Human platelets generate phospholipid-esterified prostaglandins via cyclooxygenase-1 that are inhibited by low dose aspirin supplementation

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Abstract

Oxidized phospholipids (oxPLs) generated nonenzymatically display pleiotropic biological actions in inflammation. Their generation by cellular cyclooxygenases (COXs) is currently unknown. To determine where platelets generate prostaglandin (PG)-containing oxPLs, then characterize their structures and mechanisms of formation, we applied precursor scanning-tandem mass spectrometry to lipid extracts of agonist-activated human platelets. Thrombin, collagen, or ionophore activation stimulated generation of families of PGs comprising PGE$_2$ and D$_2$ attached to four phosphatidylethanolamine (PE) phospholipids (16:0/18:1/18:0/18:0). They formed within 2 to 5 min of activation in a calcium-dependent manner (28.1 ± 2.3 pg/2 × 10$^8$ platelets). Unlike free PGs, they remained cell associated, suggesting an autocrine mode of action. Their formation was inhibited by in vivo aspirin supplementation (75 mg/day) or in vitro COX-1 blockade. Inhibitors of fatty acyl reesterification blocked generation significantly, while purified COX-1 was unable to directly oxidize PE in vitro. This indicates that they form in platelets via rapid esterification of COX-1 derived PGE$_2$/D$_2$ attached to PE. In summary, COX-1 in human platelets acutely mediates membrane phospholipid oxidation via formation of PG-esterified PLs in response to pathophysiologically agonists.—Aldrovandi, M., V. J. Hammond, H. Podmore, M. Hornshaw, S. R. Clark, L. J. Marnett, D. A. Slatter, R. C. Murphy, P. W. Collins, and V. B. O’Donnell. Human platelets generate phospholipid-esterified prostaglandins via cyclooxygenase-1 that are inhibited by low dose aspirin supplementation. J. Lipid Res. 2013. 54: 3085–3097.

Supplementary key words Oxidized phospholipids • atherosclerosis • PGE$_2$/D$_2$-PEs

Prostaglandins (PGs) are a family of lipid signaling mediators generated by cyclooxygenase (COX) enzymes, COX-1 and -2. They play central roles in health, as well as in diseases including cancer and atherosclerosis. Until recently, they were considered to only exist as free acid mediators, secreted from cells to activate G-protein coupled receptors in a paracrine manner. In 2005, Kozak et al. (1, 2) showed prostaglandins E$_2$ and D$_2$ (PGE$_2$, PGD$_2$) were generated in macrophase cell lines from COX-2 oxidation of endogenous arachidonyl-glycerol (2-AG) and arachidonyl-ethanolamide (AEA). The products signal differently to free PGE$_2$ and D$_2$; for example, PGE$_2$G mobilizes calcium rapidly in a PGE$_2$-independent manner, indicating they are chemically and functionally distinct from their free acid analogs (3, 4).

Oxidized phospholipids (oxPLs) were originally characterized as nonenzymatically-generated species present in atheromatous plaque that display potent immunomodulatory activities (5–7). Recent studies have indicated that they are also generated in a highly specific manner by cellular lipoxygenases (LOXs) in neutrophils, monocytes and platelets (8–14). Also, oxidized cholesteryl esters formed by 15-LOX in macrophase can transfer the oxidized fatty acyl group to phospholipids (15). However, oxPLs generated by COX isoforms have not been described.

Abbreviations: AA, arachidonate; ACD, acid citrate dextrose; AEA, arachidonylethanolamine; 2-AG, 2-arachidonoyl glycerol; BEL, bromoelactone; COX, cyclooxygenase; cPA$_{2i}$, cytosolic phospholipase A$_{2i}$ inhibitor; DTPA, diethylenetriaminepentaacetic acid; HETE, 12-hydroxyeicosatetraenoic acid; LOX, lipoxygenase; MRM, multiple reaction monitoring; OPEPC, oleoyloleoylphosphocholine; oxPL, oxidized phospholipids; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, prostaglandin; PG$_D_2$, prostaglandin D$_2$; PGE$_2$, prostaglandin E$_2$; PL, phospholipid; SAPE, 1-stearoyl-2-arachidonyl-PE; TXA$_2$, thromboxane A$_2$.

*Author’s Choice—Final version full access.

Manuscript received 24 June 2013 and in revised form 19 July 2013.

Published, JLR Papers in Press, July 24, 2013

DOI 10.1194/jlr.M041533
Furthermore, whereas COX-2 can oxidize AEA and 2-AG, the constitutive isoform COX-1 has not been shown to be a source of any esterified eicosanoids before. Herein, we used a targeted lipidomic approach to demonstrate that human platelets generate phospholipid (PL)-esterified PGE$_2$ and PGD$_2$ on agonist activation. Their synthesis is highly regulated, involving receptors and a number of key intracellular signaling pathways. Thus, oxPLs can be generated acutely via COX-1 and represent a new family of lipids from this important vascular signaling pathway.

**MATERIALS AND METHODS**

**Materials**

Lipid and fatty acid standards were purchased from Avanti Polar Lipids (Alabaster, AL) or Cayman Chemical (Cayman Islands). HPLC grade solvents were from Thermo Fisher Scientific (Hemel Hempstead, UK). Protease-activated receptor (PAR)-1 and -4 agonists were from Tocris Biosciences (Bristol, UK). Tri(Hemel Hempstead, UK). Protease-activated receptor (PAR)-1 (Waters Ltd. Herts, UK) with a gradient of 50–100% B over 25 min.

**Precursor LC-MS/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 mmol/L ammonium acetate, 60:20:20; B, methanol, 1 mmol/L ammonium acetate) with a flow rate of 200 μl·min$^{-1}$. Settings were DP -50 V, CE -45 V. Spectra were acquired scanning Q1 from 650 to 950 atomic mass units (amu) over 5 s with Q3 set to m/z 351.2. Absorbance was monitored at 205 nm and products identified using a mixture of standard phospholipids (Sigma-Aldrich). Fractions were collected at 30 s intervals for subsequent analysis by direct injection ESI/MS/MS on a Sciex 4000 QTrap. This was performed by injecting 20 μl of each fraction under flow (1 ml·min$^{-1}$) in methanol into the electrospray source, with specific multiple reaction monitoring (MRM) transitions monitored as parent of m/z 770.6, 796.6, 798.6 and 814.6 [M-H]$^-$, fragmenting to daughter of m/z 351.2. Settings were DP -140 V, CE -45 V.

**Reverse-phase LC-MS/MS of esterified eicosanoids**

For analysis of esterified prostaglandins in MRM mode, lipid extracts were separated by reverse-phase HPLC using a Luna 3 μm C18 (2) 150 mm × 2 mm column (Phenomenex) with a gradient of 50%–100% B over 10 min followed by 15 min at 100% B, then resetting to starting conditions over 5 min (A, methanol:acetonitrile:water, 1 mmol/L ammonium acetate, 60:20:20; B, methanol, 1 mmol/L ammonium acetate) with a flow rate of 200 μl·min$^{-1}$. Analysis was performed using heated ESI in negative ion mode at sheath, auxiliary, and sweep gas flows of 30, 10, and 0, respectively. The capillary and source heater temperatures were set to 275 and 250°C, respectively. Resolving power of 30,000 in FTMS mode was used. Negative MS/MS spectra were acquired using higher energy collision-induced-dissociation. Data-dependent MS (3) of m/z 351 was carried out in ITMS mode on the LTQ in negative mode.

**Isolation and activation of human platelets**

All blood donations were approved by the Cardiff University School of Medicine Ethics Committee, were with informed consent (SMRECG 12/37, SMRECG 12/10), and were according to the Declaration of Helsinki. For studies on isolated platelets, whole blood was collected from healthy volunteers free from nonsteroidal anti-inflammatory drugs for at least 14 days into acid-citrate-dextrose (ACD; 85 mmol/L trisodium citrate, 65 mmol/L citric acid, 10 µM 1,2-bis(2-aminooxy)ethane-N,N,N′,N′-tetraacetic acid tetrasodium-acetoxymethyl ester (BAPTA/AM), 100 nM wortmannin, 100 nM/L G6 6850, 50 µmol/L PD98059, 50 µmol/L PP2, 100 µmol/L p38 mitogen-activated protein kinase inhibitor, and 5 µM U-73122) included a 10 min preincubation at room temperature. Calcium in some experiments, calcium was omitted from Tyrode’s buffer (134 mmol/L NaCl, 12 mmol/L NaHCO$_3$, 2.9 mmol/L KCl, 0.34 mmol/L Na$_2$HPO$_4$, 1.0 mmol/L trisodium citrate, 65 mmol/L citric acid, 10 µM 1,2-bis(2-aminooxy)ethane-N,N,N′,N′-tetraacetic acid tetrasodium-acetoxymethyl ester (BAPTA/AM), 100 nM wortmannin, 100 nM/L G6 6850, 50 µmol/L PD98059, 50 µmol/L PP2, 100 µmol/L p38 mitogen-activated protein kinase inhibitor, and 5 µM U-73122) included a 10 min preincubation at room temperature. In some experiments, calcium was omitted from Tyrode’s buffer (134 mmol/L NaCl, 12 mmol/L NaHCO$_3$, 2.9 mmol/L KCl, 0.34 mmol/L Na$_2$HPO$_4$, 1.0 mmol/L MgCl$_2$, 10 mmol/L Hepes, 5 mmol/L/L glucose, pH 7.4) containing ACD (9:1, v/v). Platelets were centrifuged at 800 g for 10 min then resuspended in Tyrode’s buffer at 2 × 10$^8$·ml$^{-1}$. Platelets were activated at 37°C in the presence of 1 mmol/L CaCl$_2$ for varying times, with 0.2 unit·ml$^{-1}$ thrombin, 10 µg/ml collagen, 10 µg/L A23187, 20 µg/L TFFLR-NH$_2$, or 150 µmol/L AY-NH$_2$ before lipid extraction as below. Experiments involving signaling inhibitors (1 mmol/L aspirin, 1 mmol/L SC-560, 10 µmol/L indomethacin, 2 µmol/L OOEPC, 50 µmol/L BEL, 50 µmol/L cPLA$_2$, 75 µM thimerosal, 7 µM trisacrin C, 1 mM EGTA, 10 µM 1,2-bis(o-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid tetrakis-acetoxymethyl ester (BAPTA/AM), 100 nM wortmannin, 100 nM/L G6 6850, 50 µmol/L PD98059, 50 µmol/L PP2, 100 µmol/L p38 mitogen-activated protein kinase inhibitor, and 5 µM U-73122) included a 10 min preincubation at room temperature. In some experiments, calcium was omitted from buffers. For separation of cells from microparticles, platelets were centrifuged at 970 g for 5 min then supernatants resuspended at 16,060 g for 5 min. For aspirin supplementation, blood samples were first obtained following a 14-day NSAID-free period for aspirin.
baseline determinations of eicosanoids. Subjects were administered 75 mg/day aspirin for 7 days then provided a second blood sample. Platelets were isolated and activated in vitro using 0.2 U/ml thrombin, as described above, then lipids extracted as described below. Exclusion criteria was a known sensitivity to aspirin.

**Lipid extraction**

5 ng PGE₂-d₄, PGD₂-d₄, and di-14:0-phosphatidylethanolamine were added to samples before extraction as internal standards. Lipids were extracted by adding a solvent mixture [1 mol/L acetic acid, isopropyl alcohol, hexane (2:20:30, v/v/v)] to the sample at

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**Fig. 1.** Identification of esterified PGs in human platelets and analysis of PGE₂/D₂-PE using LC/MS/MS. A: Precursor scanning demonstrates lipids with m/z 351.2 eluting during LC/MS/MS. Total lipid extracts from washed human platelets activated with 0.2 U/ml of thrombin for 30 min at 37°C were separated on the Q-Trap platform using LC/MS/MS as described in Materials and Methods, with online negative precursor scanning for m/z 351.2. *, region of LC trace where ions appear that are elevated by thrombin stimulation. Control, broken line. B: Identification of ions that generate m/z 351.2 daughter ions. Shown is a negative MS scan of region marked * in A. Scan shows ions eluting between 19 and 24 min. C: Characterizing phospholipid headgroups of esterified PL. Lipid extracts from thrombin-activated platelets were separated on normal-phase HPLC, as described in Materials and Methods, with fractions collected at 30 sec intervals. Twenty microliters of each fraction was analyzed specific parent → m/z 351.2 MRM transitions. PL class elution was determined using commercial phospholipid standards. Panels D–G: LC/MS/MS of PGE₂/D₂-PEs. Platelet lipid extracts were separated using LC/MS/MS as described in Materials and Methods and detected on the Q-Trap platform by parent → m/z 271.2.
Fig. 2. Generation of free and esterified PGs by agonist-activated platelets. Generation of PGE₂/D₂-PEs and free prostaglandins in response to pathophysiological agonists. Washed platelets were activated for varying times as shown, and lipids were extracted and analyzed using LC/MS/MS as described in Materials and Methods. Platelets were activated using 0.2 U/ml thrombin and PGE₂/D₂-PEs were determined (A). Platelets were activated using 10 µg/ml of collagen (B). Platelets were activated using 10 µg/ml of collagen and 0.2 U/ml of thrombin (C). Platelets were activated using 10 µmol/L A23187 (D). Levels of PGE₂/D₂-PE are expressed as ratio analyte to internal standard with experiments repeated at least three times on different donors (n = 3, mean ± SEM).

Scheme 1. Structures of PGE₂/D₂-PE molecules identified in human platelets.

Reverse-phase LC-MS/MS of free eicosanoids
Lipids were separated on a C18 Spherisorb ODS2, 5 µm, 150 x 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.
Fig. 3. Esterified prostaglandins are retained by platelets while free PGE$_2$ and PGD$_2$ are primarily secreted, and generation of free and esterified PGs is sensitive to COX-1 inhibition in vitro and in vivo. A, B: Esterified PGs are retained by platelets. Washed human platelets were activated with 0.2 U/ml thrombin for 30 min before centrifugation at 970 g. The supernatant was centrifuged at 16,060 g to pellet microparticles before lipid extraction and analysis by LC/MS/MS. C, D: Esterified and free PGE$_2$/D$_2$ generation is sensitive to aspirin
ionization on a Sciex 4000 Q-Trap using parent-to-daughter transitions of m/z 351.2 [MH]+ to m/z 271 for PGE2 and PGD2, m/z 355.2 to 275.3 for PGE2-d4 and PGD2-d4 with declustering potential of -55 and collision energies of -26 V. Products were identified and quantified using standards run in parallel under the same conditions. The following transitions were monitored: m/z 351.2 → 271 (PGE2 and PGD2), m/z 355.2 → 275.3 (PGE2-d4 and PGD2-d4).

Reverse-phase LC-MS/MS of esterified eicosanoids

For analysis of PGE2-d2/PGD2-phosphatidylethanolamines (PEs) in MRM mode, lipid extracts were separated by reverse-phase HPLC using a Luna 3 μm C18 (2) 150 mm × 2 mm column (Phenomenex) with a gradient of 50–100% B over 10 min followed by 30 min at 100% B (A, methanol/acetonitrile/water, 1 mM/L ammonium acetate, at 60:20:20; B, methanol, 1 mM ammonium acetate) with flow rate 200 μl/min. MS was carried out using a Sciex 4000 Q-Trap, using declustering potential -140 V, collision energy -45 V. Lipids were monitored as parent m/z to daughter (m/z 271.2, as appropriate), with dwell time 200 ms.

Phospholipase A2 hydrolysis

Platelet lipid extracts were dried using N2 then resuspended in 1 ml buffer [150 mmol/L NaCl, 5 mmol/L CaCl2, 10 mmol/L Tris (Trizma base), pH 8.9]. Two hundred micrograms snake venom phospholipase A2 (PLA2) from Sigma-Aldrich was added and incubated for 60 min at 37°C. Lipids were reextracted as described above using hexane/isopropanol/acetic acid.

Oxidation of free and phospholipid-esterified arachidonate by purified/recombinant COX-1 and COX-2

Apo-COX-1 was purified from ram seminal vesicles and stored at 3.83 mg·ml−1 in 80 mMTRIS, pH 7.8, at −80°C (17, 18). Wild-type murine COX-2 (recombinant) was generated and purified as described (10.61 mg·ml−1) (19). Both enzymes were quantified using Bicinchoninic Acid (BCA) Protein Assay Kit (Thermo Fisher Scientific), according to manufacturer’s instructions. For heme reconstitution, Apo-COX-1 or -2 (35 μg) was preincubated on ice for 20 min with 2 molar equivalents of hematin in phosphate buffer (100 mM potassium phosphate buffer, pH 7.4). Then, 3.5 μg of the reconstituted enzyme was added to 1 ml phosphate buffer and 500 μmol/L phenol and incubated for 3 min at 37°C in the presence of 150 μM arachidonate (AA, or AA-d8). In some experiments, the same amount of AA was replaced with 1-stearyl-2-araachidonoyl-PE (SAPE). The reaction was stopped by addition of ice-cold lipid extraction solvent and immediate extraction of lipids, after addition of 5 ng each of PGE2-d4 and PGD2-d4 as internal standards, as described earlier. PGE2 and PGD2 were quantified by LC-MS/MS analysis as described earlier. In some experiments, 10 μmol/L of the metal chelator diethylenetriaminepentaacetic acid (DTPA) was added to the reaction just before the addition of holoCOX-1. The following transitions were monitored: m/z 634.4 → 227.2 (di-14:0-phosphatidylethanolamine), m/z 814.7 → 271.2 (PGE2/D2-PE), m/z 822.7 → 278.2 (PGE2/D2-PE-d8), m/z 351.2 → 271 (PGE2 and PGD2), m/z 359.2 → 278.2 (PGE2-d8 and PGD2-d8), m/z 355.2 → 275.3 (PGE2-d4 and PGD2-d4).

RESULTS

Precursor scanning LC/MS/MS identifies esterified PGs in lipid extracts from thrombin-activated platelets

To identify PGs attached to larger functional groups, washed human platelets were activated using thrombin, then lipids extracted and analyzed using precursor LC/MS/MS for m/z 351.2, the carboxylate anion of several PG species. Multiple ions eluted between 16 and 24 min that elevated on thrombin activation (Fig. 1A). Spectra acquired in this time window demonstrated four prominent ions at m/z 770, 796, 798, and 814, (Fig. 1B). These could represent either PE or phosphatidylcholine (PG), with PGs attached (20). Next, lipids were separated into PE and PC fractions using normal-phase HPLC and analyzed by flow injection LC/MS/MS, using parent-to-daughter transitions (parent m/z 770, 796, 798 and 814, daughter m/z 351.2). All four coeluted with the same retention time as a PE standard in the 7-9 min fraction (Fig. 1C). Thus, the ions are proposed as PEs containing 16:0p, 18:1p, 18:0p, and 20:4p at sn1 and a PG at sn2, and are termed prostaglandin-PEs (PG-PEs). Further analysis using MS/MS, MS^n and saponification followed by PG analysis demonstrated that one family of the lipids represents PGE2 and PGD2 esterified to PE (Scheme 1) (see supplementary data).

As we were unable to use m/z 351.2 for MRM detection of PGE2 and PGD2-PE (carboxylate daughter ion did not survive collision-induced-dissociation, as described in full in supplementary data), a method was established using m/z 271.1 as daughter ion (Fig. 1D–G). However, because PGE2 and PGD2-PE lipid coelute on our system, in subsequent studies, each PGE2 and PGD2-PE pair (i.e., the same PE species) are reported as a single species.
Human platelets acutely generate PGE$_2$/D$_2$-PEs on agonist activation that remain cell-associated

Temporal studies showed that PGE$_2$/D$_2$-PEs formed on activation with thrombin, collagen, or ionophore, similar to free PGE$_2$ and PGD$_2$ (Fig. 2A–D). While 16:0p-, 18:0p-, and 18:1p- species were always found, 18:0a/PGE$_2$/D$_2$-PE was sometimes under the limit of detection. Both free and esterified PGE$_2$ and PGD$_2$ were already detectable after 2 min activation. However, PGE$_2$/D$_2$-PEs levels peaked around 10 to 30 min before starting to decline, unlike free PGs, which remained stable or continue to increase up to 3 h after platelet activation (Fig. 2A–D). As standards are not yet available, we isolated PGE$_2$/D$_2$-PEs from platelets and quantified PG attached following hydrolysis and LC/MS/MS. Using this, we determined after 30 min of thrombin activation, mean values for esterified PGE$_2$/D$_2$-PE were 7.05 ± 0.7, 8.2 ± 0.9, 9.5 ± 0.5 and 3.3 ± 0.2 pg/2 × 10^8 platelets (mean ± SEM, five genetically unrelated donors) for the 16:0p/, 18:1p/, 18:0p, and 18:0a/ forms, respectively, with a total for all four PE species of 28.1 ± 2.3 pg/2 × 10^8 cells. PGE$_2$/D$_2$-PE was primarily retained (~85%) by the platelets with small amounts appearing in either microparticles or supernatant (Fig. 3A). In contrast, ~95% of free PGE$_2$ and PGD$_2$ was released into the supernatant (Fig. 3B).

PGE$_2$/D$_2$-PE generation is blocked by COX-1 inhibition in vitro and in vivo

In vitro, several COX inhibitors including indomethacin, aspirin, and the COX-1 inhibitor, SC 560, completely inhibited PGE$_2$/D$_2$-PE formation as well as free PGE$_2$/D$_2$ (Fig. 3C–F). Furthermore, a 7 day supplementation with COX-1 selective low dose aspirin in vivo inhibited generation of both free and esterified PGE$_2$/D$_2$ by washed platelets in response to thrombin (Fig. 3G, H).

PGE$_2$/D$_2$-PE forms in platelets via esterification of free PGE$_2$/D$_2$ into PE

To determine whether PGE$_2$/D$_2$-PE generation by platelets is via direct oxidation of PE or fast esterification of newly formed eicosanoid, PLAs, and fatty acyl reacylation pathways were inhibited and the ability of purified and recombinant COX isoforms to oxidize AA-containing PE in vitro tested. Inclusion of several PLAs inhibitors demonstrated requirement for cPLA$_2$ (cPLA$_{2\alpha}$), but not iPLA$_2$ BEL or sPLA$_2$ OOEPC (Fig. 4A, C). Similar results were seen for free PG (Figs. 4B, D). Inhibition of fatty acylation using thimerosal or triasin C showed approximately 50% inhibition of PGE$_2$/D$_2$-PE generation (Fig. 4E, F). In separate experiments, platelets were supplemented with PGE$_2$/44 or AA-d8, at amounts similar to those generated during platelet activation, to determine whether exogenous lipids are incorporated into PE during the timescale of platelet activation. However, platelets never generated deuterated-PGE$_2$/D$_2$-PEs in our experiments (not shown).

Purified COX isomers generated PGE$_2$ and D$_2$ from AA with a 2:1 predominance of PGE$_2$ over PGD$_2$, due to decomposition of enzymatically-generated PGH$_2$, similar to what is observed in platelets (Figs. 2, 4G, H). A small amount of PGE$_2$/D$_2$-PE was detected in 18:0a/20:4-PE (SAPE), even though it was a freshly opened vial, and this was increased by hematin (the COX-1 cofactor, added alone as control) through nonenzymatic oxidation (Fig. 4I). However, COX-1 did not elevate PGE$_2$/D$_2$-PE further, indicating it cannot directly oxidize SAPE. However, when SAPE was added during COX-1 oxidation of AA, a small formation of PGE$_2$/D$_2$-PEs was observed (Fig. 4I). Where AA-d8 was used instead of AA, deuterated forms of esterified PGs were not detected, indicating that esterified PGs originated directly via SAPE oxidation (not shown). Metal chelation (DTPA) did not inhibit formation, demonstrating that Fenton chemistry was not involved (Fig. 4I). Thus, it is likely that AA-derived radicals escaping from the COX-1 active site during turnover oxidize PE in a metal-independent manner generating isoprostane-PEs that include PGE$_2$/D$_2$-PEs. This may also occur in platelets as a minor pathway for esterified isoprostane formation, but it is unlikely to account for the majority of the PGE$_2$/D$_2$-PE formation as esterified isoprostanes were not detected in our experiments.

Collectively, the requirement for cPLA$_2$ and COX-1 for platelet PGE$_2$/D$_2$-PE generation indicates that COX-1 oxidation of AA is required. Inhibition by triasin C and thimerosal suggest that PGE$_2$/D$_2$-PE generation in platelets occurs via reesterification of PGs. However, only endogenously generated PG is utilized, suggesting tight coupling between PGE$_2$/D$_2$ synthesis and incorporation into PE.

Fig. 4. Generation of PGE$_2$/D$_2$-PEs requires cPLA$_2$ and esterification of free eicosanoids. A-D: Generation of free and esterified PGE$_2$/D$_2$ requires cPLA$_2$ but not the other PLAs isoforms. Washed human platelets were incubated for 10 min with each phospholipase A$_2$ inhibitor prior to activation (0.2 U/ml for 30 min) followed by lipid extraction and analysis by LC/MS/MS. Inhibitors: cytosolic PLA$_2$ (cPLA$_{2\alpha}$) inhibitor (cPLA$_{2\alpha}$i, 50 nmol/L), Ca$^{2+}$-dependent secretory PLA$_2$ (sPLA$_2$) inhibitor (OOEP2C, 2 µmol/L), Ca$^{2+}$-independent intracellular PLA$_2$ (iPLA$_2$) inhibitor (BEL, 50 nmol/L) or vehicle (DMSO, 0.5%). E, F: Inhibition of PGE$_2$/D$_2$-PE, by thimerosal or triasin C. Washed platelets were incubated for 30 min at 37°C with 75 µmol/L thimerosal or 7 mmol/L triasin C prior to thrombin activation (0.2 U/ml for 30 min) followed by lipid extraction and analysis using LC/MS/MS. For all experiments, n = 3; mean ± SEM; data are representative of three independent experiments. *P < 0.05, **P < 0.01, and ***P < 0.001 versus control using ANOVA and Bonferroni post hoc test. G–H: COX isoforms generate a 2:1 ratio of PGE$_2$/PGD$_2$ in vitro. 3.5 µg of purified ovine COX-1 and 3.5 µg recombinant murine COX-2 were incubated at 37°C for 3 min with 150 µM of AA before lipid extraction and analysis using LC/MS/MS as described in Materials and Methods. PGE$_2$ and PGD$_2$ are expressed as micrograms/3.5 µg enzyme formed over 3 min (n = 3, mean ± SEM). 1:PE is oxidized to PGE$_2$/D$_2$-PE during oxidation of AA by COX-1. Purified ovine Apo-PGHS-1 was reconstituted with hematin, ratio 2:1 (hematin: Apo-PGHS-1). 3.5 µg of purified ovine COX-1 was incubated at 37°C for 3 min with the following substrates: 150 µmol/L of AA; 150 µmol/L SAPE; liposomes containing AA and SAPE, in the presence or absence of 10 µM DTPA, before lipid extraction and analysis using LC/MS/MS as described in Materials and Methods. Levels of PGE$_2$/D$_2$-PE are expressed as ratio analyte to internal standard/3.5 µg enzyme generated over 3 min. PGE$_2$ and PGD$_2$ are expressed as micrograms/3.5 µg enzyme generated over 3 min (n = 3, mean ± SEM).
Generation of PGE$_2$/D$_2$-PE requires PARs, intracellular calcium mobilization, src-tyrosine kinases, and MEK1, but not P13-kinase, whereas PKC exerts negative feedback inhibition on generation.

Thrombin activates platelets via PAR1 and PAR4. Peptide agonists for either receptor, TFFLR-NH$_2$ (PAR1) or AV-NH$_2$ (PAR4), stimulated generation of both esterified and free PGE$_2$/D$_2$ (Fig. 5A, B). Generation of PGE$_2$/D$_2$-PE and free PGE$_2$/D$_2$ in response to thrombin was inhibited by the cytosolic calcium chelator BAPTA/AM, but not by chelation of extracellular calcium by EGTA, implicating calcium mobilization from intracellular stores (Fig. 5C, D).

Inhibition of P13-kinase (wortmannin) was without effect, while blocking PKC (Gö 6850) significantly enhanced PGE$_2$/D$_2$-PE formation (Fig. 5E,F). This indicates that PKC exerts a negative feedback effect on free and esterified PGE$_2$/D$_2$ formation. Several agents effectively blocked generation including: U-73122, PD98059, PP2 and p38 MAP kinases inhibitor, implicating phospholipase C, MEK1, src-tyrosine kinases and p38 MAP kinases, respectively (Fig. 5E-H). Collectively, the data indicate a highly coordinated receptor and intracellular signaling pathway that is similar for both free and PE-esterified prostaglandins (Scheme 2).

**DISCUSSION**

Herein, we showed that agonist-activated human platelets generate families of oxPLs via COX-1 that comprise esterified PGs, specifically PGE$_2$ and PGD$_2$ attached to PE. They form through a coordinated sequence of receptor and intracellular signaling pathways. We defined a new group of oxPLs and COX products, which form in platelets in response to pathophysiological agonists. OxPLs are families of bioactive lipids generated by both enzymatic and nonenzymatic pathways in vascular and immune cells (3, 7, 9–11, 13, 14). Up to now, it has not been considered that COX is a source of these; thus, this represents the first example of cross-talk between these two key lipid signaling pathways. It is also a new finding for COX-1, which has not been shown as a source of esterified PGs before.

PGE$_2$/D$_2$-PEs originated from COX-1 as their generation was sensitive to pharmacological inhibitors of this pathway both in vitro and in vivo. The primary product of COX-1, PGH$_2$, is unstable in aqueous milieu, and in platelets, is either rapidly transformed to thromboxane A$_2$ (TXA$_2$) or undergoes rearrangement to PGE$_2$ and D$_2$ (21, 22). The PGD synthase inhibitor HQL-79 did not block PGD$_2$ formation (data not shown). Platelets express mPGES-2 and cPGEs, which catalyze PGE$_2$ generation from PGH$_2$, while mPGEs-1 is not detectable (21). Because no selective mPGES-2 and cPGEs inhibitors were available, involvement of mPGES-2 and cPGEs could not be investigated. However, as free PGE$_2$/D$_2$ ratios are similar for both platelets and purified COXs, their generation in platelets from PGH$_2$ prior to esterification is most likely nonenzymatic (Figs. 2, 4G, H).

The levels of these lipids are lower than for free PGs generated by platelets (28.1 ± 2.3 pg/2 × 10$^8$ platelets). However, they are not secreted from the cells and thus maybe concentrated in intracellular membranes, leading to considerably higher local concentrations. Furthermore, bioactive phospholipids can signal at extremely low concentrations; for example, PAF that can cause life-threatening airway inflammation and inflammatory activation at only 10–1,000 pM concentrations (23).

PGE$_2$/D$_2$-PEs are generated within the first 2 min of platelet activation, similar to the timescale for generation of free PGE$_2$/D$_2$ (Fig. 2A–D). This indicates that formation is fast, coordinated, and a common event in response to several agonists. Their temporal generation (peaking around 10–30 min) could indicate further metabolism of esterified PGs through PLA$_2$ hydrolysis or membrane remodeling pathways. The involvement of several signaling pathways indicates that this is a highly regulated event and further underscores their likely relevance to platelet biology. All these act upstream, stimulating cPLA$_2$ and COX-1 and leading to generation of both free and esterified PGE$_2$/D$_2$ (Scheme 2). Partial inhibition using thimerosal or triasin C coupled with in vitro experiments showing that COX-1 cannot directly oxidize PE suggests that PGE$_2$/D$_2$-PEs form via reesterification of newly formed PGE$_2$/D$_2$ (Scheme 2). This idea is also supported by the absence of 8-iso-PGE$_2$ and 11β-PGE$_2$ in LC-MS/MS chromatograms of PGs hydrolyzed from platelet PE (supplementary Fig. III G). This mechanism is fully consistent with aspirin inhibition in vitro and in vivo. Due to the short timescales involved and the inability of PGE$_2$d4 to become esterified during platelet activation, it is likely that the proteins involved in formation and reesterification are closely associated such that AA hydrolysis, oxidation, and esterification...
are coordinated. We note that little is known regarding how oxidized fatty acids are esterified into phospholipids, and whether the enzymes involved display preferences for different fatty acids or eicosanoids. To address this, in separate experiments, rat liver microsomes were used as a model system to study PGE2 esterification into PE. However, although AA was efficiently esterified to either PE or PC, we were unable to detect PGE2-PE or -PC formation. We note that previous studies have shown that CoA independent transacylation reactions involved in ether lipid coupling are present in platelets but not rat liver microsomes, indicating that these systems likely contain very different complement of enzymes involved in fatty acid acylation (24).

The CoA-synthetases and lysophospholipid acyltransferases likely responsible for prostaglandin esterification are localized at the endoplasmic reticulum, mitochondrial membrane, and peroxisomal membranes (25). Furthermore, COX-1 is localized to dense tubule structures in platelets (26). Thus, PGE2/D2-PE generation may occur on intracellular membranes (Scheme 2). In this case, exogenously added PGE2 must enter the platelet in order to be esterified into PL. Thus, the lack of PGE2-d4 esterification might also be due to an inability of platelets to take up this lipid through the absence of prostaglandin transporters on the cell surface. To date, nothing is known regarding expression of these proteins by platelets nor how they utilize oxidized fatty acids as substrates (27).

PGE2/D2-PEs belong to a growing family of phospholipid-esterified eicosanoids that have been described in platelets and other circulating vascular cells over the last 5 years. Up to now, all were generated enzymatically by LOXs and in platelets include families of PE and PC that contain 12-hydroxyeicosatetraenoic acid (HETE) or 14-hydroxydocosahexaenoic acid (13, 14). Additional LOX-derived PE-esterified HETEs and keto-eicosatetraenoic acids have also been characterized in human neutrophils and monocytes (3, 9, 10). Platelet HETE-phospholipids are also generated following PAR receptor triggering, but the intracellular signaling cascade is partially distinct, involving sPLA2 and extracellular calcium, but not PLC. This likely reflects the different signaling pathways involved in 12-LOX versus COX-1 activation in platelets.

Unlike free eicosanoids, PGE2/D2-PEs remain membrane-bound, indicating that they are likely to act locally (Fig. 3A,B). This is similar to other enzymatically-generated oxPLs, such as HETE-phospholipids generated by LOXs, which regulate coagulation and immune cell signaling (3, 9–11, 14). Oxidized PLs contain polar groups that can protrude from the cell membrane surface. In the case of oxidized PCs, this has led to the “Lipid Whisker Hypothesis”, where the sn2 fatty acid derivatives coat the outside of the cell and act as scavenger receptor ligands (28). Due to their shape and polarity, PGE2 and PGD2 attached to PE are also likely to protrude from the intracellular membrane surface.

Scheme 2. Proposed mechanisms for formation of PGE2/D2-PE by human platelet COX-1. First, AA is oxidized by COX-1, then esterified into PE. Formation of the lipids takes place in intracellular membranes where COX-1 and esterification enzymes are localized.
where they could interact with cytosolic proteins. Additionally, they could perturb membrane dynamics during platelet activation, through causing thinning or increasing water permeability, as shown to occur during chemical oxidation of membranes (29). This may play a role in vesiculation or degradation, both events that involve significant membrane perturbations. Once sufficient quantities of these lipids can be generated, these ideas will all be tested in future studies.

In summary, COX-1 was found to generate a new family of oxPLs in platelets. The identification of these new metabolites opens the way for the study of how phospholipid-bound PGs may regulate membrane behavior during platelet function and whether these might be a distinct target for modulation in platelet-dependent pathologies.

REFERENCES