysis of variance (ANOVA) with Tukey-Kramer multiple comparison post hoc test, which was used to compare the means of each condition. Statistical significance is denoted as follows: *P < 0.05; **P < 0.01; ***P < 0.001. Statistical analysis was performed using GraphPad Prism 6. Unless otherwise stated in figure legends, data were displayed as Tukey boxplots, where whiskers represent 1.5 the lower and upper interquartile range, and data not included within the whiskers were displayed as individual values. The correlation plot (Fig. 7B) was created in R using the corplot package (56, 57).

**SUPPLEMENTARY MATERIALS**

Fig. S1. Primary data for the generation of eoxPLs by washed wild-type and ALOX12⁻/⁻ platelets.

Fig. S2. Analysis of HC and APS demographics for effects on eoxPL generation shows no effect of age, gender, or arterial thrombosis on platelet 12-HETE-PEs.

Fig. S3. Primary data for the generation of eoxPLs by washed platelets from HC and APS patients.

Fig. S4. Correlation plots for lipids from all groups show that lipids group according to enzymatic origin.

Fig. S5. Correlation plots for lipids from either HC or APS patients show that lipids group according to enzymatic origin.

Table S1. Patient demographics and relevant medical details.

Table S2. Age and gender demographics of study participants.

Movie S1. Simulation of the movement of HETE-PC in a lipid biomembrane.

**REFERENCES AND NOTES**


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Networks of enzymatically oxidized membrane lipids support calcium-dependent coagulation factor binding to maintain hemostasis


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Lipid networks and blood clotting

Blood coagulation is important for preventing blood loss (hemostasis) and bacterial invasion. Coagulation depends on factors presented on the plasma membranes of activated platelets and leukocytes. Through biochemical and lipidomic analyses, Lauder et al. showed that the exposure of enzymatically oxidized phospholipids (eoxPLs) derived from lipoxygenases in platelets and leukocytes was required for hemostasis. Compared to platelet eoxPLs from healthy controls, eoxPLs were increased in abundance in platelets from patients with the thrombotic disorder antiphospholipid syndrome (APS). The addition of hydroxyeicosatetraenoic acid–phospholipids (HETE-PLs) to mice deficient in lipoxygenases promoted blood coagulation and restored hemostasis. Together, these data suggest that platelets and leukocytes generate networks of eoxPL molecules, which enhance blood coagulation and contribute to the excessive clotting observed in patients with APS.