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Phospholipid-esterified Eicosanoids Are Generated in Agonist-activated Human Platelets and Enhance Tissue Factor-dependent Thrombin Generation*[S]

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Here, a group of specific lipids, comprising phosphatidylethanolamine (PE)- or phosphatidylcholine (PC)-esterified 12*S*-hydroxyeicosatetraenoic acid (12*S*-HETE), generated by 12-lipoxygenase was identified and characterized. 12*S*-HETE-PE/PCs were formed within 5 min of activation by thrombin, ionophore, or collagen. Esterified HETE levels generated in response to thrombin were 5.85 ± 1.42 (PE) or 18.35 ± 4.61 (PC), whereas free was 65.5 ± 17.6 ng/4 × 10^7 cells (n = 5 separate donors, mean ± S.E.). Their generation was stimulated by triggering protease-activated receptors-1 and -4 and signaling via Ca^{2+} mobilization secretory phospholipase A2, platelet-activating factor-acetylhydrolase, *src* tyrosine kinases, and protein kinase C. Stable isotope labeling showed that they form predominantly by esterification that occurs on the same time scale as free acid generation. Unlike free 12*S*-HETE that is secreted, esterified HETEs remain cell-associated, with HETE-PEs migrating to the outside of the plasma membrane. 12-Lipoxygenase inhibition attenuated externalization of native PE and phosphatidylserine and HETE-PEs. Platelets from a patient with the bleeding disorder, Scott syndrome, did not externalize HETE-PEs, and liposomes supplemented with HETE-PC dose-dependently enhanced tissue factor-dependent thrombin generation *in vitro*. This suggests a role for these novel lipids in promoting coagulation. Thus, oxidized phospholipids form by receptor/agonist mechanisms, not merely as an undesirable consequence of vascular and inflammatory disease.

Eicosanoids are lipid signaling mediators generated by the action of lipoxygenase (LOX),[2] cyclooxygenase, or cytochrome P450 on free arachidonic acid (AA) hydrolyzed from membrane phospholipids (PLs) by phospholipase A2 (PLA2). They play important roles in both physiology and disease processes, including asthma, atherosclerosis, hypertension, inflammation, and cancer. Recently, their generation as part of larger complex molecules was indicated by observations that prostaglandin D2-glycerol is synthesized by cyclooxygenase-2 and that ionophore-stimulated monocytes generate 15-hydroxyeicosatetraenoic acid (15-HETE) attached to phosphatidylethanolamine (PE) (1–3). The formation of esterified eicosanoids suggests that they represent a new paradigm in eicosanoid biology whereby the complex molecule mediates signaling actions that are distinct from the free acid. In support, prostaglandin E-glycerol stimulates calcium mobilization, unlike free prostaglandin E2 (4). Thus, the identification and characterization of new esterified eicosanoids in mammalian cells represent important and clinically relevant goals.

PLs are the major structural constituent of mammalian cell membranes where they provide a permeability barrier as well as acting as important regulators of numerous cell functions, including signaling, secretion, motility, and internalization. Their spatial organization is tightly controlled and defines the structure and function of the membrane. Several years ago, PL peroxidation was observed in tissue from human diseases, including Alzheimer disease, atherosclerosis, and ischemia-reperfusion (5–9). This led to the idea that it was a common event in diverse inflammatory diseases, responsible for pathological features, including cell dysfunction and death. In support, a role for oxidized PLs in apoptosis has been suggested (10). PL peroxidation products generated *in vitro* by chemical oxidation comprise hundreds of related structures, assumed to

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Materials and Methods, Results, and Figs. S1–S5.

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[2] The abbreviations used are: LOX, lipoxygenase; AA, arachidonic acid; PL, phospholipid; sPLA2, secretory phospholipase A2; HETE, hydroxyeicosatetraenoic acid; 12-HpETE, precursor to 12*S*-HETE; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PS, phosphatidylserine; HPLC, high pressure liquid chromatography; PAR, protease-activated receptor; OOEPC, oleyloxyethylphosphocholine; PACOCF3, palmityl trifluoromethyl ketone; BEL, bromoenol lactone; MAFP, methyl arachidonyl fluorophosphonate; ACD, acid-citrate-dextrose; DMPE, di-14:0-phosphatidylethanolamine; DMPC, di-14:0-phosphatidylcholine; LC, liquid chromatography; MS/MS, tandem mass spectrometry; amu, atomic mass units; Z, benzyloxycarbonyl; NHS, *N*-hydroxysuccinimide; SAPE, stearoyl arachidonyl phosphatidylethanolamine; SAPS, stearoyl arachidonyl phosphatidylserine; $cPLA_2$, calcium-dependent PLA_2; $iPLA_2$, calcium-independent PLA_2.

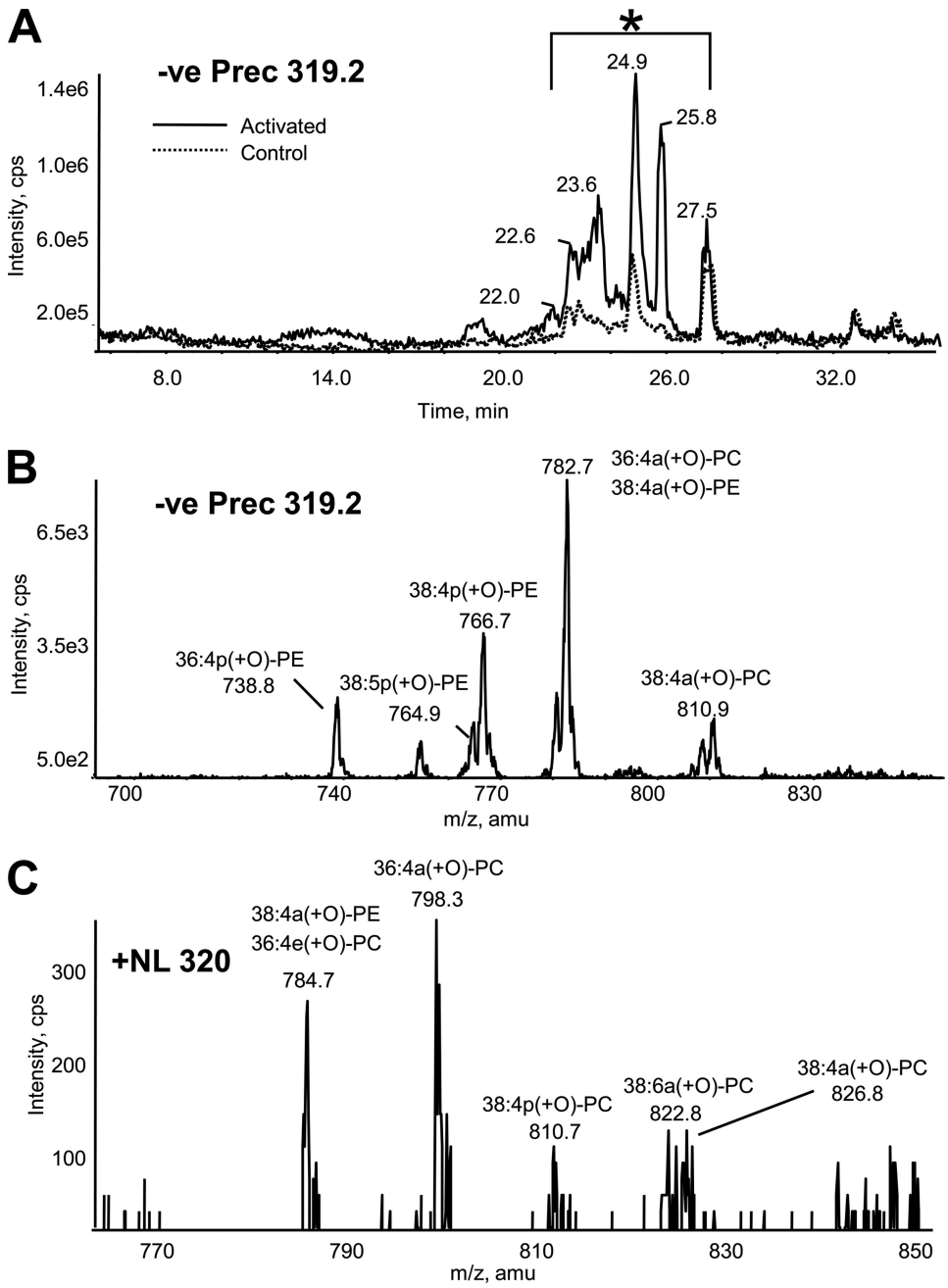


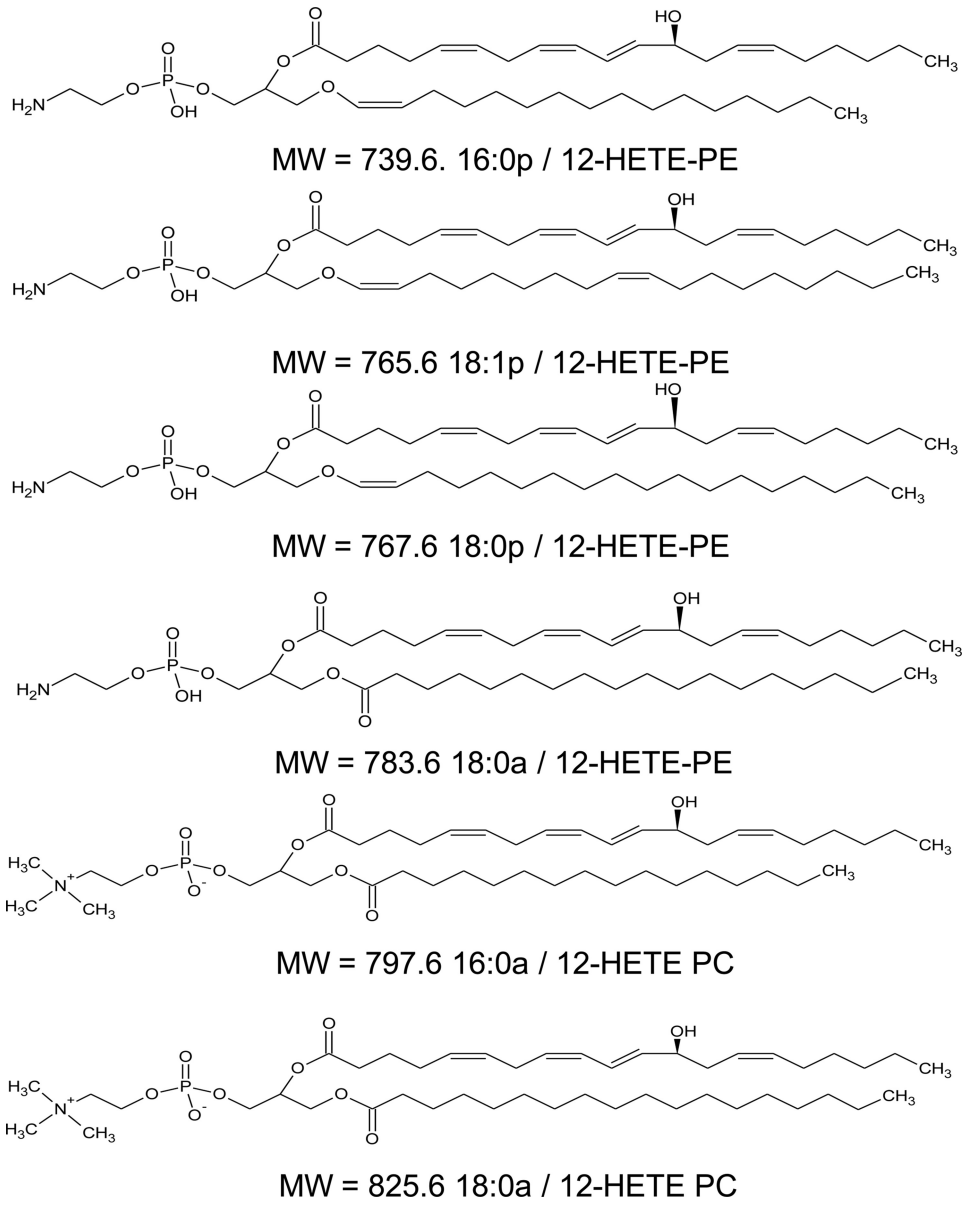
FIGURE 1. **Precursor scanning of platelet lipid extract identifies several ions that generate daughter ions with *m/z* 319.2 or neutral loss of 320 amu.** *A*, representative LC/MS/MS showing negative precursor scans of control (*dashed line*) or thrombin-activated (*solid line*) platelet lipid extracts. Total lipid extracts from washed human platelets activated with 0.2 unit/ml thrombin for 30 min at 37 °C were separated using LC/MS/MS as described under "Experimental Procedures," with online negative precursor scanning for *m/z* 319.2. *, region of LC trace where ions appear that are elevated by thrombin stimulation. *B*, identification of ions that generate *m/z* 319.2 daughter ions. Shown is a negative MS scan of region marked * in *A*. *C*, identification of ions that lose 320 amu in positive mode. Positive neutral loss LC/MS/MS of thrombin-activated platelet lipid extracts was carried out. Scan shows ions eluting between 21–27 min.

form via decomposition of PL hydroperoxides. Some possess potent signaling actions, including activation of intracellular signaling pathways, whereas others are ligands for lipid scavenger receptors, including CD36 (9, 11–14). Most studies on *ex vivo* diseased tissue measure short chain aldehydes such as 4-hydroxynonenal, which are end products of hydroperoxide decomposition, and the primary products formed are uncharacterized. Thus, formation of oxidized PLs *in vivo* has been generally considered an uncontrolled and undesirable pathological event, and the initial mechanisms responsible are unknown. We recently showed that human monocytes and murine macrophages generate oxidized PLs in response to agonist activation (2, 3). In these studies, preliminary identification of analogous lipids from agonist-stimulated platelets was presented. Here, we extend those earlier studies by presenting a detailed structural characterization of this family of lipids along with cellular localization and potential biological function.

EXPERIMENTAL PROCEDURES

Materials—Lipid and fatty acid standards were purchased from Avanti Polar Lipids (Alabaster, Alabama) or Cayman Chemical (Cayman Islands). HPLC grade solvents were from Fisher Chemicals. PAR-1 and -4 agonists were from Tocris Biosciences (Bristol, UK), platelet signaling inhibitors, PP2, rottlerin, staurosporine, oleyloxyethylphosphocholine (OOEPC), palmityl trifluoromethyl ketone (PACOCF3), bromoenol lactone (BEL), U73112, wortmannin, and methyl arachidonyl fluorophosphonate (MAFP) were from Calbiochem. All other reagents were from Sigma-Aldrich unless otherwise stated.

Isolation and Activation of Human Platelets—All blood donations were approved by the Cardiff University School of Medicine Ethics Committee and were with informed consent. Whole blood was collected from healthy volunteers or from a patient with Scott syndrome, free from nonsteroidal antiinflammatory drugs for at least 14 days into acid-citrate-dextrose (ACD; 85 mM trisodium citrate, 65 mM citric acid, 100 mM glucose) (blood:ACD, 8.1:1.9, v/v) and centrifuged at 250 × *g* for 10 min at room temperature. The platelet-rich plasma was collected and centrifuged at 900 × *g* for 10 min, and the pellet was resuspended in Tyrode's buffer (134 mM NaCl, 12 mM $NaHCO_3$, 2.9 mM KCl, 0.34 mM Na_2HPO_4, 1.0 mM $MgCl_2$, 10 mM Hepes, 5 mM glucose, pH 7.4) containing ACD (9:1, v/v). The platelets were washed by centrifuging at 800 × *g* for 10 min then resuspended in Tyrode's buffer at a concentration of $2 \times 10^8 \cdot ml^{-1}$. Platelets were acti-



SCHEME 1

vated at 37 °C in the presence of 1 mM $CaCl_2$ for specified time periods, with 0.2 unit·ml^{-1} thrombin, 10 μg/ml collagen, 10 μM A23187, 100 μM TFLLR-NH_2, or 150 μM AY-NH_2 before lipid extraction as below. Experiments involving signaling inhibitors included a 10-min preincubation step at 37 °C before activation with thrombin. In some experiments, $H_2{}^{18}O$ (CK Gas Products) was used to generate Tyrode's buffer, or 50 ng of 12-HETE-d8 was added to incubations. For separation of cells from microparticles and supernatants, activated samples were first centrifuged at 970 × *g*, then supernatants were re-spun at 16,060 × *g*. Concentrations of agonist used were at physiological or standard levels (15, 16).

Lipid Extraction—Where quantified, 10 ng each of 12-HETE-d8, di-14:0-phosphatidylethanolamine (DMPE), and/or di-14:0-phosphatidylcholine (DMPC) was added to samples before extraction, as internal standards. In some experiments hydroperoxides were reduced to their corresponding stable alcohols by the addition of 1 mM $SnCl_2$ for 10 min at room temperature. Lipids were extracted by adding a solvent mixture (1 M acetic acid, isopropyl alcohol, hexane (2:20:30, v/v/v)) 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 (17). After vortexing and centrifugation, lipids were recovered in the upper hexane layer. The samples were then reextracted by addition of an equal volume of hexane. The combined hexane layers were dried and analyzed for free or esterified HETEs using LC/MS/MS as below.

Precursor and Neutral Loss Scanning MS—Lipid extracts were separated by reverse phase HPLC using a Luna 3 μm C18 (2) 150 × 2-mm column (Phenomenex, Torrance, CA) with a gradient of 50–100% B over 10 min followed by 30 min at 100% B (A, methanol:acetonitrile:water, 1 mM ammonium acetate, 60:20:20; B, methanol, 1 mM ammonium acetate) with a flow rate of 200 μl min^{-1}. Settings were DP −140 V, CE −45 V. Spectra were acquired scanning Q1 from 600–1100 atomic mass units (amu) over 5 s with Q1 set to daughter ion of interest. For positive neutral loss scanning, polarity was reversed.

Free Acid HETE Quantitation Using LC/MS/MS—Samples were separated on a C18 Spherisorb ODS2, 5 μm, 150 × 4.6-mm column (Waters, Hertfordshire, UK) using a gradient of 50–90% B over 10 min (A, water:acetonitrile:acetic acid, 75:25:0.1; B, methanol:acetonitrile:acetic acid, 60:40:0.1) with a flow rate of 1 ml·min^{-1}. Products were quantitated by LC/MS/MS electrospray ionization on an Applied Biosystems 4000 Q-Trap using parent-to-daughter transitions of *m*/*z* 319.2 (HETE, $[M\text{-}H]^-$) to *m*/*z* 179 (12-HETE), 115 (5-HETE), 155 (8-HETE), 167 (11-HETE), 219 (15-HETE) and *m*/*z* 327 to 184 for 12-HETE-d8, with collision energies of −20 to −30 V. Products were identified and quantified using HETE positional isomers and 12-HETE-d8 standards run in parallel under the same conditions.

Synthesis of 18:0/HETE-PE and 16:0/HETE-PC Standards for Quantitation—64 μl 10 mM pentamethylchromanol (Sigma) in chloroform was added to 5 mg 16:0a/20:4-PC or 18:0a/20:4-PE in 1.5 ml of chloroform and then dried under N_2. The sample was incubated at 37 °C for 24 h, resuspended in 200 μl of methanol, and then the hydroperoxides were reduced using $SnCl_2$. Lipids were extracted by the addi-

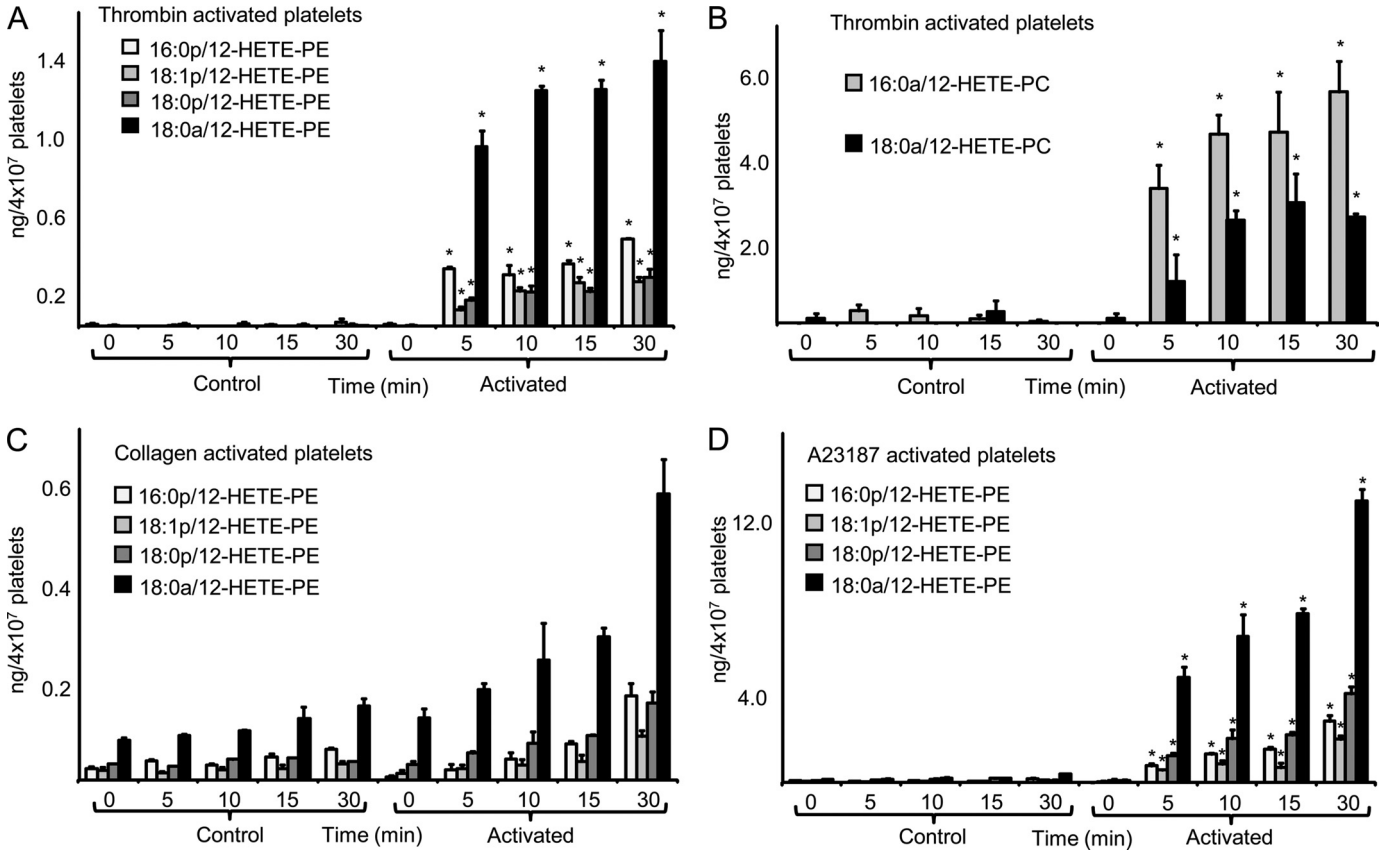


FIGURE 2. **12*S*-HETE-PE and -PCs are acutely generated in response to agonist stimulation of platelets.** Washed platelets were activated for varying times as described in Fig. 1, and lipids were extracted and analyzed using LC/MS/MS as described under "Experimental Procedures" for the presence of specific 12*S*-HETE-containing isomers. Levels are expressed as nanograms/4 × 10^7 platelets (n = 3, mean ± S.E.), with experiments repeated at least three times on different donors. *A*, platelets were activated using 0.2 unit/ml thrombin, and 12*S*-HETE-PEs were determined. *B*, platelets were activated using thrombin, and 12*S*-HETE-PCs were determined. *C*, platelets were activated using 10 μg/ml collagen. *D*, platelets were activated using 10 μM A23187.

tion of 800 μl of methanol, 2 ml of chloroform, 0.5 ml of water, with vortexing and centrifugation, dried, and then resuspended in methanol. 16:0a/HETE-PC and 18:0a/HETE-PE (containing six positional isomers) was purified using reverse phase HPLC. In the case of HETE-PEs, integration of peaks during elution was used to determine relative amounts of each isomer. Samples were purified on a Discovery® C18, 5-μm, 250 × 4.6-mm column (Supelco Analytical; Sigma-Aldrich) using a gradient of 50–100% B over 20 min (A, 100% water with 1 mM ammonium acetate; B, 100% methanol with 1 mM ammonium acetate) with a flow rate of 1 ml·min^{-1}. Quantitation of total HETEs or in each standard preparation was by absorbance at 235 nm, using $E_{1\ mM/1\ cm}$ = 28.1.

Synthesis of Biotinylated Standards—SAPE, DMPE, SAPS, and DMPS solutions were evaporated to dryness before the addition of 220 μl of chloroform and 110 μl of methanol. 6 mg of NHS-biotin was added followed by 3.3 μl of triethylamine. The resulting solution was left at room temperature for 30 min before purification by reverse phase HPLC as above. The percentage yield of each compound, SAPE-B, DMPE-B, SAPS-B, and DMPS-B, was found to be 69.4, 73.8, 55.3, and 49.6%, respectively.

Reverse Phase LC/MS/MS of PLs—Lipid extracts were separated by reverse phase HPLC using a Luna 3 μm C18 (2) 150 × 2-mm column (Phenomenex, Torrance, CA) with a gradient of 50–100% B over 10 min followed by 30 min at 100% B (A, methanol:acetonitrile:water, 1 mM ammonium acetate, 60:20:20; B, methanol, 1 mM ammonium acetate) with a flow rate of 200 μl min^{-1}. Settings were DP −140 V, CE −45 V, (AA-PE/PC and HETE-PE/PC lipids), DP −165 V, CE −64 V (biotinylated AA-PE) and DP −185 V, CE −62 V (biotinylated AA-PS). 12-HETE-PE and PC lipids were quantitated using standard curves generated by varying 18:0/HETE-PE or 16:0/HETE-PC, with a fixed amount of DMPE/DMPC, and using the daughter ion (179.2) specific for the 12-HETE positional isomer. Biotinylated PE and phosphatidylserine (PS) were also quantified using a standard curve, generated by varying SAPE-B and SAPS-B, with a fixed of amount DMPS-B, DMPE, and DMPE-B. The following transitions were monitored: 782.6 → 179.2, (18:0a/12-HETE-PE, 16:0a/12-HETE-PC), 810.9 → 179.2 (18:0a/12-HETE-PC), 766.6 → 179.2, (18:0p/12-HETE-PE), 764.6 → 179.2, (18:1p/12-HETE-PE), 738.6 → 179.2 (16:0p/12-HETE-PE), 762.6 → 343.2 634.5 → 227.2, (DMPE) 1008.6 → 319.2, (18:0a/HETE-PE-B), 992.6 → 319.2, (18:0p/HETE-PE-B), 990.6 → 319.2, (18:1p/HETE-PE-B), 964.6 → 319.2, (16:0p/HETE-PE-B), 766.6 → 303.2, (18:0a/AA-PE), 750.6 → 303.2, (18:0p/AA-PE) 748.6 → 303.2, (18:1p/AA-PE), 722.6 → 303.2, (16:0p/AA-PE), 992.6 → 303.2, (18:0a/AA-PE-B), 976.6 → 303.2, (18:0p/AA-PE-B), 974.6 → 303.2, (18:1p/AA-PE-B), 948.6 → 303.2 (16:0p/AA-PE-B), 1036.6 → 303.2 (18:0a/AA-PS-B), 860.6 → 227.2 (DMPE-B) and 904.6 → 227.2 (DMPS-B). For identification, product ion spectra were obtained at the

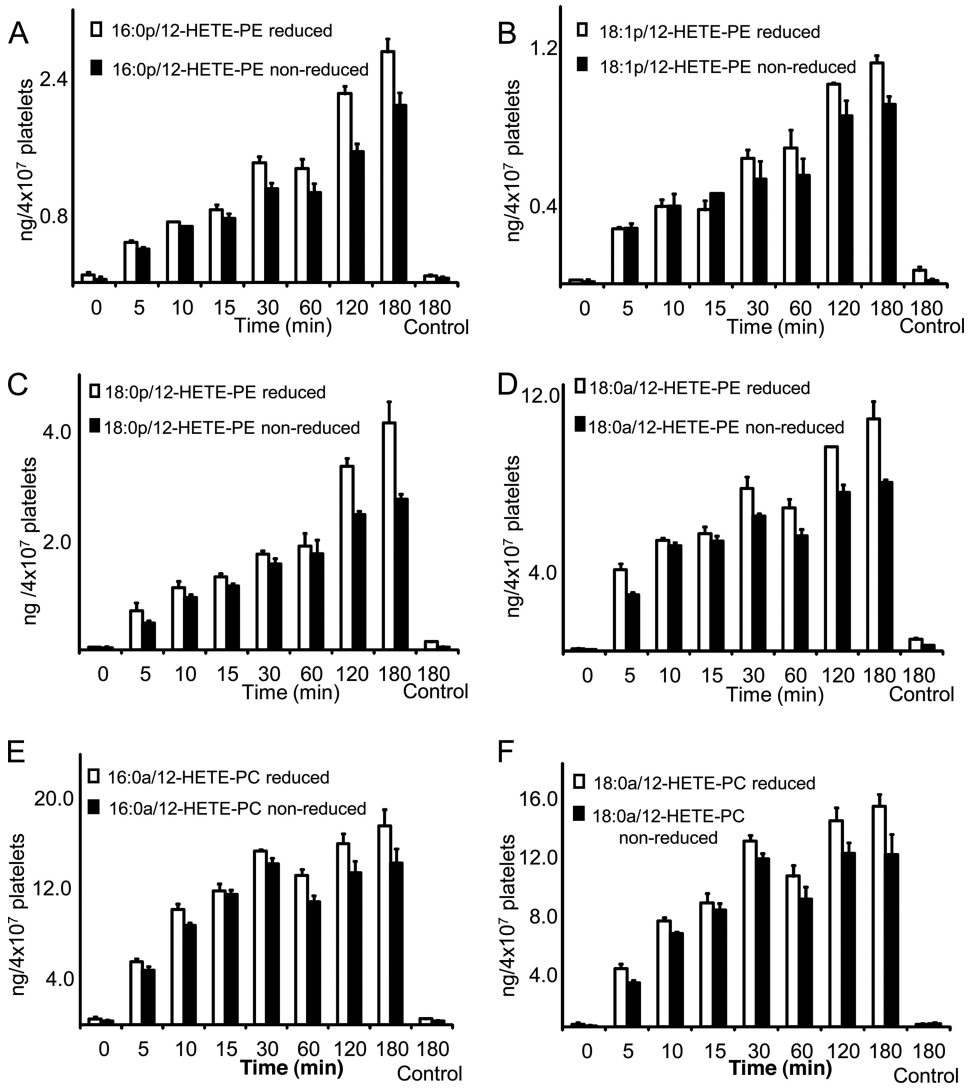


FIGURE 3. **Platelets efficiently reduce PL hydroperoxides to hydroxides following their generation.** *A–F*, platelets were activated using 0.2 unit/ml thrombin for 15 min, then lipids were extracted with or without $SnCl_2$ reduction, as described under "Experimental Procedures." Levels are expressed as nanograms/4 × 10^7 platelets ($n = 3$, mean ± S.E.).

apex of the MRM transitions, with the MS operating in ion trap mode. Scans were acquired over 0.65 s, with a linear ion trap fill time of 200 ms and Q0 trapping.

Determination of PE and PS Externalization by Biotinylation—Washed human platelets were activated using 0.2 unit/ml thrombin as before for 30 min, before addition of 1.5 mg/ml sulfo-NHS-biotin (Pierce) for 10 min at room temperature. The platelets were then centrifuged at 970 × *g*, the supernatant removed, and cells were resuspended in fresh Tyrode's buffer and lipids extracted as before, after the addition of 10 ng of biotinylated PL standards. Biotinylated 12-HETE-PE, AA-PE, and AA-PS were detected at 226.3 amu higher than native lipid and were identified based on characteristic daughter ions for Sn-1 and Sn-2 lipids on MS/MS spectra obtained at apex of peaks during LC separation.

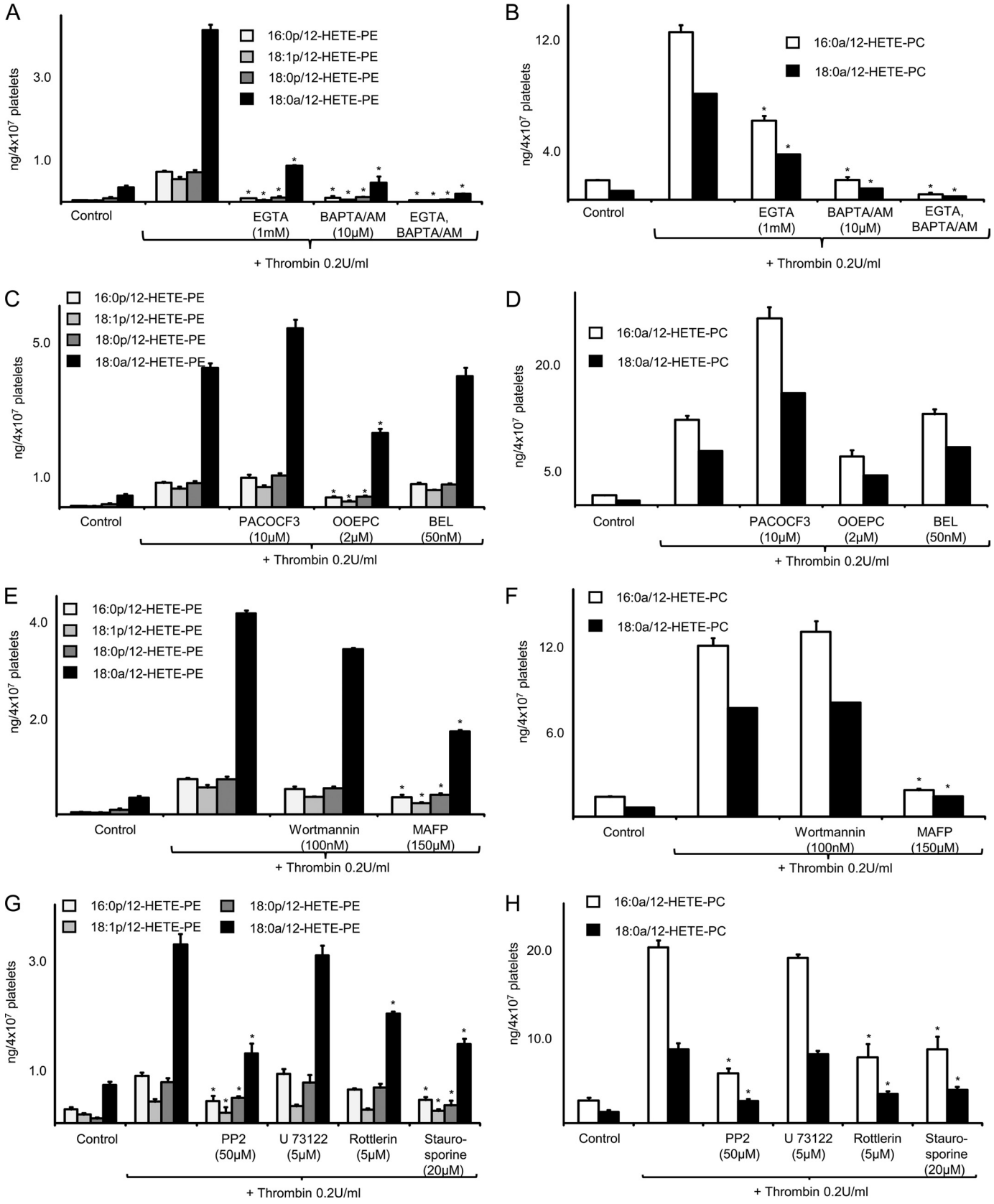
Calibrated Automated Thrombography—Whole human blood was taken into corn trypsin inhibitor (20 μg/ml) and sodium citrate (4%). Plasma was obtained by centrifugation at 3000 rpm for 15 min. The plasma was aspirated and spun again to obtain platelet-rich plasma. Liposomes were generated as follows. Varying amounts of 16:0/20:4-PC or 16:0/HETE-PC generated by air oxidation and purified as described above were added to the lipid extract from unactivated control platelets (human platelets were isolated as above and immediately extracted) in a glass vial, and then the solvent was evaporated under N_2. 20 mM HEPES, 140 mM NaCl buffer was added, and the sample was vortexed. Liposomes were then prepared by 10 freeze-thaw cycles followed by passing through Liposofast miniextruder using 100-nm pore membranes (Avestin, Ottawa, ON, Canada) 29 times. Trigger solution was prepared by mixing 30 μl of 1 nM Innovin tissue factor, 870 μl of working buffer (5 g/liter bovine serum albumin in Hepes/NaCl buffer as above), and 100 μl of liposomes. The final concentration of lipid in the assay was adjusted to be equivalent to 2×10^8 platelets·ml^{-1}. To determine thrombin generation, 80 μl of platelet-rich plasma was added per well, in a 96-well plate. 20 μl of trigger solution, with or without 16:0/20:4-PC or 16:0/HETE-PC, was added in triplicate. For each sample set, a separate calibrator well, containing thrombin calibrator (α_2-macroglobulin-thrombin) instead of liposomes was used. The 96-well plate was placed into a Fluoroskan Ascent reader (Thermo Electron Corporation), and automated addition of 20 μl of fluorogenic substrate for thrombin (Z-Gly-Gly-Arg) was initiated. The thrombin concentration reported represents the maximum amount of thrombin present during the assay period.

Statistical Analysis—The figures show representative data sets ($n = 3$, mean ± S.E.) with experiments conducted at least three times on different donors. Combining data sets from three donors was not feasible because of the high level of intra-donor variation in absolute levels of HETE-PLs. Statistics used Students' *t* test, with $p < 0.05$ being considered significant.

RESULTS

Precursor-LC/MS/MS Scanning of Platelet Lipid Extracts—To characterize esterified eicosanoids generated by agonist-receptor signaling, platelets were chosen because they oxidize AA using either cycloxygenase-1 or 12-LOX. Lipid extracts from thrombin-activated platelets were subject to precursor-LC/MS/MS, which fragments using collision-in-

duced dissociation and scans for a functional group of interest in Q3. With Q3 set at 319.2, the carboxylate anion for HETE, a series of ions that were decreased/absent in control platelets were observed (Fig. 1*A*). Fig. 1*B* shows the *m*/*z* values of these ions, comprising a series of lipids between *m*/*z* 738 and 810. These are consistent with PE or PC, PLs that contain



HETE, although for some ions there are more than one candidate, as indicated on Fig. 1*B* (18). Most PL classes form facile negative ions, but some ionize better in positive mode (*e.g.* PC). Thus, positive neutral loss scanning for the loss of the eicosanoid (320 amu) was also performed. Here, ions corresponding to a number of PE or PC PLs containing HETE are again observed, as shown on Fig. 1*C*. The *m*/*z* 798 and 826 are consistent with 16:0a/12-HETE-PC and 18:0a/HETE-PC $[M+H]^+$, which appear in negative precursor 319 scans as *m*/*z* 782 and 810 $[M\text{-}CH_3]^-$, respectively.

Structural Characterization of Esterified HETEs—Several approaches were utilized which are detailed in the supplementary material. These included normal phase separation of platelet PL classes followed by LC/MS/MS to determine headgroup of each ion containing HETE, then generation of MS/MS spectra during online LC separation of each ion in negative or positive mode. Using these methods, the esterified HETEs were identified as 16:0p/12*S*-HETE-PE, 18:1p/12*S*-HETE-PE, 18:0p/12*S*-HETE-PE, 18:0a/12*S*-HETE-PE, 16:0a/12*S*-HETE-PC, and 18:0a/12*S*-HETE-PC (Scheme 1). The positional and enantiomeric specificity of the HETE implicates platelet 12-LOX in their generation.

Temporal Generation of Esterified 12S-HETEs by Human Platelets—12*S*-HETE-PEs and -PCs formed immediately on thrombin activation, with ~100-fold elevations by 15 min and levels continuing to rise at a slower rate up to 30 min, similar to free 12*S*-HETE (Fig. 2, *A* and *B*, and data not shown). Their levels were of the same magnitude as free 12*S*-HETE, indicating that this is a significant metabolic pathway for 12-LOX (5.85 ± 1.42 (PE), 18.35 ± 4.61 (PC), 65.5 ± 17.6 (free) ng/4 × 10^7 cells, $n = 5$ separate donors, mean ± S.E.). The high S.E. values indicate a high degree of variation between genetically unrelated donors. The lipids were formed from the six most abundant AA-containing PE and PC molecular species present (data not shown). Thus, the pattern of products is logically dictated by the substrates available in the plasma membrane. Ionophore also stimulated PL oxidation, although collagen was less effective (Fig. 2, *C* and *D*) (2). The enzymatic precursor to esterified 12*S*-HETE, the hydroperoxy 12-HpETE, is unstable and may decompose to multiple products, including esterified carbonyls, isoprostanes, epoxides, and short chain aldehydes. To determine whether platelets generated 12-HpETE-PLs, yields of esterified 12*S*-HETE with/without reduction of lipid extracts were compared. Up to 60 min after activation, all esterified 12-H(p)ETE was already reduced (to HETE), presumably via glutathione peroxidases, thus limiting its decomposition (Fig. 3). However, beyond that time, reduction was slower, and at later time points formation and decomposition of HpETE-PLs may occur.

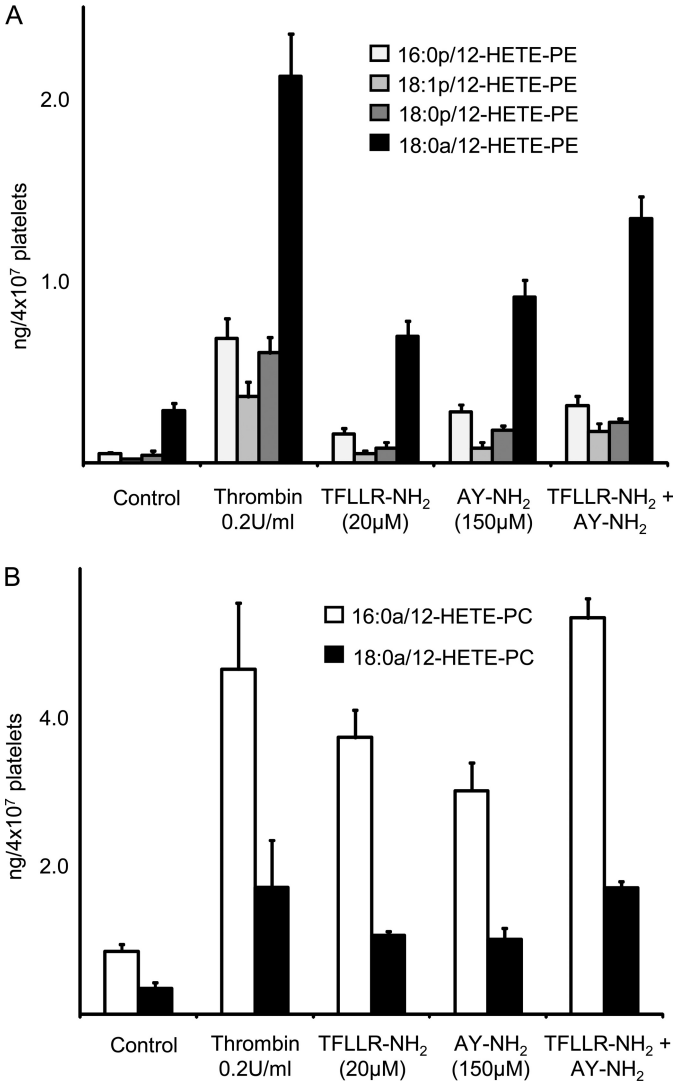


FIGURE 5. **12*S*-HETE-PEs and -PCs are generated via PAR-1 and PAR-4 receptor stimulation.** Washed human platelets were activated with a PAR-1 agonist, TFLLR-NH_2 (100 μM) and/or a PAR-4 agonist AY-NH_2 (150 μM) for 30 min before lipid extraction and LC/MS/MS analysis as described under "Experimental Procedures." Activation using 0.2 unit/ml thrombin for 30 min acted as a positive control. Levels are expressed as nanograms/4 × 10^7 platelets ($n = 3$, mean ± S.E.). *B*, 12*S*-HETE-PC generation.

Receptor and Signaling Pathways Regulating Esterified 12S-HETE Formation—The receptor-dependent pathways regulating acute PL peroxidation were identified using pharmacological inhibitors. Thrombin stimulation of 12*S*-HETE-PE or -PC generation was inhibited by blockers of calcium mobilization (EGTA/BAPTA-AM), *src*-tyrosine kinases (PP2, staurosporine), protein kinase C (rottlerin), or sPLA2 (OOEPC), whereas inhibition of c-PLA2 or i-PLA2 (PACOCF3, BEL), PLC (U73112), or phosphoinositol 3-kinase (wortmannin) was

FIGURE 4. **Ca^{2+}, *src* tyrosine kinases, protein kinase C, and sPLA2 are required for thrombin-stimulated generation of 12*S*-HETE-PE and -PC.** Washed human platelets were incubated for 10 min with each inhibitor prior to thrombin activation (0.2 unit/ml for 30 min) before lipid extraction and analysis as described under "Experimental Procedures." Levels are expressed as nanograms/4 × 107 platelets ($n = 3$, mean ± S.E.), with experiments repeated at least three times on different donors. *A*, 12*S*-HETE-PE generation by platelets incubated with 1 mM EGTA and/or 10 μM BAPTA/AM. *B*, 12*S*-HETE-PC generation by platelets incubated with 1 mM EGTA and/or 10 μM BAPTA/AM. *C*, 12*S*-HETE-PE in platelets incubated with 10 μM PACOCF3, 2 μM OOEPC, or 50 nM BEL. *D*, 12*S*-HETE-PC in platelets incubated with 10 μM PACOCF3, 2 μM OOEPC, or 50 nM BEL. *E*, 12*S*-HETE-PE in platelets incubated with 100 nM wortmannin or 150 μM MAFP. *F*, 12*S*-HETE-PC in platelets incubated with 100 nM wortmannin or 150 μM MAFP. *G*, 12*S*-HETE-PE generated by platelets incubated with 50 μM PP2, 5 μM U73122, 5 μM rottlerin, or 20 μM staurosporine. *H*, 12*S*-HETE-PC generated by platelets incubated with 50 μM PP2, 5 μM U73122, 5 μM rottlerin, or 20 μM staurosporine prior to activation.

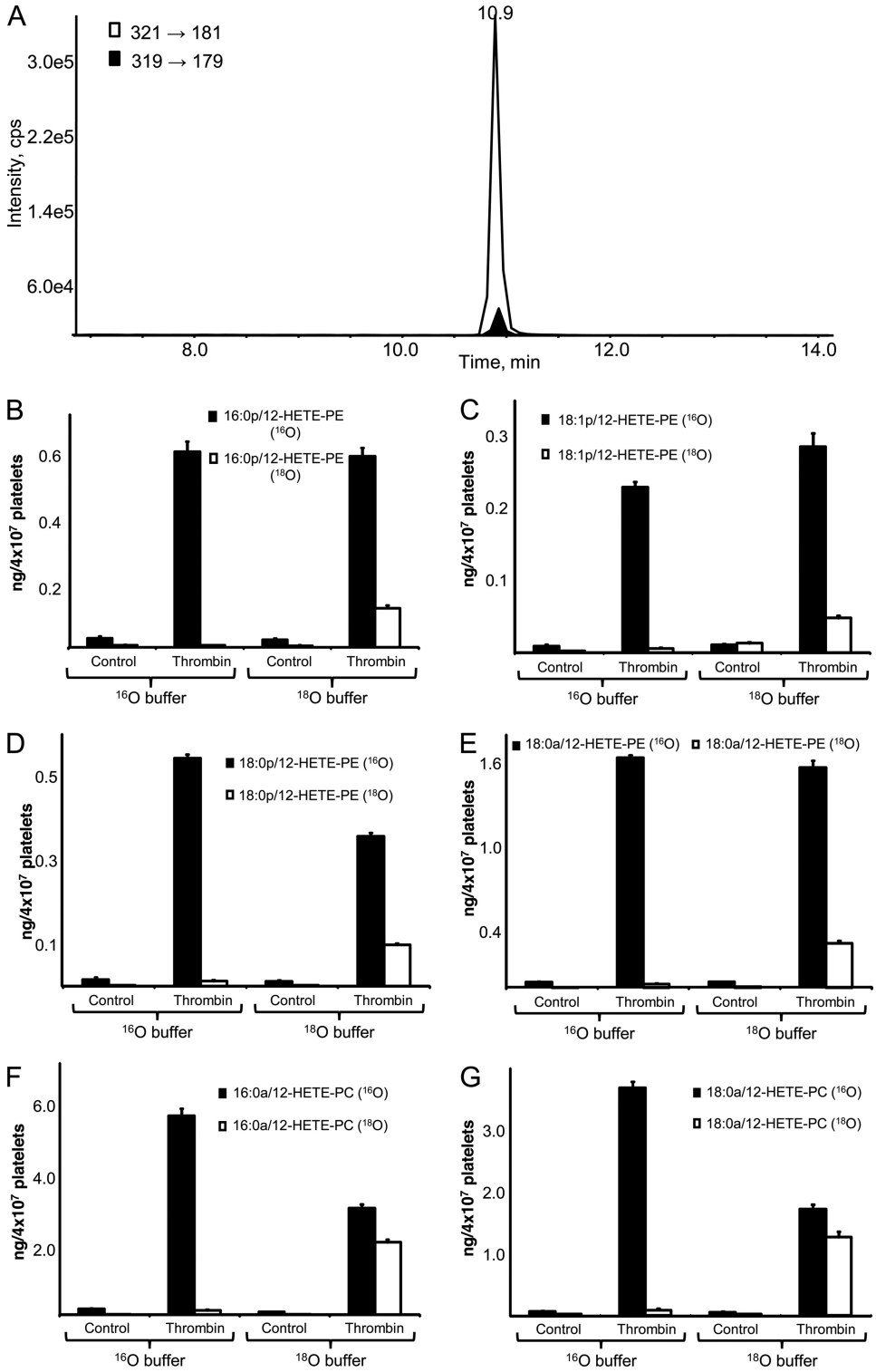


FIGURE 6. **Investigations into the mechanism of esterified HETE formation.** Platelets were activated by thrombin (0.2 unit/ml for 30 min) in Tyrode's buffer made with either $H_2{}^{16}O$ or $H_2{}^{18}O$, as described under "Experimental Procedures," before lipid extraction and analysis using LC/MS/MS. Levels are expressed as nanograms/4 × 10^7 platelets (n = 3, mean ± S.E.). *A*, free 12-HETE generated by thrombin-activated platelets contains predominantly ^{18}O. Representative LC/MS/MS trace of 12-[^{16}O]HETE (m/z 319–179) and [^{18}O]HETE (m/z 321–181) from platelets activated in $H_2{}^{18}O$-containing buffer is shown. *B–E*, 12*S*-HETE-PEs were analyzed as described under "Experimental Procedures" with transitions corresponding to an increase of 2 amu to detect ^{18}O containing lipids. *F* and *G*, 12*S*-HETE-PCs were analyzed as described under "Experimental Procedures" with transitions corresponding to an increase of 2 amu for ^{18}O detection.

without effect (Fig. 4). MAFP, an inhibitor of several enzymes with PLA2 activity (including platelet-activating factor-acetylhydrolase, fatty acid amylhydrolase, cPLA2, and iPLA2) significantly inhibited 12*S*-HETE-PLs (Fig. 4, *E* and *F*). Finally, the PAR-1 agonist TFLLR-NH_2 and the PAR-4 agonist AY-NH_2 stimulated 12*S*-HETE-PE and -PC to a similar level, with additive effects implicating both of the platelet thrombin receptors (Fig. 5). The data indicate that formation of these lipids is tightly regulated by receptor and intracellular signaling pathways.

Studies on Mechanism of 12-LOX Oxidation of Free or Esterified AA in Human Platelets—Esterified 12*S*-HETEs could form either by direct oxidation of PL or by esterification of 12*S*-HETE into lyso-PL. To distinguish, experiments were conducted in buffer containing $H_2{}^{18}O$ (19). When AA is hydrolyzed from PLs by PLA2, one atom of oxygen from H_2O is incorporated at the carboxyl group. Thus, 12-LOX-derived free 12*S*-HETE will have a mass increase of 2 amu. During fatty acid reesterification into PL, one oxygen atom is lost. Thus, if formed by esterification of free HETE, ~50% of the resulting PL will demonstrate a mass increase (19). In contrast, little incorporation should occur if the lipids are generated by direct oxidation of intact PL. As expected, 95% of free 12*S*-HETE was labeled with ^{18}O (Fig. 6*A*). In contrast 40–50% of HETE-PC and 15–20% of the HETE-PEs were labeled (Fig. 6, *B–E*). In addition, we and others found that recombinant platelet 12-LOX was unable to oxidize intact PE directly to any appreciable extent (20) (data not shown). These results suggest that the lipids form primarily via esterification of newly formed 12*S*-HETE. Finally, platelets were spiked with exogenous 12-HETE-d8, at amounts similar to what is generated during platelet activation. Little was incorporated into either PE or PC (Fig. 7). The data indicate the existence of a very tight coupling between endogenous

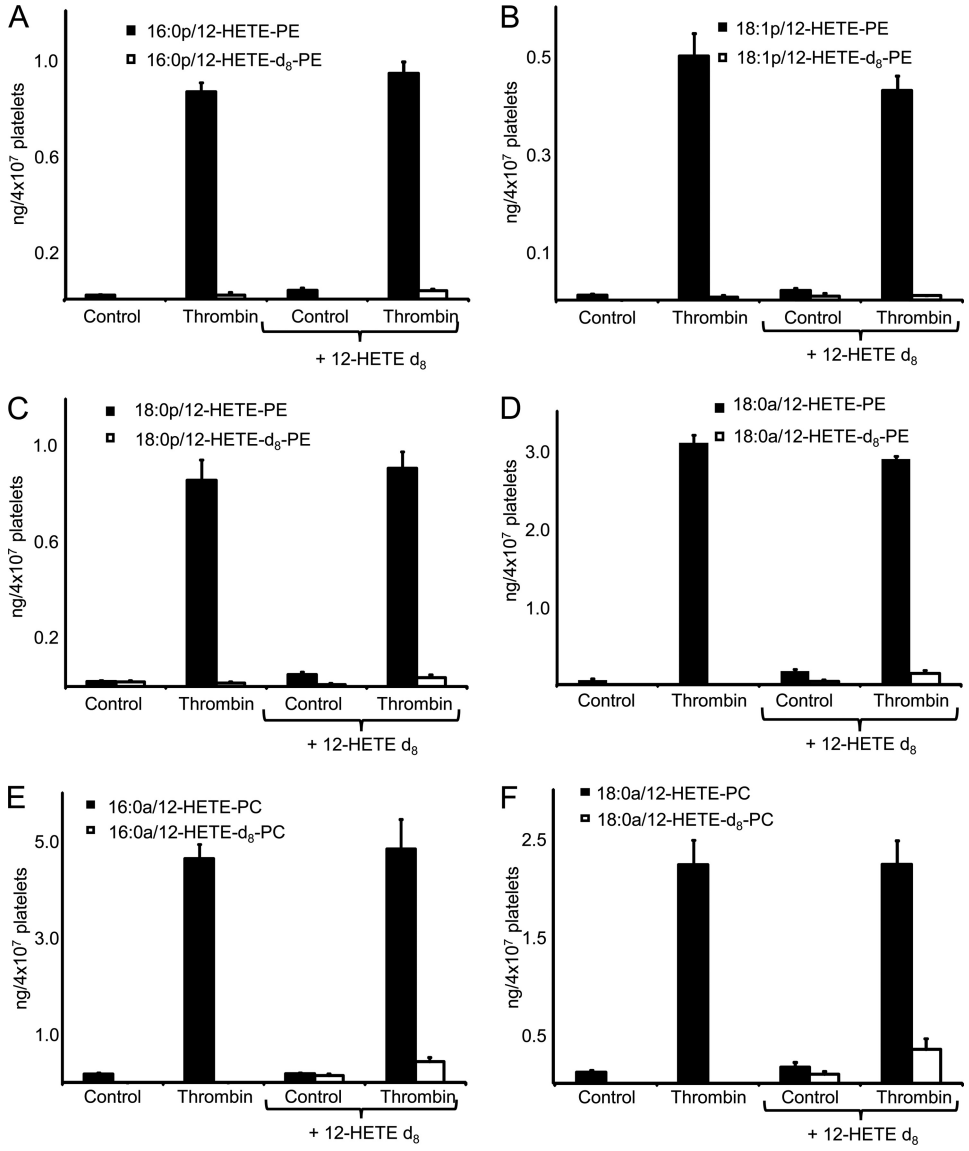


FIGURE 7. **12-HETE-d8 is only slightly incorporated into PC or PE during thrombin activation of platelets.** Platelets were activated with thrombin in the presence of 50 ng of 12-HETE-d8 before lipid extraction and analysis. Levels are expressed as nanograms/4 × 10^7 platelets (n = 3, mean ± S.E.). *A–D*, 12*S*-HETE-PEs generated by platelets contain negligible amounts of exogenous 12-HETE-d8. *E* and *F*, 12*S*-HETE-PCs generated by platelets also incorporate a low proportion of 12-HETE-d8.

generation of 12-HETE and its esterification into PC, with which exogenous 12-HETE-d8 cannot effectively compete.

Cellular Localization and Externalization of Esterified 12S-HETE in Healthy and Scott Syndrome Platelets—The majority of the PL-esterified 12*S*-HETE was retained by the cells, with only small amounts appearing in either microparticles or supernatant (Fig. 8, *A* and *B*). This is different from free 12-HETE, of which ~80% was released and suggests a distinct function for these lipids (Fig. 8*C*).

In most mammalian cells, PC is the major external facing PL, whereas aminophospholipids including PE and PS are on the inner leaflet. PE accounts for the vast majority of the inner membrane PL, with PS being a relatively minor component. During platelet activation, aminophospholipids translocate to the outer leaflet, where they contribute to development of a surface that binds coagulation factors (21–24). Specifically, fibrin is formed by the cleavage of fibrinogen by thrombin, which in turn is generated by cleavage of prothrombin by the prothrombinase complex (FXa/FVa). Similarly, FX is converted to FXa by the tenase complex (FIXa/FVIIIa). All of these factors bind to aminophospholipids externalized on platelet activation, substantially enhancing their activity. We wondered whether HETE-PLs could also participate in this event because chemical oxidation of a leukemic cell line was recently reported to enhance thrombogenesis *in vitro* (25). Although 12*S*-HETE-PCs may be generated on the external side of the cell, translocation of PE, PS, and 12*S*-HETE-PEs must occur if they are to support coagulation. To examine this, extracellular-facing aminophospholipid was derivatized by a cell-impermeable biotinylation reagent, sulfo-NHS-biotin, then detected using LC/MS/MS with mass addition of 226.3 amu. The identities of the adducts were verified by their characteristic MS/MS spectra, which in the case of 18:0a/12*S*-HETE-PE-biotin, contained ions at m/z 179, 319 (12-HETE $[M\text{-}H]^-$), 283 (stearate $[M\text{-}H]^-$), 366 (biotin-PE-phosphate $[M\text{-}H]^-$), 688 $[M\text{-}H\text{-}R^2CHCO_2]^-$, and 706 $[M\text{-}H\text{-}R^2CH{=}C{=}O]^-$ (Fig. 9*A*). Using this assay, we found that thrombin stimulated formation of the four biotinylated-12*S*-HETE-PE, indicating their exposure on the outer leaflet, and this was inhibited by the 12-LOX inhibitor esculetin as expected (Fig. 9*C*).

Scott syndrome is a rare bleeding disorder characterized by the inability to support coagulation on the surface of the platelet, with the mechanism proposed to be due to lack of aminophospholipid externalization, as measured by annexin V binding (26–29). As expected, we found that thrombin-stimulated Scott syndrome platelets externalized far less PE and did not externalize PS, but also did not externalize 12-HETE-PE, even though these lipids were generated in amounts fairly similar to platelets from healthy volunteers, at 4.4 ng of 12-HETE-PE and 9.78 ng of 12-HETE-PC (nanograms/4 × 10^7 cells, n = 2 independent experiments on different occasions) (Figs. 9, *C* and *D*, 10, *A* and *B*). Finally, esculetin also significantly inhibited externalization of non-oxidized PE and PS molecular species in 50% of our healthy donor pool (n = 10 separate donors) (Fig. 10, *A* and *B*).

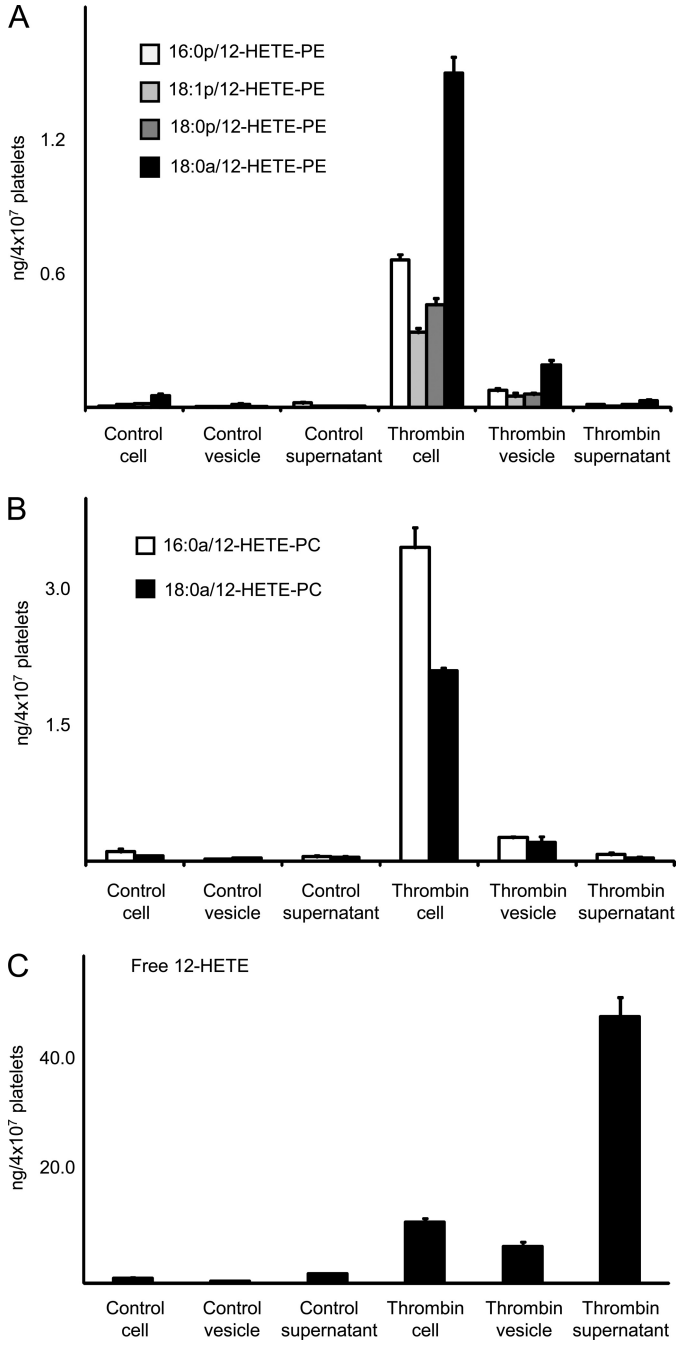


FIGURE 8. **12*S*-HETE-PLs are retained by platelets, and free 12-HETE is primarily secreted from the cell.** Washed human platelets were activated with 0.2 unit/ml thrombin for 30 min before centrifugation at 970 × *g*. The supernatant was then centrifuged at 16,060 × *g* to pellet microparticles before lipid extraction and analysis by LC/MS/MS ($n = 3$, mean ± S.E.). *A* and *B*, 12-HETE-PE and -PCs remain cell-associated. *C*, the majority of free 12-HETE is secreted from platelets.

Therefore, 12-LOX may also regulate native aminophospholipid externalization, although the reasons for this variation remain to be explored.

Role of Oxidized Phospholipids in Coagulation—Thrombin generation was measured in the presence of liposomes made from unactivated platelet lipids, supplemented with amounts of either 16:0/20:4-PC or 16:0/HETE-PC found in activated human platelets. HETE-PC was chosen for this experiment because HETE-PE might stimulate coagulation due to the PE headgroup rather than the HETE moiety. The experiments used a mixed isomer preparation that included equal amounts of all six positional isomers because purified 12-HETE-PE is not yet available. Total lipid concentrations were adjusted to be equivalent to a typical platelet concentration of $2 \times 10^8 \cdot ml^{-1}$ plasma. Thus, we generated liposome preparations that represent both the lipid amount and content of human platelets and contained amounts of 16:0/HETE-PC that are found in thrombin-stimulated cells. Human platelet-poor plasma was used as a source of factors, with the coagulation reactions initiated by addition of tissue factor (5 pM) in the presence of corn trypsin inhibitor to prevent contact activation. Liposomes containing 16:0a/HETE-PC caused a dose-dependent increase in thrombin generation, which was not seen with the control liposomes, containing 16:0/20:4-PC (Fig. 10*C*). This indicates that physiological concentrations of HETE-containing PCs enhance the activity of the prothrombinase complex.

DISCUSSION

In this study, a lipidomic approach identified novel families of esterified eicosanoids, comprising 12-HETE attached to either PE or PC, and their cell biology was characterized. The lipids belong to a class of molecule termed oxidized phospholipid, previously only found in diseased tissue such as atheroma. Up until now, they were assumed to be generated by nonspecific oxidation reactions as a consequence of tissue-damaging inflammation. In contrast, specific molecular families of oxidized PL form on acute receptor/agonist-dependent activation of human platelets, indicating that they are physiological products and of likely importance in cell signaling during both health and disease.

Formation of analogous lipids from human monocytes and murine macrophages was recently described (2, 3). However, in those cells that contain a different LOX isoform, generation in response to physiological agonist/receptor dependent signaling was not shown. Furthermore, 12/15-LOX (15-LOX1) generated HETE-PEs by direct oxidation of membrane PL rather than by esterification as found for platelets. The 12/15-LOX has long been known capable of oxidation of PLs *in vitro*, in contrast to the platelet enzyme (30, 31). A further distinction relates to the formation of HETE-PCs in platelets, but not monocytes or macrophages. This may be due to the distinct mechanisms of formation in different cell types. Specifically, in monocytes 12/15-LOX directly oxidizes PE, the predominant substrate available on the inner leaflet of the plasma membrane. In contrast, as PC is a major source of AA in platelets, lyso-PC may be the predominant substrate available for reesterification of 12-HETE following its formation. Our earlier studies indicated that 12-HETE-PEs are formed by human platelets in response to collagen; however, those studies focused on the PE class and did not describe HETE-PCs (2). Furthermore, this study includes the full structural characterization of the platelet lipids, along with biological and functional data.

The predominance of esterified 12*S*-HETE over other positional or stereoisomers indicates that the lipids are generated by platelet 12-LOX, an enzyme that belongs to a family of lipid-oxidizing enzymes expressed in mammals predominantly by immune cells and platelets (32). They are traditionally known as a source of free eicosanoids, oxidizing AA that has been released by PLA2,

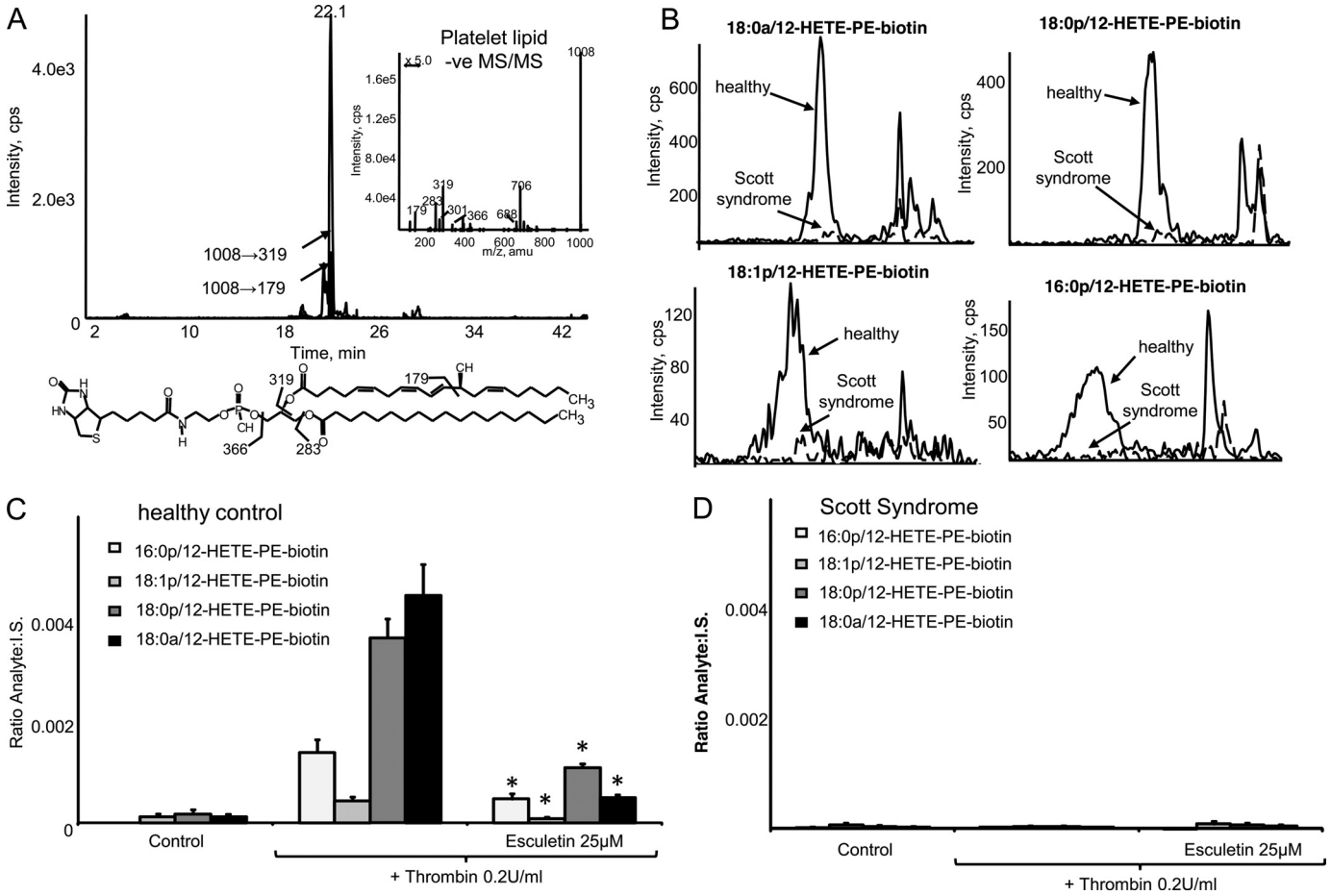


FIGURE 9. **Externalization of PE, PS, and HETE-PEs in healthy and Scott syndrome patients.** Washed human platelets were activated using 0.2 unit/ml thrombin for 30 min before the addition of 1.5 mg/ml sulfo-NHS-biotin for 10 min at room temperature. Platelets were then centrifuged at 970 × *g*, the supernatant was removed, and then cells were resuspended in fresh Tyrode's buffer and lipids extracted ($n = 3$, mean ± S.E.). *A*, negative ion LC/MS/MS of *m/z* 1008 → 319 and 1008 → 179 from biotinylated thrombin-activated platelet lipid extract, showing detection of 18:0a/12-HETE-PE-biotin. *Inset*, negative ion MS/MS spectrum at 22.1 min from biotinylated platelet lipid. *B*, representative LC/MS/MS traces showing the absence of biotinylated lipids in platelets with Scott syndrome versus healthy subject. Samples were generated from either healthy or Scott syndrome platelets and analyzed using LC/MS/MS as in *B*. *C*, externalization of oxidized PE in platelets from a healthy donor. Biotinylated PE was measured in activated platelet lipid extracts with and without 10-min preincubation with 25 μM esculetin ($n = 3$, mean ± S.E.), using LC/MS/MS as described under "Experimental Procedures." *D*, defective externalization of oxidized PE in platelets from a patient with Scott syndrome. Scott syndrome platelets were activated and analyzed using LC/MS/MS as in *B* ($n = 3$, mean ± S.E.).

forming bioactive lipids, including leukotrienes and HETEs, and acute formation of esterified eicosanoids is not well characterized, particularly in response to receptor-dependent agonists.

The lipids were generated via activation of PAR-1/4 receptors and utilizing several intracellular signaling intermediates including Ca^{2+}, protein kinase C, sPLA2, and *src* tyrosine kinases. A previous study showed that cPLA2 is required for generation of free 12*S*-HETE; however, this was not the case for 12-HETE-PLs because PACOCF3 was without effect (Fig. 4) (33). Inhibition by MAFP suggests that AA destined for HETE-PL generation is hydrolyzed by a PLA2 that could include platelet-activating factor-acetylhydrolase, fatty acid amylhydrolase, cPLA2, or iPLA2; however, the latter two enzymes were excluded by the use of PACOCF3 and BEL. This suggests that distinct phospholipase enzymes are involved in generation of free *versus* esterified 12*S*-HETE. The fast temporal generation also indicates a tight coupling between pathways that generate PL-bound 12*S*-HETE by esterification (*e.g.* a phospholipase, 12-LOX and fatty acyl-CoA ligase). In support, little exogenously added 12*S*-HETE-d8 was incorporated into PLs during the time scale of 12*S*-HETE-PE or -PC generation even though esterification was fast, following the same time scale as free 12*S*-HETE generation (Fig. 6 and data not shown). Although 12*S*-HETE-PC appeared to form exclusively by esterification of free 12*S*-HETE, it was less clear how 12*S*-HETE-PE generation occurred (Fig. 6). However, we believe it to be unlikely that these lipids form via direct oxidation because recombinant platelet 12-LOX did not efficiently oxidize 18:0a/20:4-PE *in vitro* (20) (data not shown). The sPLA2 inhibitor OOEPC inhibited generation of both free and esterified 12-HETE (Fig. 4, *C* and *D*). This enzyme can modulate platelet activation via AA-independent mechanisms and was suggested not to be a source of free fatty acid for thromboxane generation in these cells, although this has not been conclusively shown (34, 35). Thus, sPLA2 may be involved either through mediating AA release or by formation of lyso-PC, a known inhibitor of platelet activation (36).

Beyond 1 h after activation, platelets became less able to reduce 12*S*-HpETE-PE or -PC to the more stable HETE forms (Fig. 3). Hydroperoxylipids are unstable and considered the precursors for most decomposition products of lipid peroxida-

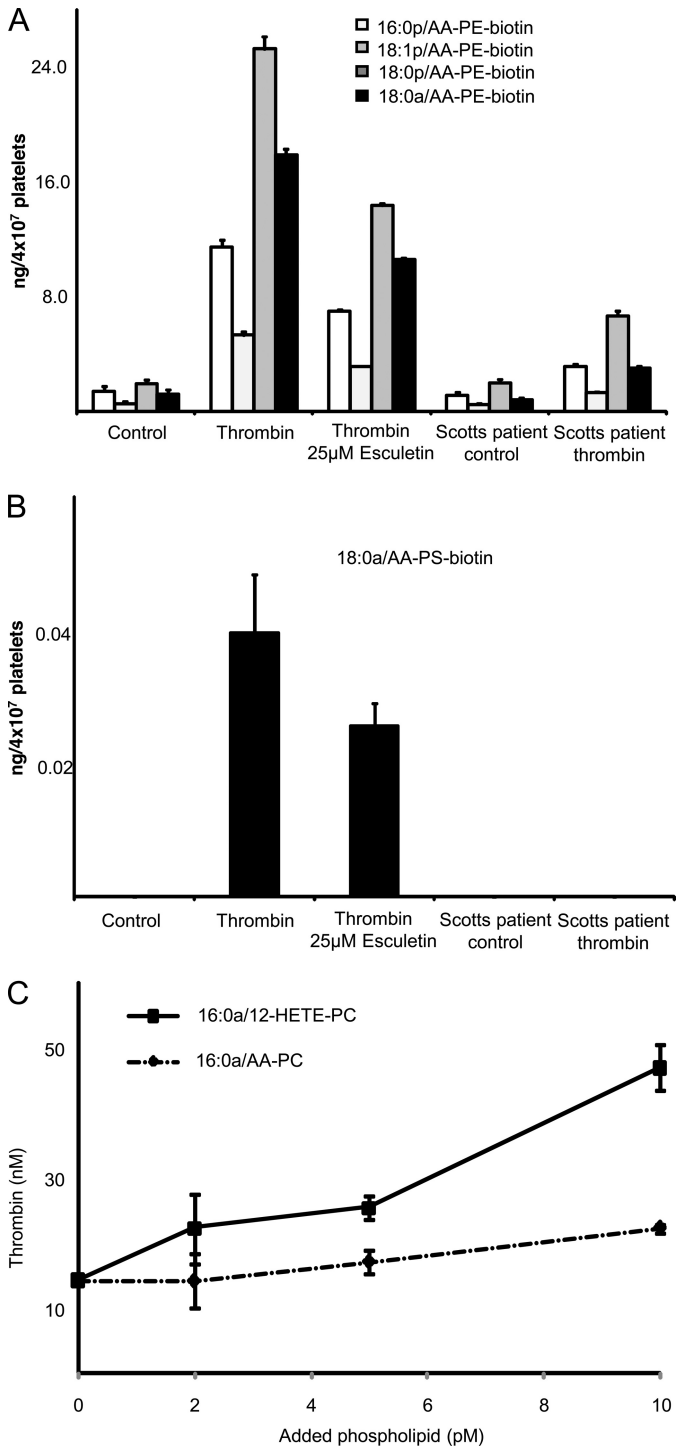


FIGURE 10. **Aminophospholipid externalization is partially 12-LOX-dependent, and 12*S*-HETE-PC stimulates thrombin generation.** *A* and *B*, externalization of native PE and PS lipids following thrombin activation of platelets. Washed human platelets were incubated with or without esculetin from 10 min before activation with 0.2 unit/ml thrombin for 30 min followed by the addition of 1.5 mg/ml sulfo-NHS-Biotin for 10 min at room temperature. Platelets were then centrifuged at 970 × *g*, the supernatant removed, then cells resuspended in fresh Tyrode's buffer and lipids extracted ($n = 3$, mean ± S.E.). Biotinylated PE or PS was measured in activated platelet lipid extracts with and without 10-min preincubation with 25 μM esculetin ($n = 3$, mean ± S.E.). *C*, HETE-PCs stimulate thrombin generation in human plasma. Liposomes generated from human platelet lipid extracts containing 16:0/HETE-PC or 16:0/20:4-PC were added to platelet-poor plasma, and thrombin generation was activated using tissue factor and measured using calibrated automated thrombography as described under "Experimental Procedures."

tion, including scavenger receptor ligands such as oxovaleryl phosphatidylcholine (12). Thus, 12-LOX may become a source of these secondary bioactive PLs in situations where platelet activation *in vivo* is elevated, *e.g.* cardiovascular disease. Currently, *in vivo* pathways that generate such lipids are not known. Recent studies have proposed that extracellular exposure of oxidized PLs, including those containing hydroxy/oxy short chain aldehydes derived from hydroperoxide decomposition, facilitates their physical contact with pattern recognition receptors (21). This is termed the lipid whisker model. Thus, it is possible that 12-LOX-derived oxidized PLs, formed and then externalized in response to agonist activation, may participate in similar processes, although this remains to be determined.

Unlike free eicosanoids, 12*S*-HETE attached to PE or PC remained cell-associated with 12*S*-HETE-PE also able to translocate to the outer membrane (Figs. 8, *A–C*, and 9*C*). Interestingly, we recently found that murine resident peritoneal macrophages contain 12-HETE-PE even without agonist activation; however, PE externalization requires ionophore treatment and is enhanced in 12/15-LOX-deficient mice (3). The reason for this difference is unclear, but it may be species- or LOX isoform-dependent.

Because the majority of the PC is already on the outside in resting cells, it is likely that 12*S*-HETE-PC is also facing the external side. Thus, we hypothesized that esterified HETEs could participate in events on the outer side of the plasma membrane. Indeed, HETE-PC dose-dependently stimulated coagulation when incorporated into liposomes generated from platelet lipids (Fig. 10*C*). This is consistent with a recent study that found that nonenzymatic oxidation of PLs could enhance thrombin generation on the surface of lymphocyte cell lines (25). Results using the 12-LOX inhibitor, esculetin, indicated that externalization of un-oxidized PE at least partly depended on 12-LOX (Fig. 10). As expected, esculetin also blocked externalization of HETE-PEs, presumably through preventing their formation (Fig. 9). These results indicate that inhibition of LOX could decrease coagulant activity through inhibition of the external presentation of either PE or through blocking formation of HETE-PCs or -PEs.

PE and PS facilitate coagulation factor interactions with the membrane via calcium coordination to their amino headgroups. Although PC is unable to mediate this phenomenon, the hydroxyl group introduced by 12-LOX will have anionic character that could act in a similar manner. Although C12 of AA attached to PLs is normally buried in the membrane hydrophobic region, introduction of a polar group will cause the lipid to bend, exposing the hydroxyl where it should be accessible for interactions with extracellular proteins and calcium. Calcium coordination with hydroxyl groups within a bilayer structure has been shown previously by x-ray diffraction (37). Further experiments using purified clotting factors and lipids will examine whether this is the case.

Platelets from a patient with the bleeding disorder Scott syndrome externalized ~25% of the level of PE as healthy platelets, but exposure of PS or 12*S*-HETE-PEs was not detected (Figs. 9, *B* and *D*, and 10, *A* and *B*). Scott syndrome platelets do support a low rate of coagulation, which may be partly due to the presence of 12-HETE-PCs that are generated

at normal levels by the cells (data not shown) and would be expected to be on the outer leaflet. Thus, the lack of 12-HETE-PE externalization in Scott syndrome may also contribute to the bleeding phenotype. These observations may also be relevant to thrombotic disorders, for example, patients with antiphospholipid syndrome demonstrate elevated lipid peroxidation along with antibodies against oxidized PLs *in vivo*, although the epitopes are not characterized (38, 39). Future work will characterize the role of these new lipids in regulating hemostasis *in vivo* and thrombosis in human vascular disease, including antiphospholipid syndrome.

Esterified eicosanoids belong to a class of molecule termed oxidized PL, which includes hundreds of structures including decomposition products of PL hydroperoxides that can act as scavenger receptor ligands (11–14). Up until now, this has been generally assumed to be an uncontrolled pathological event forming multiple bioactive lipids that play deleterious roles in vascular disease and inflammation. In contrast, we show that it is triggered by acute agonist-receptor signaling in human platelets, resulting in generation of specific groups of molecular species. The data indicate that their formation is physiological and represents a new signaling paradigm for eicosanoids through formation of membrane-associated complex lipids that are structurally distinct from traditional free acid products. Of interest, we recently showed that a structurally similar lipid (18:0a/15-HETE-PE) generated by human monocytes can inhibit lipopolysaccharide stimulation of cytokine generation, as can other related oxidized PCs (3, 40).

In summary, the physiological formation of a family of esterified HETE lipids, including the involvement of receptor and intracellular signaling pathways in their generation, has been characterized. The lipids enhanced thrombin generation in an *in vitro* assay, indicating a potential role in hemostasis. Thus, esterified eicosanoids represent new families of lipids that may be of importance in platelet function in health and disease.

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