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Lloyd Morgan, Christopher P Thomas, Hartmut Kuhn, V B O'Donnell. Thrombin activated human platelets acutely generate oxidized docosahexanoic acid-containing phospholipids via 12-lipoxygenase.. Biochemical Journal, 2010, 431 (1), pp.141-148. 10.1042/BJ20100415 . hal-00517250

HAL Id: hal-00517250

<https://hal.science/hal-00517250>

Submitted on 14 Sep 2010

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Thrombin activated human platelets acutely generate oxidized docosahexanoic acid-containing phospholipids via 12-lipoxygenase.

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Running Head: Human platelets acutely generate phospholipid-esterified hydroxy-docosahexanoic acid

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SYNOPSIS

Arachidonate-containing oxidized phospholipids are acutely generated by 12-lipoxygenase (LOX) in agonist-activated platelets. In this study, formation of structurally-related lipids by oxidation of docosahexanoic acid (DHA)-containing phospholipids is demonstrated using lipidomic approaches. Precursor scanning reverse phase LC/MS/MS identified a new family of lipids that comprise phospholipid-esterified hydroxydocosahexanoic acid (HDOHE). Two diacyl and two plasmalogen phosphatidylethanolamines containing predominantly the 14-HDOHE positional isomer (18:0p/14-HDOHE-PE, 18:0a/14-HDOHE-PE, 16:0a/14-HDOHE-PE, 16:0p/14-HDOHE-PE) were structurally characterized using MS/MS and by comparison with biogenic standards. An involvement of 12-LOX was indicated since purified recombinant human 12-LOX also generated the 14-HDOHE isomer from DHA. Pharmacological studies using inhibitors and recombinant platelet 12-LOX indicate that they form via esterification of newly formed free HDOHE. HDOHE-PEs formed at significant rates (2 - 4 ng/4 x 10⁷ cells) within 2 - 180 min of thrombin stimulation, and their formation was blocked by calcium chelation. In summary, a new family of oxidized phospholipid was identified in thrombin-activated human platelets.

Keywords: lipoxygenase, platelets, phospholipid, mass spectrometry, thrombin, docosahexanoic acid

INTRODUCTION

Lipoxygenases (LOX), cyclooxygenases (COX) or cytochrome P450 (CYP) oxidize free arachidonate to form eicosanoids, mediators that play important roles in both physiology and disease in virtually all organs. Several years ago, eicosanoid esterification, where the lipids become attached to phospholipids was studied by addition of radiolabeled hydroxyeicosatetraenoic acids (HETEs) to cells followed by characterization of their fate by radio-TLC [1-6]. In those studies, HETEs became esterified to phospholipids over periods of several hours and could be released by subsequent cell activation. Although phospholipid classes that contained HETE were determined, specific molecular species could not be identified. More recently, using lipidomic methods, we have found that acute activation of several immune cells, including monocytes, platelets and macrophages leads to formation of distinct families of phospholipid-esterified HETEs. The lipids form via LOX catalysis, within 2-5 min, in response to pathophysiological agonists and remain cell-associated suggesting an autocrine mode of action. This phenomenon is distinct from earlier work since the eicosanoids are endogenously generated and incorporation is acute taking place over very short timeframes of 2-5 min. Each cell type generates 4-6 unique molecular structures, including diacyl and plasmalogen forms, belonging to either phosphatidylethanolamine (PE) or phosphatidylcholine (PC) classes. The positional isomers generated depend exclusively on the LOX isomer expressed. For example, IL-4-stimulated human monocytes and IL-13-stimulated human bronchial epithelial cells generate 15-HETE-PEs, while murine peritoneal macrophages and human platelets generate predominantly 12-HETE-PLs [7-10].

To date, studies have concentrated on oxidized phospholipids that originate from arachidonate oxidation, and lipids containing other unsaturated fatty acids have not been characterized. These could include linoleate and omega-3 species such as docosahexanoic acid (DHA), derived from fish oil. Since fish oil-derived fatty acids are reported to possess anti-platelet activities, this study examined for the presence of analogs of oxidized phospholipid containing monohydroxy forms of this lipid, hydroxydocosahexanoic acid (HDOHE) generated by human platelets following acute activation with the pathophysiologically-relevant agonist, thrombin.

EXPERIMENTAL PROCEDURES

Isolation and activation of human platelets. All blood donations were approved by the Cardiff University School of Medicine and Dentistry Ethics Committee. Whole blood was collected, with informed consent, from healthy volunteers free from non-steroidal anti-inflammatory 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 resuspended in Tyrode's buffer (134 mM NaCl, 12 mM NaHCO₃, 2.9 mM KCl, 0.34 mM Na₂HPO₄, 1.0 mM MgCl₂, 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 $2 \times 10^8 \text{ ml}^{-1}$. Platelets were activated using 0.2 U/ml thrombin at 37°C in the presence of 1 mM CaCl₂ for varying times.

Lipid extraction. For quantitation, 10 ng 13-HODE-d₄ and/or dimyristoyl-PE (DMPE) was added before extraction. Lipids were extracted by adding a solvent mixture (1 M acetic acid, 2-propanol, hexane (2:20:30, v/v/v)) to the sample at a ratio of 2.5 ml solvent mixture/ml sample, vortexing, and then adding 2.5 ml hexane [11]. After vortex and centrifugation, lipids were recovered in the upper hexane layer. The samples were then re-extracted 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 described below. Inhibitors of calcium mobilization were added 10 min before activation.

Precursor scanning mass spectrometry. Lipid extracts were separated by reverse phase HPLC using a Luna 3 µm C18 (2) 150 x 2 mm column (Phenomenex, Ltd, 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. Mass spectrometry was carried out using an Applied Biosystems 4000 Q-Trap. Settings were DP -140 V, CE -45 V. Spectra were acquired scanning Q1 from 600 – 1100 amu over 5 seconds with Q1 set to daughter ion of interest.

Phospholipid separation and analysis by LC/MS/MS. Phospholipid separation was carried out as described for precursor scanning. Analytes were monitored in Single or Multiple Reaction Monitoring (SRM, MRM) modes using parent – carboxylate anion or internal daughter ion for specific positional isomers. Product ion spectra were obtained at the apex of elution of each lipid, 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.

Normal phase HPLC-UV of phospholipid classes and HDOHE isomer determination. Fractionation of platelet lipid classes was undertaken on a Spherisorb S5W 150 x 4.6 mm column (Waters Ltd. Herts, UK) with a gradient of 50-100 % B over 25 min (A, hexane:propan-2-ol, 3:2; B, solvent A:water, 94.5:5.5) at a flow rate of 1.5 ml.min⁻¹. Absorbance was monitored at 205 nm and lipids identified using a mixture of standard phospholipids (Sigma, Poole, UK). Fractions corresponding to PE (approx 6 min RT) or PC (approx 20 min RT) were collected for subsequent analysis by ESI/MS/MS. PE or PC lipids were then analyzed for the *m/z* of interest using reverse phase separation described above. For determination of HDOHE positional isomer composition in PE, the purified phospholipid fraction was saponified as described below, then resuspended in 50 µl methanol, and HDOHE isomers analyzed by reverse phase LC/MS/MS as described below.

Saponification. Lipids were suspended in 1.5 ml of propan-2-ol, and then fatty acids released by base hydrolysis with 1.5 ml NaOH at 60°C for 30 min under N₂. Hydrolyzed samples were acidified to pH 3.0 with 0.5 M HCl, and fatty acids were extracted twice with 3 ml hexane. The combined hexane layers were dried, resuspended in 100 µl MeOH and stored under N₂ at -80°C until analysis by LC/MS/MS.

Free HDOHE isomer analysis using LC/MS/MS. HDOHE isomers were separated on a Discovery® C18, 5 µm, 250 x 4.6 mm column (Supelco Analytical, Sigma-Aldrich, UK) using a gradient of 50-90 % B over 15 min (mobile phase as above) with a flow rate of 1 ml.min⁻¹. Products were quantitated by LC/ESI/MS/MS on an Applied Biosystems 4000 Q-Trap using parent to daughter transitions of *m/z* 343.2 (HDOHE, [M-H]⁻) to *m/z* 101.1 (4-HDOHE), 141.1 (7-HDOHE), 109.1 (8-HDOHE), 153.1 (10-HDOHE), 149.1 (11-HDOHE), 193.1 (13-HDOHE), 205.1 (14-HDOHE), 233.1 (16-HDOHE), 273.1 (17-HDOHE) and 187.1 (20-HDOHE). 12-HETE-d₈ was monitored at *m/z* 327 to 184. All collision energies were between -20 to -30 V. Products were identified and quantified using HDOHE positional isomers, and 13-HODE-d₄ standards (all from Cayman Chemical) run in parallel under the same conditions.

Oxidation of PE and PC using recombinant LOXs. 10mM brain L-α-phosphatidylethanolamine (Avanti Polar Lipids, Alabaster, AL), 18:0a/22:6-PC or -PE (Sigma) was incubated for 30 min at 37 °C in 0.1 M KH₂PO₄ buffer, pH 7.4, 0.2 % sodium cholate, with 100 µl of I428A mutant rabbit 15-lipoxygenase, that oxidized primarily at C12 [12]. Samples were then reduced using 1 mM SnCl₂ for 10 min, 20 °C, and extracted as before for LC/MS/MS analysis. DHA or 18:0a/22:6-PE was incubated for 30 min at 37 °C in 0.1 M KH₂PO₄ buffer, pH 7.4, 0.2 % sodium cholate, with 50 µl recombinant human platelet 12-LOX. Samples were then reduced using 1 mM SnCl₂ for 10 min, 20 °C, and extracted as before for LC/MS/MS analysis.

Quantitation of HDOHE-PEs. Mixed isomer HDOHE-PE standards were generated using air oxidation, and purified using reverse phase HPLC. A standard curve was constructed by analyzing varying (1pg-10ng on column) amounts of standard with fixed amount of DMPE (100pg). Ratio of analyte/internal standard (integrated from chromatograms) was calculated and converted into amount of HDOHE-PE using the standard curve. As platelets contain a mixture of positional isomers, total HDOHE-PE was determined using *m/z* 343.2 daughter ion which quantifies all 10 isomers as a single group.

RESULTS

Precursor LC/MS/MS scanning of platelet lipid extracts. To identify lipids that contain oxidized DHA, lipid extracts from thrombin-activated platelets were subject to precursor LC/MS/MS, which fragments using collision-induced-dissociation (CID), and scans for a functional group of interest in Q3. With Q3 set at m/z 343.2 (i.e. the carboxylate anion of HDOHE), negative precursor LC/MS/MS demonstrates several ions that increase on activation, marked by * (Fig 1 A). Four major ions predominate, m/z 762.6, 778.7, 790.6, 806.7, and they are largely absent in unactivated platelets (Fig 1 A,B). A fifth large ion was observed to elute after these lipids (27 min), marked by ** (m/z 818.6). Although this lipid appears to be a significant species in this figure, it was only observed in about a third of our donor pool isolates. Its m/z is consistent with two potential phospholipids, 18:0p/HDOHE-PC or 20:0p/HDOHE-PE. However, we were unable to assign it to either since the retention time is too late for a PC, and the unoxidized parent lipid 20:0p/22:6-PE is not detected in our cells in precursor scans for m/z 327, equivalent to DHA (m/z 802.6, Fig 3 C). Thus, we believe that neither is a likely candidate for this lipid. LC/MS/MS spectra were generated for this ion in both positive and negative mode (assuming that $[M+H]^+$ will be either 820 or 834 for PE or PC, respectively). However, neither were consistent with the potential molecular species as listed above, and furthermore, due to its late retention time, unrelated ions from co-eluting isobaric species were also detected (data not shown). Thus we have been unable to structurally characterize this peak. Precursor scans for DHA (m/z 327.2) demonstrate four major lipids that are 18 amu less than those for HDOHE-containing ions shown in Fig 1 A (Fig 1 C). This indicates that both DHA and HDOHE are generally contained within the same phospholipid pool in human platelets.

Structural characterization of esterified HDOHEs marked by *. Several approaches were utilized for elucidating structure. First, phospholipids were separated by class using normal phase LC. This was followed by reverse LC/MS/MS to determine which phospholipid classes contained esterified HDOHE. Separately, platelet lipid extracts were compared with standards using LC/MS/MS. In this case, standards were generated by oxidation of DHA-containing phospholipids using a recombinant mutant rabbit 15-LOX, that oxidizes at C12 of arachidonate and is capable of phospholipid oxidation [12].

1. Normal phase separation of PL classes. To determine PL class for each ion, PE and PC was purified from thrombin-activated platelets and analyzed for the presence of the four most abundant m/z 343.2-containing ions. All were detected in the PE but not the PC fraction, indicating that they all belong to this phospholipid class (Fig 2).

2. Structural determination of HDOHE-PEs using LC/MS/MS. The four predominant masses observed in precursor m/z 343.2 scans (Fig 1) were analyzed using LC/MS/MS, monitoring for parent to m/z 343 $[M-H]^-$. They elute as broad peaks from 20-25 min, suggesting they contain a mixture of positional isomers of HDOHE (Figs 3, 4). The abundance of platelet HDOHE-containing lipids was below the limit of sensitivity for generation of online ion trap MS/MS spectra. Thus, structural assignment was made based on comparison of MRM retention time with synthetic standards generated by oxidation of brain PE (that contains a high proportion of DHA) using a mutant rabbit 12-LOX (Ile418Ala). Unfortunately, for most of these lipids, purified substrates were unavailable at the time. As shown, the major ions for m/z 762.2, 778.6, 790.6 and 806.6 co-elute with standards for 16:0p/HDOHE-PE, 16:0a/HDOHE-PE, 18:0p/HDOHE-PE and 18:0a/HDOHE-PE, respectively (Figs. 3, 4, Scheme 1). MS/MS spectra demonstrate several ions that confirm structural assignment of the standards. For example, all show m/z 343 (HDOHE $[M-H]^-$), and 281 (from internal

fragmentation of HDOHE). Parent ions at m/z 778 and 806 have daughter fragments at m/z 255 and 283, indicating sn1 fatty acids of 16:0 and 18:0, respectively (Figs 3D, 4D insets). Finally, all show loss of the sn2 HDOHE (344 amu) at: m/z 418, 434, 446 and 462 for m/z 762, 778, 790 and 806. They also demonstrate loss of the corresponding sn2 ketene at +18 amu above these. In addition to HDOHE-PEs, platelet lipids analyzed for m/z 790 and 806 showed fairly large ions eluting just after 26 min (Fig 4A,C). Although these also generate a m/z 343 daughter ion, they appear too late to be HDOHE-PEs, and are uncharacterized isobaric lipids present in this complex extract.

3. Saponification and analysis of HDOHE positional isomers. To determine the positional isomer(s) of HDOHE contained in the lipids, total PE was isolated from activated platelet lipids by normal phase HPLC and then saponified to release all HDOHE. The sample was then analyzed using LC/MS/MS, in comparison with standards. This showed that 14-HDOHE is the most abundant positional isomer contained in the PE phospholipids, with smaller amounts of 17- and 20-HDOHE (Fig 5 A). This was similar to the isomer distribution of free HDOHE generated by thrombin-activated platelets, with 14-HDOHE also predominating (Fig 5 B). We were unable to determine S/R enantiomers since standards are not available.

4. Studies using human recombinant platelet 12-LOX. To determine whether these lipids may be generated by platelet 12-LOX, human recombinant enzyme was incubated with DHA, and formed primarily the 14-HDOHE positional isomer (Fig 5 C). In contrast it did not oxidize either AA or DHA when attached to PE (not shown). This indicates that the enzyme does not directly oxidize phospholipids.

HDOHE-PEs are generated acutely, in a calcium-dependent manner and requires hydrolysis of DHA. Activation of platelets using 0.2 U/ml thrombin for varying times showed that the lipids are generated in an acute manner, with elevations apparent by as early as 2 min, then continuing to slowly increase to at least 3 hr (Fig 6 A). Similar temporal increases were apparent for free HDOHE isomers, with 14-HDOHE predominating (Fig 6 B). Separately, platelets were incubated with 10 ng 14-HDOHE, then studied for formation of HDOHE-PEs (others not shown were low/undetected). To determine whether exogenously added HDOHE could be incorporated into these phospholipids, 10 ng 14-HDOHE was incubated with platelets for up to 30 min. However, during this time, HDOHE-PEs were not observed to form, indicating that their generation during platelet activation is a tightly-coupled process, with HDOHE destined for esterification being incorporated extremely fast into the phospholipid pool (Fig 6 C). Inclusion of BAPTA-AM or EGTA abolished generation of HDOHE-PEs, indicating a dependence on calcium mobilization, as expected for platelet 12-LOX (Fig 7 A). Finally, generation of either free or esterified HDOHE was inhibited by blocking either sPLA2 (OOEPC) or cPLA2 (cPLA2i), but not iPLA2 (BEL) (Fig 7 B,C). These observations are consistent with the idea that HDOHE-PE formation requires hydrolysis of DHA and formation of free HDOHE before its esterification into phospholipid.

DISCUSSION

In this study, a novel family of oxidized phospholipid comprising HDOHE attached to PE was identified in thrombin-activated human platelets. This is the first demonstration of esterified oxidation products of DHA formed by an agonist-activated cell type, and represents a new family of oxidized phospholipid. We recently identified formation of esterified hydroxyeicosatetraenoic acids (HETE) in human platelets and monocytes, and murine peritoneal macrophages, and showed that they form by the action of cell-specific LOXs [7, 8, 10]. HDOHE-PEs are a related but distinct family, containing instead oxidized DHA, a major fatty acid in fish oil.

In this study, the predominance of esterified 14-HDOHE over other positional isomers suggests these lipids are also generated by platelet 12-LOX, similar to esterified HETEs (Fig 5 A). In support, recombinant human 12-LOX formed primarily free 14-HDOHE from free DHA (Fig 5 C). LOXs are a family of lipid oxidizing enzymes expressed in mammals predominantly by immune cells and platelets [13]. They are traditionally known as a source of free eicosanoids, oxidizing arachidonate that has been released by PLA₂, forming bioactive lipids including leukotrienes and HETEs. Thrombin-activated platelets also generated free 14-HDOHE from endogenous DHA, consistent with a previous report of this positional isomer being formed on incubation of exogenous DHA with platelets (Fig 5 B)[14]. However, generation of free 14-HDOHE from endogenous platelet phospholipid-derived DHA has not been reported before. Due to limitations in amount of material and unavailability of enantiomerically pure standards, we were unable to determine the enantiomeric composition of either the free or esterified 14-HDOHE generated.

In platelets, HDOHE was esterified into PE, with four distinct molecular species detected (16:0p, 16:0a, 18:0p, 18:0a) (Scheme 1). A further unidentified lipid was detected in extracts from a subset of donors at *m/z* 818.6. This distribution is different from platelet phospholipid-esterified HETEs, which comprise two PC and four PEs. Also, HDOHE, but not HETE is found attached to 16:0a-PE. The reason for selective generation of a 16:0a-PE species and absence of HDOHE-PCs may relate to the distribution of DHA vs AA-containing parent phospholipids in the membrane. In support, precursor scans for *m/z* 327.2 (DHA carboxylate anion) show the same distribution of lipids as HDOHE (Fig 1). The inability of recombinant 12-LOX to oxidize DHA-containing PE suggests that the lipids form via sequential PLA₂ hydrolysis of DHA-PE, followed by 12-LOX oxidation, then finally re-esterification of HDOHE into PE. This idea is further supported by the observation that inhibition of cPLA₂ (cPLA₂i) blocks their generation, and is similar to the mechanism of HETE-PL formation recently proposed (Fig 7 B) [10]. In the earlier study, we used a different cPLA₂ inhibitor (PACOCF₃, 10 μ M) and did not reveal a role for this isoform in formation of HETE-PLs. However, we since found out that 10 μ M was probably not sufficient for full inhibition (IC₅₀ for cPLA₂: 45 μ M) ([15] and data not shown). Thus, it is likely that hydrolysis of DHA or AA for oxidation and re-esterification following oxidation does involve cPLA₂. On the other hand, sPLA₂ may act in a different manner, for example through generation of lyso-PC that signals independently of fatty acid mobilization [16, 17]. Time course experiments showed that HDOHE-PE formation was fast, paralleling that of free HDOHE, and that it required calcium mobilization (Fig 6 A,B & 7 A) [10]. This is further consistent with a role for 12-LOX, which is known to be activated acutely in a calcium-dependent manner. Levels continued to rise, even up to 3 hrs post activation, although in some donor isolates, a small decrease by later timepoints was noted and ascribed to donor variability (data not shown). Finally, added free 14-HDOHE was not incorporated into platelet phospholipids during this timescale (Fig 6 C). This suggests a tight coupling of pathways that synthesize and esterify

HDOHE, such that eicosanoid destined for export as free HDOHE is formed from a different pool.

HETE-PCs were recently found to promote tissue factor-dependent thrombin generation in vitro suggesting a pro-coagulant role [10]. In contrast, omega-3 fatty acids from fish oil, including DHA have long been suggested to possess antiplatelet activities [18]. Although studies have been somewhat contradictory, reported effects include inhibition of thrombus and vascular lesion formation in vivo [18, 19]. How fish oil derived fatty acids mediate these effects is unknown, but one proposed mechanism involves formation of omega-3 based prostaglandins by cyclooxygenase that may oppose the action of proaggregatory lipids generated from arachidonate. Our studies identify new DHA-derived lipids that may also regulate coagulation through antagonizing the actions of HETE-PEs or through acting as a reservoir for free HDOHE. In this case, 14-HDOHE could be released by PLA₂ and then act as a free acid mediator to antagonize platelet activation and smooth muscle constriction, as previously shown [20, 21] These ideas will be tested as soon as sufficient quantities of purified HDOHE-PEs become available.

Esterified eicosanoids and fatty acids belong to a class of molecule termed oxidized phospholipid, which includes hundreds of structures including decomposition products of phospholipid hydroperoxides that can act as scavenger receptor ligands [22-25]. Up to 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 in our recent study, and herein, that it is triggered by acute agonist-receptor signaling in human platelets, resulting in generation of specific groups of molecular species[10]. This new study further supports the idea that acute phospholipid oxidation by platelets is physiological and distinct to formation of free acid eicosanoids through formation of structurally-distinct complex lipids.

FUNDING The studies were funded by Wellcome Trust and European Commission (LSHM-CT-2004-0050333).

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FIGURE LEGENDS

Figure 1. Precursor scanning of platelet lipid extract identifies several ions that generate daughter ions with m/z 343.2 *Panel A. Representative LC/MS/MS showing negative precursor scans of thrombin-activated platelet lipid extracts for lipids that lose an ion with m/z 343.2. Total lipid extracts from activated human platelets were separated using LC/MS/MS as described in Experimental procedures, with online negative precursor scanning for m/z 343.2. * marks region of LC trace where ions appear. Control: dashed line. Panel B. Identification of ions that generate m/z 343.2 daughter ions. Negative MS scan of region marked * in Panel A. Panel C. Precursor scan of m/z 327.2 shows lipids containing DHA. Thrombin-activated platelet lipid extracts were separated on LC/MS/MS and ions that generate m/z 327.2 daughter ions identified.*

Figure 2. HDOHE is esterified into PE phospholipids PE and PC fractions were generated by normal phase separation of platelet lipids, and then analyzed for the four most abundant HDOHE-containing transitions, by monitoring parent \rightarrow 343.2 MRM transitions using LC/MS/MS as described for phospholipids. *Panels A,C,E,G. Analysis of PC fractions for ions as listed on panels. Panels B,D,F,H. Analysis of PE fractions for ions as listed on panels.*

Figure 3. Structural identification of m/z 762.6 and 778.6 as 16:0p/HDOHE-PE and 16:0a/HDOHE-PE, using MS/MS. *Panel A. Negative ion LC/MS/MS elution profile of m/z 762 \rightarrow 343 from platelets. Lipid extracts from thrombin-activated platelets were separated as described in Experimental procedures, monitoring m/z 762 \rightarrow 343. Panel B. Negative ion LC/MS/MS elution profile and MS/MS spectrum of m/z 762 \rightarrow 343 from standard. Brain PE was oxidized using rabbit 12-LOX as described in Experimental Procedures, then separated using LC/MS/MS. Spectra were obtained in ion trap mode at the apex of elution at 21.2 min. Panel C. Negative ion LC/MS/MS elution profile of m/z 778 \rightarrow 343 from platelets. Lipid extracts from thrombin-activated platelets were separated as described in Experimental procedures, monitoring m/z 778 \rightarrow 343. Panel D. Negative ion LC/MS/MS elution profile and MS/MS spectrum of m/z 778 \rightarrow 343 from standard. Brain PE was oxidized using rabbit 12-LOX as described in Experimental procedures, then separated using LC/MS/MS. Spectra were obtained in ion trap mode at the apex of elution at 20.0 min*

Figure 4. Structural identification of m/z 790.6 and 806.6 as 18:0p/HDOHE-PE and 18:0a/HDOHE-PE, using MS/MS. *Panel A. Negative ion LC/MS/MS elution profile of m/z 790 \rightarrow 343 from platelets. Lipid extracts from thrombin-activated platelets were separated as described in Experimental procedures, monitoring m/z 790 \rightarrow 343. Panel B. Negative ion LC/MS/MS elution profile and MS/MS spectrum of m/z 790 \rightarrow 343 from standard. Brain PE was oxidized using rabbit 12-LOX as described in Experimental procedures, then separated using LC/MS/MS. Spectra were obtained in ion trap mode at the apex of elution at 23.5 min. Panel C. Negative ion LC/MS/MS elution profile of m/z 806 \rightarrow 343 from platelets. Lipid extracts from thrombin-activated platelets were separated as described in Experimental procedures, monitoring m/z 806 \rightarrow 343. Panel D. Negative ion LC/MS/MS elution profile and*

MS/MS spectrum of m/z 806 \rightarrow 343 from standard. Brain PE was oxidized using rabbit 12-LOX as described in Experimental procedures, then separated using LC/MS/MS. Spectra were obtained in ion trap mode at the apex of elution at 22.4 min.

Figure 5. Identification of 14-HDOHE as the major oxidized DHA enantiomer in PE phospholipids of thrombin-activated platelets and oxidation of DHA by recombinant human platelet 12-LOX. *Panel A. Platelet generated 18:0a/HDOHE-PE preferentially contains 14-HDOHE isomer.* PE was purified from thrombin-activated platelets were separated using normal phase LC, saponified, then HDOHE positional isomers in the saponified PE fraction characterized using LC/MS/MS as described in Experimental procedures. *Panel B. 14-HDOHE is the predominant free acid isomer generated from activated platelets.* Free HDOHE isomers were analyzed using LC/MS/MS from extracts of human platelets activated with 0.2 U/ml thrombin. *Panel C. Oxidation of DHA by recombinant platelet LOX generates the 14-HDOHE isomer.* DHA was oxidized using recombinant human platelet 12-LOX as described in Experimental procedures, then isomers determined using LC/MS/MS.

Figure 6. Time course and calcium dependence of generation of HDOHE-PEs by thrombin-activated human platelets. *Panel A. Time course of HDOHE-PE generation by thrombin-activated platelets.* Platelets were activated using 0.2 U/ml thrombin for varying times, then lipids extracted and analysed using LC/MS/MS as described in Experimental procedures. HDOHE-PEs were quantified by comparison with standard curves for HDOHE-PE vs DMPE, conducted as described. *Panel B. Time course of free HDOHE generation by thrombin-activated platelets.* Platelets were activated using 0.2 U/ml thrombin for varying times, then lipids extracted and analysed using LC/MS/MS as described in Experimental procedures for 8-, 14- and 16-HDOHE. Other isomers were low/undetectable. *Panel C. Exogenously-added free HDOHE is not incorporated into phospholipids.* Platelets were incubated with 10 ng 14-HDOHE for 15 or 30 min at 37 °C before analysis for HDOHE-PE formation using LC/MS/MS.

Figure 7. Blocking HDOHE-PE generation by pharmacological inhibitors. *Panel A. Generation of HDOHE-PEs requires calcium mobilisation.* Washed human platelets were incubated for 10 min with EGTA (1 mM) and/or BAPTA/AM (10 μ M) prior to thrombin activation (0.2 U/ml for 30 min). Lipids were extracted and analysed as described in Experimental Procedures (n = 3, mean \pm SEM). *Panel B. Generation of HDOHE-PEs requires phospholipases.* Washed human platelets were incubated for 10 min with cPLA2i (1.2 μ M), OOEPc (2 μ M) or BEL (50 nM) prior to thrombin activation (0.2 U/ml for 30 min). Lipids were extracted and analysed as described in Experimental Procedures (n = 3, mean \pm SEM). *Panel C. Generation of free HDOHE-PEs requires phospholipases.* Washed human platelets were incubated for 10 min with cPLA2i (1.2 μ M), OOEPc (2 μ M) or BEL (50 nM) prior to thrombin activation (0.2 U/ml for 30 min). Lipids were extracted and analysed as described in Experimental Procedures (n = 3, mean \pm SEM).

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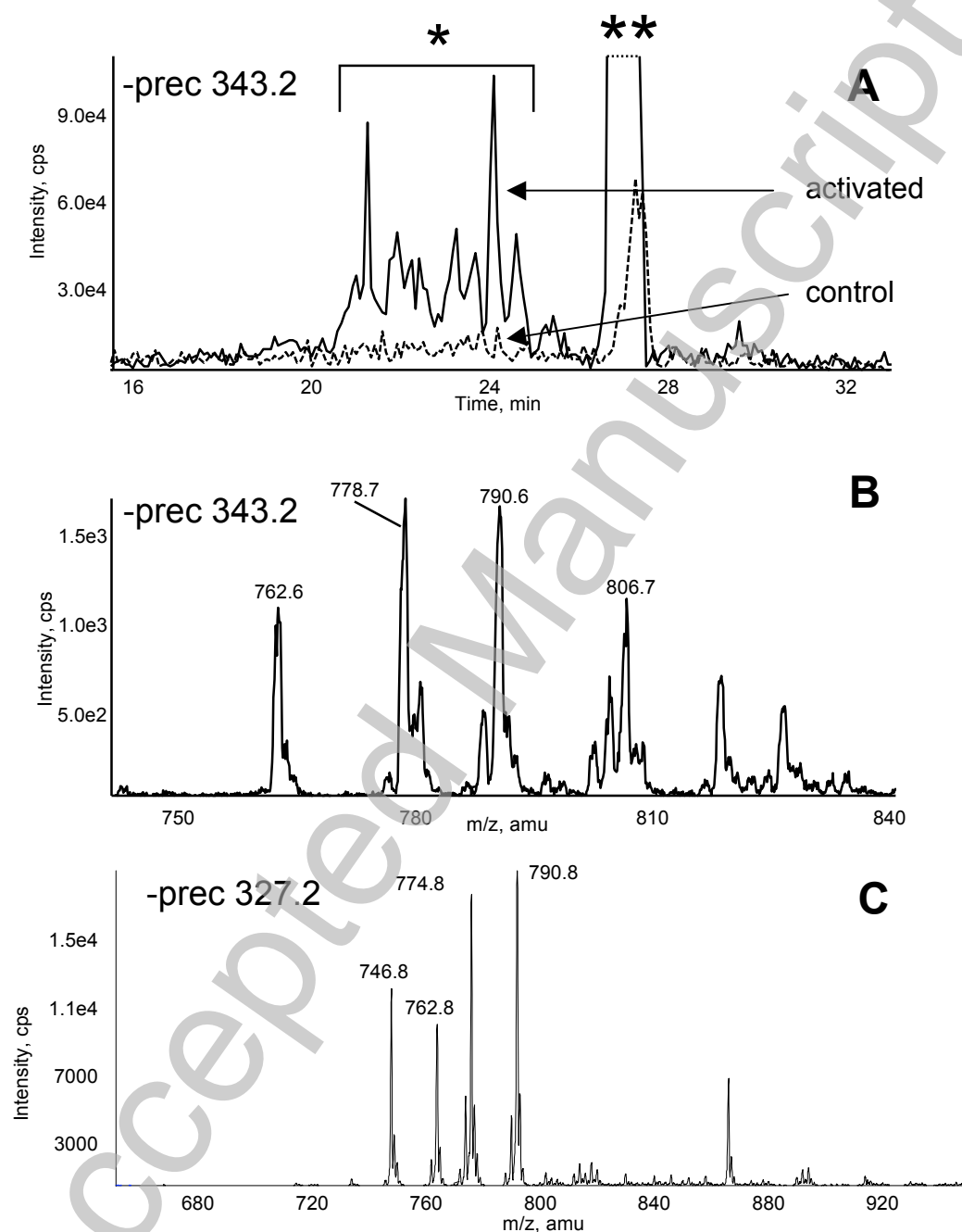
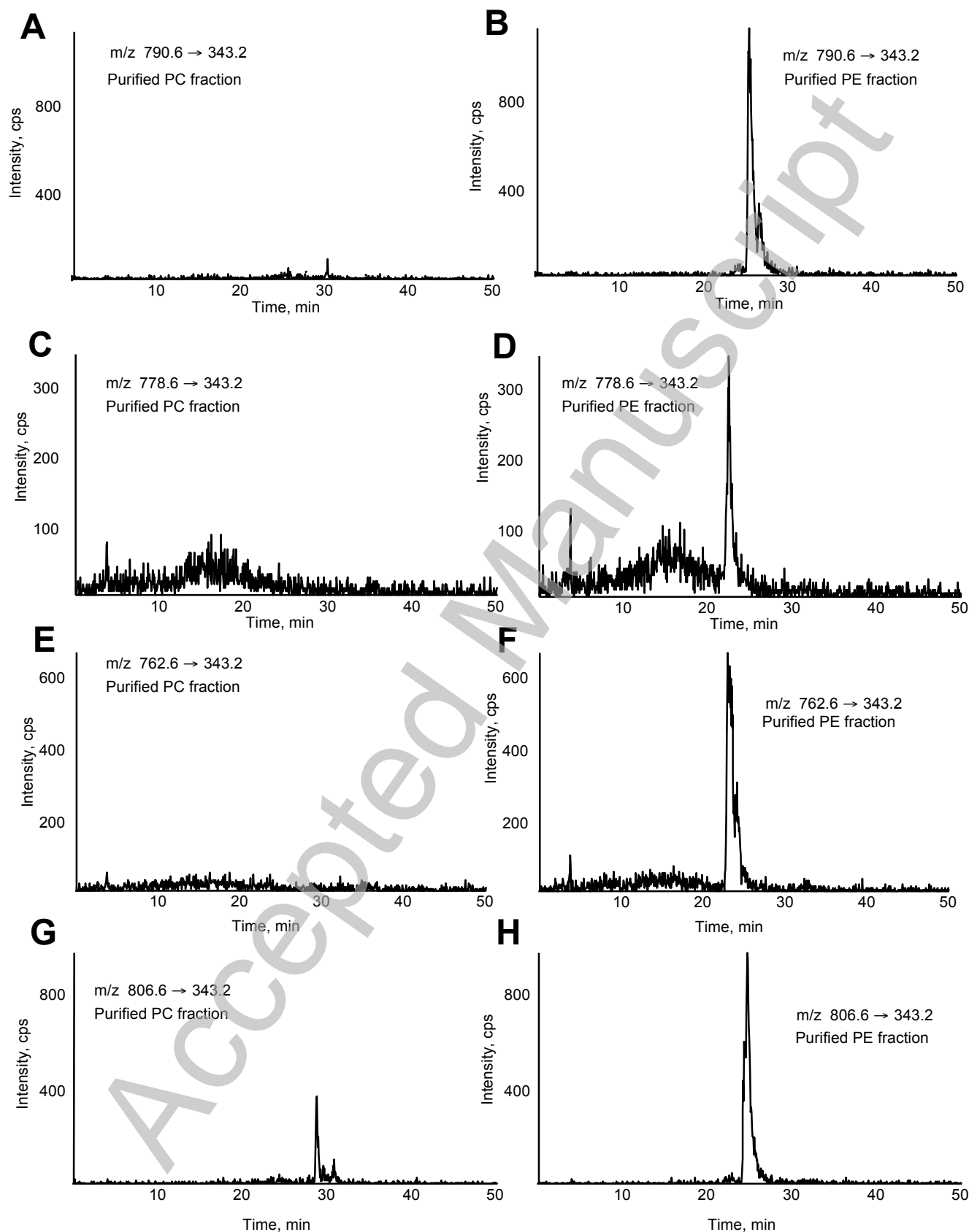


Figure 1.

**Figure 2**

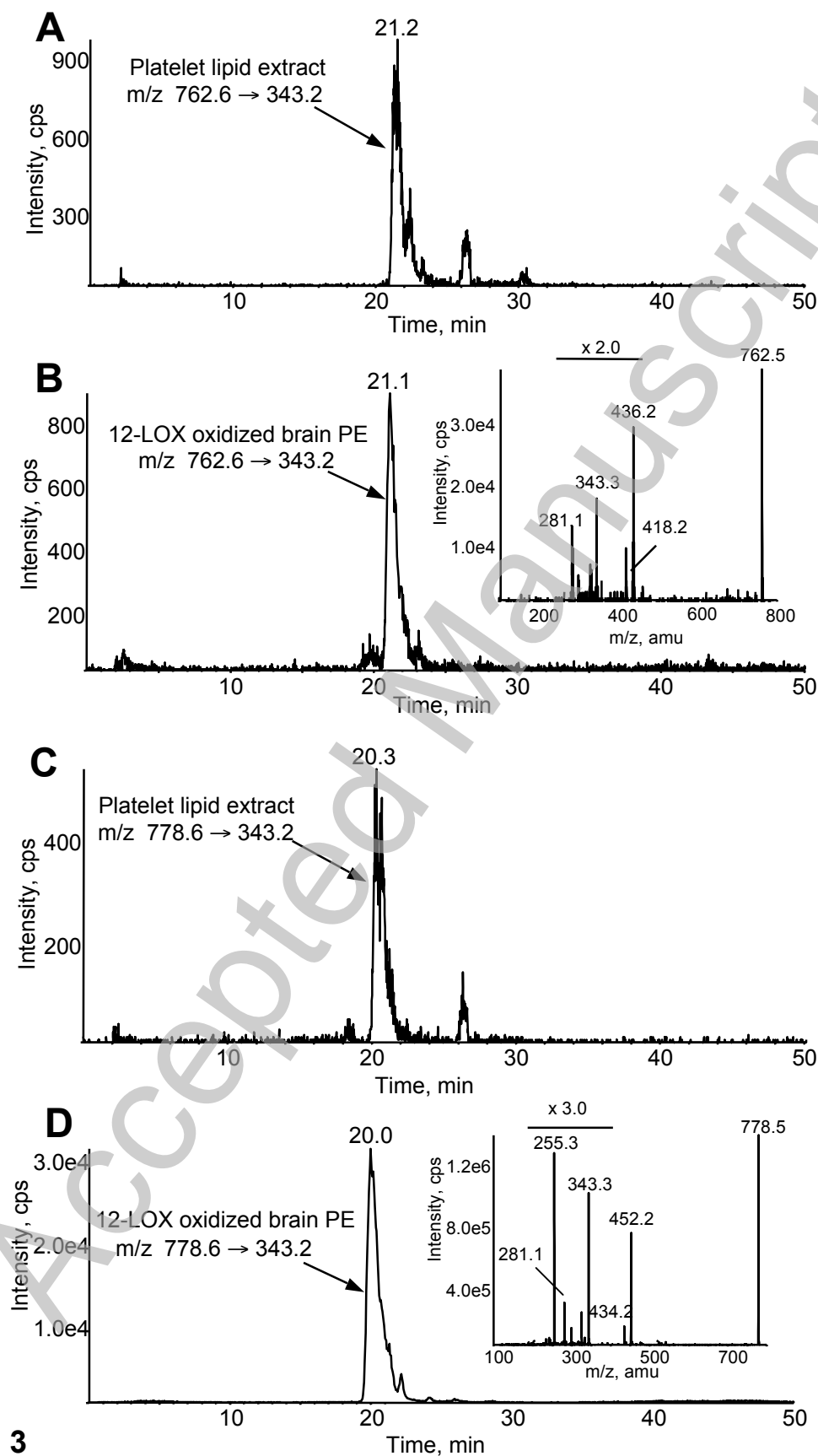


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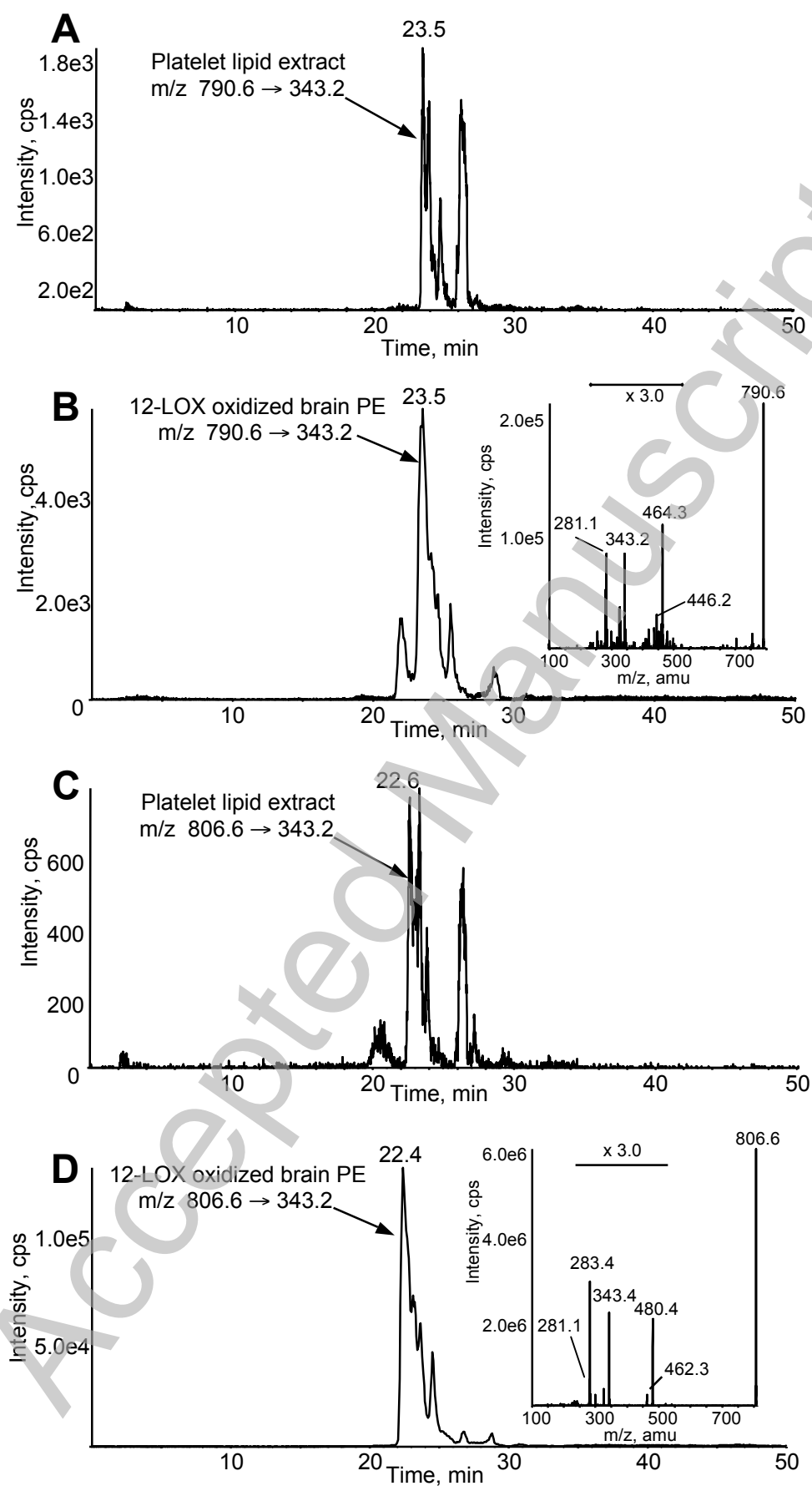
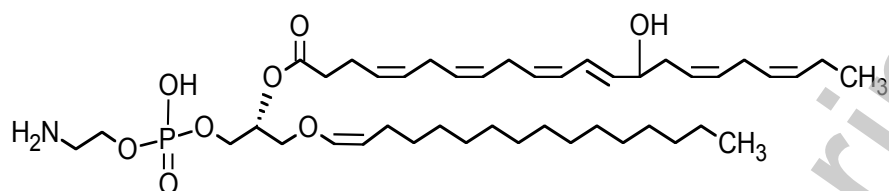
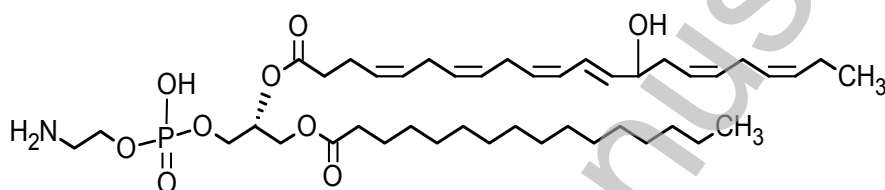


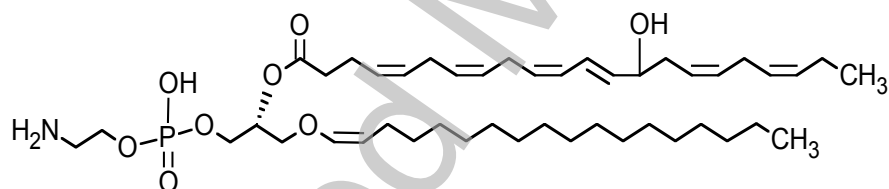
Figure 4



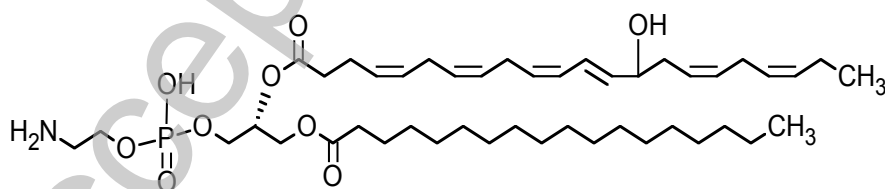
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MW = 779.6 16:0a/14-HDOHE PE



MW = 791.6 18:0p/14-HDOHE PE



MW = 807.6 18:0a/14-HDOHE PE

Scheme 1

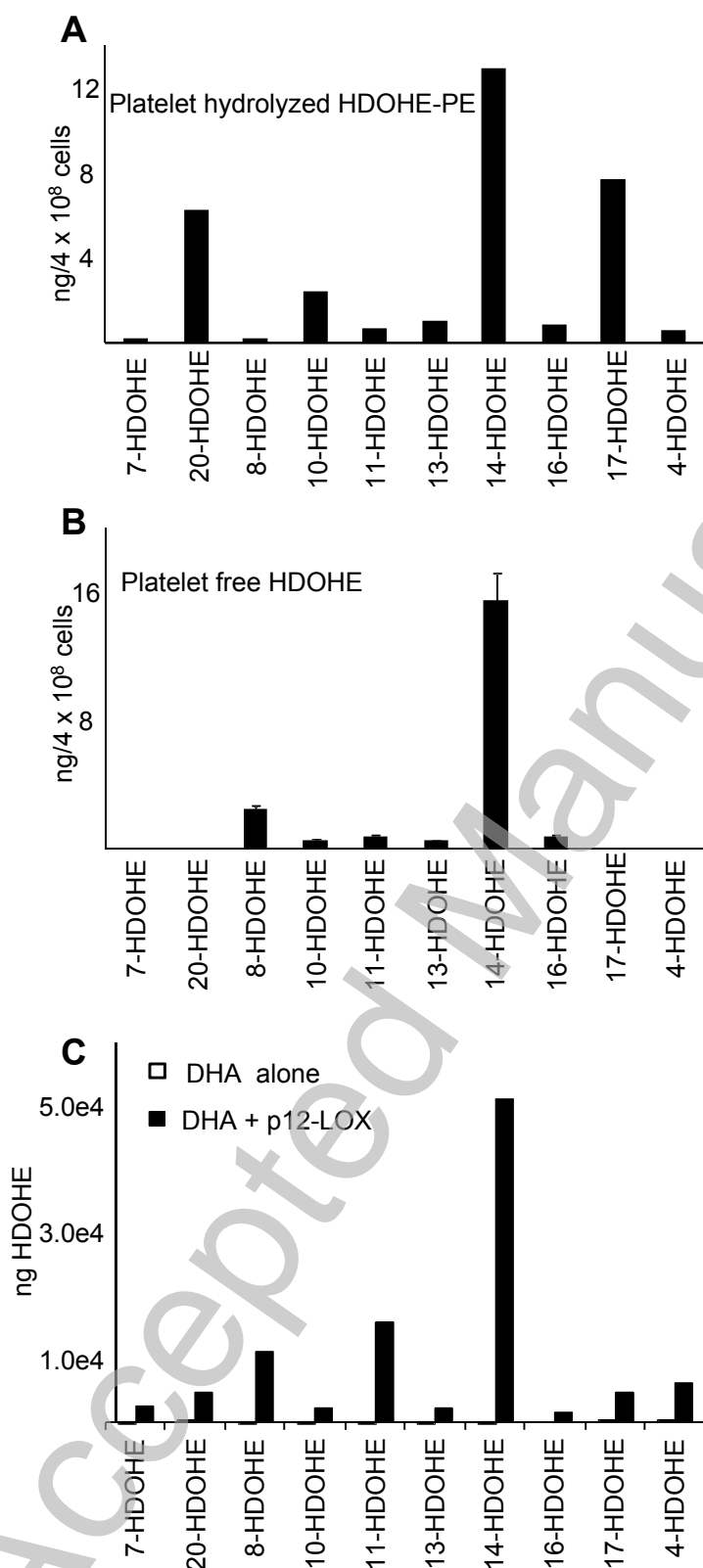


Figure 5

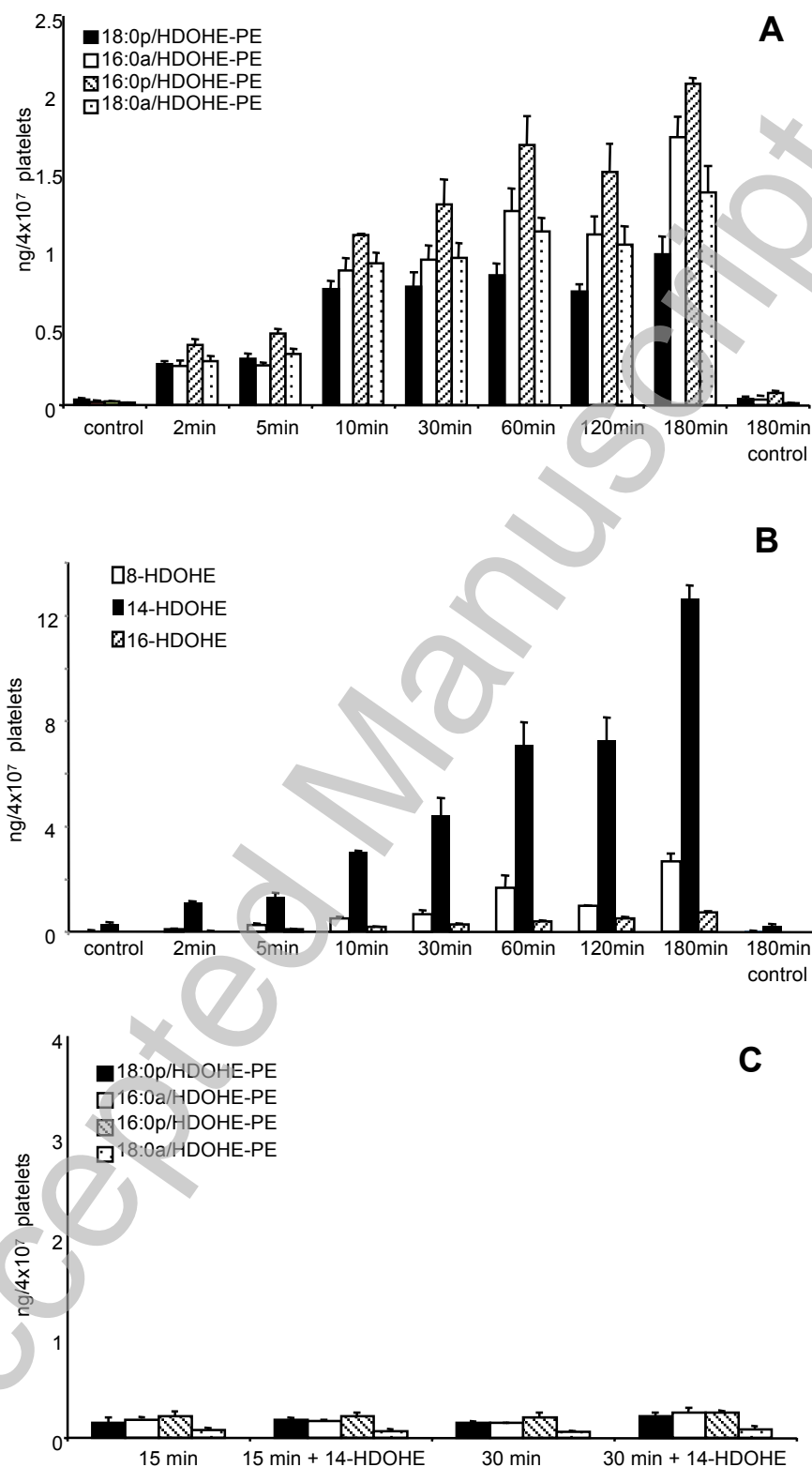


Figure 6

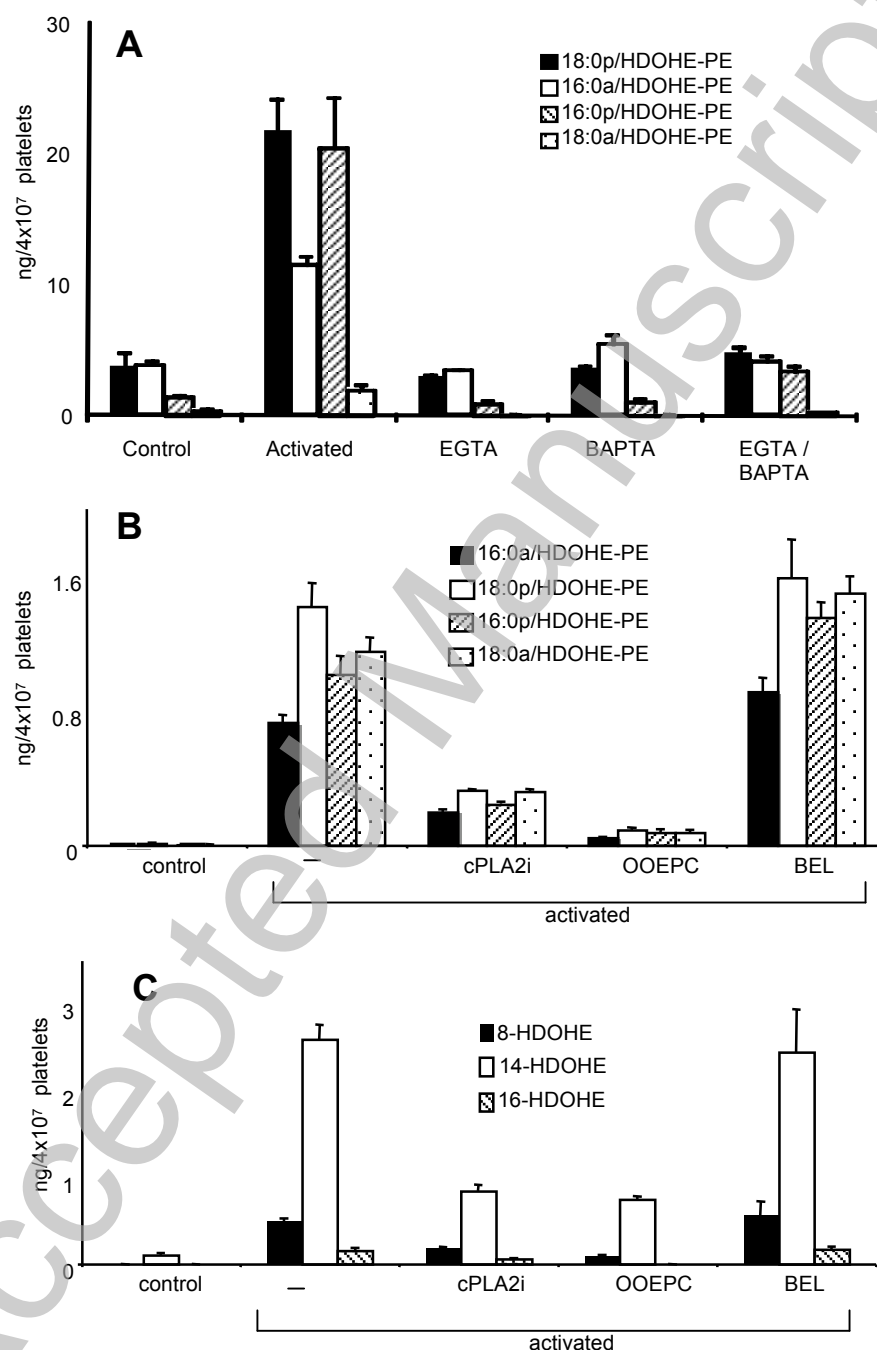


Figure 7