Endothelial Cell PAF Synthesis following Thrombin Stimulation Utilizes Ca^{2+}-Independent Phospholipase A_2[†]

Jane McHowat,* Pamela J. Kell, Hugh B. O'Neill, and Michael H. Creer

Department of Pathology, St. Louis University School of Medicine, 1402 South Grand Boulevard, St. Louis, Missouri 63104

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ABSTRACT: Platelet activating factor (PAF) is a potent lipid autocoid that is rapidly synthesized and presented on the surface of endothelial cells following thrombin stimulation. PAF production may occur via de novo synthesis or by the combined direct action of phospholipase A_2 (PLA_2) and acetyl-CoA:lyso-PAF acetyltransferase or via the remodeling pathway. This study was undertaken to define the role of PLA_2 and plasmalogen phospholipid hydrolysis in PAF synthesis in thrombin-treated human umbilical artery endothelial cells (HUAEC). Basal PLA_2 activity in HUAEC was primarily found to be Ca^{2+}-independent ($iPLA_2$), membrane-associated, and selective for arachidonylated plasmenylcholine substrate. Thrombin stimulation of HUAEC resulted in a preferential 3-fold increase in membrane-associated $iPLA_2$ activity utilizing plasmenylcholine substrates with a minimal increase in activity with alkylacyl glycerophospholipids. No change in cystolic $iPLA_2$ activity in thrombin-stimulated HUAEC was observed. The thrombin-stimulated activation of $iPLA_2$ and associated hydrolysis of plasmalogen phospholipids was accompanied by increased levels of arachidonic acid (from 1.1 ± 0.1 to 2.8 ± 0.1%) and prostacyclin release (from 38 ± 12 to 512 ± 24%) as well as an increased level of production of lysoplasmenylcholine (from 0.6 ± 0.1 to 2.1 ± 0.3 nmol/mg of protein), lysophosphatidylcholine (from 0.3 ± 0.1 to 0.6 ± 0.1 nmol/mg of protein), and PAF (from 790 ± 108 to 3380 ± 306 dpm). Inhibition of $iPLA_2$ with bromoenol lactone resulted in inhibition of $iPLA_2$ activity, plasmalogen phospholipid hydrolysis, production of choline lysophospholipids, and PAF synthesis. These data indicate that PAF production requires $iPLA_2$ activation in thrombin-stimulated HUAEC and may occur through the CoA-independent transacylase remodeling pathway rather than as a direct result of the PLA_2-catalyzed hydrolysis of membrane alkylacyl glycerophosphocholine.

Platelet-activating factor (PAF) is an acetylated alkyl ether glycerophospholipid which acts as a mediator of homotypic and heterotypic communication between cells. Biologic effects of PAF are manifest at concentrations as low as 10^{-12} M and include both physiologic and pathologic responses. PAF is produced by human endothelial cells in response to a wide variety of agonists, including thrombin, resulting in diverse effects such as neutrophil and platelet adherence, Ca^{2+} uptake, increased permeability of the endothelial cell monolayer, and production of prostacyclin (*1*−*3*).

PAF synthesis occurs via de novo synthesis, by the combined direct action of PLA_2 and acetyl-CoA:lysoPAF acetyltransferase, or by a phospholipid remodeling pathway. Evidence indicates that the remodeling pathway for PAF synthesis is activated during inflammation and hypersensitivity responses, whereas the de novo synthetic pathway is thought to be the source of PAF required for physiologic functions (*1*). The remodeling pathway involves tightly coupled phospholipase A_2 (PLA_2)-catalyzed hydrolysis of membrane phospholipids (plasmenylethanolamine) to produce a lysophospholipid [lysoplasmenylethanolamine (lyso-PlsEtn)] acyl group acceptor followed by CoA-independent transacylation to generate lyso-PAF and, finally, acetylation of lyso-PAF at the *sn*-2 position catalyzed by acetyl-CoA: lyso-PAF acetyltransferase (*4*−*7*).

This study was undertaken to define the relative contribution of these alternative pathways for PAF synthesis and the role of calcium-independent PLA_2 (group VI PLA_2 or $iPLA_2$) in the production of PAF by human endothelial cells following thrombin stimulation. We report that thrombin stimulation of human umbilical artery endothelial cells (HUAEC) activates a membrane-associated $iPLA_2$ that selectively hydrolyzes membrane plasmalogen phospholipids, leading to an increased level of production of lysoplasmalogens and arachidonic acid. Inhibition of $iPLA_2$ with bromoenol lactone [BEL, a selective inhibitor of $iPLA_2$ (*8*)] suppresses phospholipid hydrolysis, lysoplasmenylcholine production, and subsequent PAF synthesis. These data demonstrate that $iPLA_2$ is required for PAF synthesis following thrombin stimulation and suggest that $iPLA_2$ may be involved in the remodeling pathway for PAF production in thrombin-stimulated HUAEC.

MATERIALS AND METHODS

Materials. Human umbilical artery endothelial cells (HUAEC) were obtained from VEC Technologies (Rensse-

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* To whom correspondence should be addressed. Phone: (314) 577-8302. Fax: (314) 268-5649. E-mail: Mchowatj@slucare1.sluh.edu.

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laer, NY). Bromoenol lactone (BEL) was a gift from Hoffmann-La Roche (Nutley, NJ). [^{3}H]Acetic anhydride, [^{3}H]-oleic acid, and [^{3}H]arachidonic acid were purchased from NEN (Boston, MA); [^{14}C]lysophosphatidylcholine and [^{14}C]-acetic anhydride were purchased from Amersham (Arlington Heights, IL). Lysoplasmenylcholine was prepared by alkaline hydrolysis of bovine heart choline glycerophospholipids and characterized as described previously (*9*). Specific *sn*-2 radiolabeled phospholipid substrates were synthesized by acylation of choline lysophospholipids as previously described (*8*). Other reagents were purchased from Sigma Chemical Co. (St. Louis, MO).

Endothelial Cell Cultures. Endothelial cells from human umbilical arteries were grown to confluence in MCDB-131 medium with 10% fetal calf serum, 10 ng/mL epidermal growth factor, 1 μg/mg hydrocortisone, 200 μg/mL endothelial cell growth supplement, and 90 μg/mL heparin. Cells were allowed to grow to confluence, achieving a contact-inhibited monolayer of flattened, closely apposed endothelial cells in 4−5 days. After confluence had been achieved, cells were passaged in a 1:3 dilution and cells from passages 3 and 4 used for experiments.

Stimulation of Confluent Endothelial Cells. All experiments were carried out with HUAEC present as a confluent monolayer. HUAEC were washed with HEPES buffer with the following composition: 133.5 mmol/L NaCl, 4.8 mmol/L KCl, 1.2 mmol/L $MgCl_2$, 1.2 mmol/L KH_2PO_4, 10 mmol/L HEPES, 10 mmol/L glucose, and 1.2 mmol/L $CaCl_2$ (pH 7.4). Thrombin was dissolved in HEPES buffer at a stock concentration that was 100 times greater than the final concentration that was used. Where appropriate, stock solutions of BEL in DMSO were diluted with HEPES buffer and added prior to thrombin stimulation. At the end of the stimulation period, chloroform and methanol were added directly to the HUAEC monolayer for measurement of phospholipid and lysophospholipid levels. For measurement of PLA_2 activity, the surrounding buffer was removed and immediately replaced with ice-cold buffer containing 250 mmol/L sucrose, 10 mmol/L KCl, 10 mmol/L imidazole, 5 mmol/L EDTA, 2 mmol/L DTT, and 10% glycerol (pH 7.8) (PLA_2 assay buffer). For assessment of arachidonic acid or 6-keto-$PGF_{1\alpha}$ release, the surrounding medium was removed and centrifuged and the supernatant removed for assay of arachidonic acid radioactivity or 6-keto-$PGF_{1\alpha}$ content.

Phospholipase A_2 Activity. Endothelial cells suspended in ice-cold PLA_2 assay buffer were sonicated on ice six times for 10 s, and the sonicate was centrifuged at 14000*g* for 10 min. The resultant supernatant fraction was centrifuged at 100000*g* for 60 min to separate the membrane fraction (pellet) from the cystolic fraction (supernatant).

System I (modified from refs 10−14). Phospholipase A_2 activity in subcellular fractions was assessed by incubating the enzyme with 100 μM (16:0,[1-^{14}C]16:0)phosphatidylcholine (Amersham, specific activity of 120 dpm/pmol, ∼55 μCi/μmol) in either 400 μM Triton X-100, 100 mM HEPES (pH 7.5), 5 mM EDTA, and 0.1 mM ATP (EDTA buffer) or 10 mM $CaCl_2$, 100 mM KCl, and 25 mM Tris-HCl (pH 8.5, Ca^{2+} buffer) at 40 °C for 30 min in a total volume of 500 μL. The substrate was prepared by drying under nitrogen and solubilizing in EDTA or Ca^{2+} buffer with sonication until the solution clarified. Reactions were initiated by the addition of enzyme (cystolic or membrane protein) to the assay system.

System II (modified from refs 10−14). Phospholipase A_2 activity in subcellular fractions was assessed by incubating the enzyme with 30 μM (16:0,[^{3}H]20:4)phosphatidylcholine (Amersham, specific activity of 150 dpm/pmol, ∼68 μCi/μmol) in either 400 μM Triton X-100, 100 mM HEPES (pH 7.5), 5 mM EDTA, and 0.1 mM ATP or 10 mM $CaCl_2$, 100 mM KCl, and 25 mM Tris-HCl (pH 8.5) at 40 °C for 30 min in a total volume of 500 μL. The substrate was prepared by drying under nitrogen and solubilizing in EDTA or Ca^{2+} buffer with sonication until the solution clarified. Reactions were initiated by the addition of enzyme (cystolic or membrane protein) to the assay system.

System III (taken from refs 15−17). Phospholipase A_2 activity in subcellular fractions was assessed by incubating the enzyme with a synthetic 100 μM (16:0,[^{3}H]18:1)- or (16:0,[^{3}H]20:4)plasmenylcholine, phosphatidylcholine, or alkylacyl glycerophosphocholine substrate (specific activity of 150 dpm/pmol, ∼68 μCi/μmol) in assay buffer containing 100 mM Tris, 4 mM EGTA, and 10% glycerol (pH 7.0) at 37 °C for 5 min in a total volume of 200 μL. Reactions were initiated by adding the radiolabeled phospholipid substrate as a concentrated stock solution in ethanol.

Reactions in all systems were terminated by the addition of butanol, and then tubes were vortexed and centrifuged at 2000*g* for 5 min. The released radiolabeled fatty acid was isolated by application of an aliquot of the butanol phase to channeled Silica Gel G plates, development in a petroleum ether/diethyl ether/acetic acid mixture (70/30/1, v/v), and subsequent quantification by liquid scintillation spectrometry with appropriate quench correction.

Choline Lysophospholipid Production. Lysophosphatidylcholine (lysoPtdCho) and lysoplasmenylcholine (lysoPlsCho) measurements were taken using a modification of a radiometric assay method described previously (*18*, *19*). Lipids were extracted from HUAEC and the surrounding medium by the method of Bligh and Dyer (*20*), and lysophospholipids were separated from other phospholipids by HPLC. The purified lysoPtdCho and lysoPlsCho fractions were acetylated with [^{3}H]acetic anhydride using 0.33 M (dimethylamino)-pyridine (DMAP) as a catalyst. The acetylated lysophospholipid was then separated by thin-layer chromatography and radioactivity quantified by liquid scintillation spectrometry. Standard curves were constructed, and the lysoPtdCho and lysoPlsCho content was derived for all samples and normalized according to the protein content of HUAEC as described previously (*18*, *19*). [^{14}C]LysoPtdCho was added as an internal standard to all samples to correct for the loss of sample that occurred during extraction, purification, and acetylation.

PAF Production. Confluent HUAEC monolayers were washed twice with Hank's balanced salts solution containing 135 mM NaCl, 0.8 mM $MgSO_4$, 10 mM HEPES (pH 7.4), 1.2 mM $CaCl_2$, 5.4 mM KCl, 0.4 mM KH_2PO_4, 0.3 mM Na_2-HPO_4, and 6.6 mM glucose and incubated with 50 μCi of [^{3}H]acetic acid for 20 min. After thrombin stimulation for the selected time interval, lipids were extracted from the cells by the method of Bligh and Dyer (*20*). The chloroform layer was concentrated by evaporation under N_2, applied to a silica gel 60 TLC plate, and developed in a chloroform/methanol/acetic acid/water mixture (50/25/8/4, v/v). The region cor-

responding to PAF was scraped, and radioactivity was quantified using liquid scintillation spectrometry. Loss of PAF during extraction and chromatography was corrected by adding a known amount of [^{14}C]PAF as an internal standard. [^{14}C]PAF was synthesized by acetylating the *sn*-2 position of lyso-PAF with [^{14}C]acetic anhydride using 0.33 M DMAP as a catalyst. The synthesized [^{14}C]PAF was purified by HPLC. The specific activity of the final [^{14}C]-PAF product was 300 μCi/μmol. In selected experiments, the surrounding buffer was removed from the HUAEC at the end of the thrombin stimulation period and the level of PAF production was measured in the surrounding buffer and in the HUAEC separately. In these experiments, 3.6% bovine serum albumin was added to Hank's buffer.

Synthesis and Acid-Catalyzed Hydrolysis of [^{3}H]PAF Species. 1-*O*-Acyl-2-[^{3}H]acetyl-*sn*-glycero-3-phosphocholine, 1-*O*-alk-1′-enyl-2-[^{3}H]acetyl-*sn*-glycero-3-phosphocholine, and 1-*O*-alkyl-2-[^{3}H]acetyl-*sn*-glycero-3-phosphocholine were synthesized by acetylating the corresponding lysophospholipids with [^{3}H]acetic anhydride using 0.33 M DMAP and incubating overnight at 37 °C. Synthesized [^{3}H]PAF species and [^{3}H]PAF produced in thrombin-stimulated HUAEC were divided equally, and one sample was incubated with 0.1 M HCl for 20 min at 37 °C to hydrolyze the vinyl ether linkage at the *sn*-1 position of PAF species derived from plasmalogen lysophospholipids. Following acid-catalyzed hydrolysis, both samples were extracted using the method of Bligh and Dyer (*20*), and [^{3}H]PAF was separated by HPLC using a hexane/2-propanol/water mobile phase (465/465/70). Radioactivity in the eluate was collected at 1 min intervals and counted by liquid scintillation spectrometry.

Arachidonic Acid Release. The extent of arachidonic acid release was determined by measuring the amount of [^{3}H]-arachidonic acid released into the surrounding medium from HUAEC prelabeled with 3 μCi of [^{3}H]arachidonic acid per 35 mm culture dish for 18 h. Following incubation, HUAEC were washed three times with Tyrode's solution containing 3.6% bovine serum albumin to remove unincorporated [^{3}H]-arachidonic acid. Endothelial cells were incubated at 37 °C for 15 min prior to implementation of the experimental conditions. At the end of the stimulation period, the surrounding medium was removed to a scintillation vial and represented the amount of radiolabeled arachidonic acid released from the HUAEC during the stimulation interval. The amount of radiolabeled arachidonic acid remaining in the endothelial cell monolayer was measured by adding 1 mL of 10% sodium dodecyl sulfate, removing the cells from the culture well by scraping, and adding them to a scintillation vial. Radioactivity in both the surrounding medium and endothelial cells was quantified by liquid scintillation spectrometry.

PGI_2 Formation. Following thrombin stimulation, the buffer surrounding the HUAEC monolayer was removed and rapidly centrifuged. The amount of PGI_2 released from endothelial cells was measured in the resultant supernatant as its stable metabolite 6-keto-$PGF_{1\alpha}$. The amount of 6-keto-$PGF_{1\alpha}$ in the sample was measured using a commercially available immunoassay kit (R&D Systems, Minneapolis, MN).

Separation and Quantification of Individual Choline and Ethanolamine Glycerophospholipid Molecular Species. Cellular phospholipids were extracted from HUAEC by the method of Bligh and Dyer (*20*) at 0−4 °C. The chloroform layer was dried under N_2 and the lipid residue resuspended in 1 mL of a chloroform/methanol mixture (1/1, v/v). Phospholipids were separated into different classes by HPLC using gradient elution with a hexane/2-propanol/water mobile phase as described previously (*21*). Individual choline and ethanolamine glycerophospholipid molecular species were separated by reverse-phase HPLC using a gradient-elution system with an acetonitrile/methanol/water mobile phase with 20 mM choline chloride (*21*). Quantification of individual phospholipid molecular species was achieved by determination of the amount of lipid phosphorus in reverse phase HPLC column effluents by the method of Itaya and Ui (*22*).

Statistics. Statistical comparison of values was performed with the Student's *t* test or analysis of variance with the Fisher multiple-comparison test as appropriate. All results are expressed as means ± the standard error of the mean (SEM). Statistical significance was considered to be $p < 0.05$.

RESULTS

Stimulation of HUAEC with increasing concentrations of thrombin for 10 min resulted in a dose-dependent increase in the level of PAF production with an ED_{50} for thrombin of 0.06 IU/mL (data not shown). Incubation of HUAEC with 0.05 IU/mL thrombin resulted in a time-dependent increase in the level of PAF production that was significant after 1 min and maximal after 5 min and remained at maximal levels over the 30 min stimulation period (Figure 1A). PAF produced in response to thrombin stimulation remained associated with the HUAEC and was not released into the surrounding buffer (Figure 1B).

To optimize the detection of thrombin-activated PLA_2 and to characterize the PLA_2 activity in HUAEC, we employed modifications of different previously published assay systems that were initially designed to selectively measure $iPLA_2$ and/or intracellular calcium-activated PLA_2 ($cPLA_2$) activity. System I was designed to measure the activity of $iPLA_2$ by using dipalmitoylphosphatidylcholine as the substrate ($iPLA_2$ from $P388D_1$ macrophages is ATP-activated, and the activity is maximal with disaturated substrates in the presence of Triton-X-100) and by measuring activity in the absence of Ca^{2+} ($iPLA_2$ is the only isoform that does not require Ca^{2+} for catalysis or association with the phospholipid substrate). System II was designed to measure the activity of $cPLA_2$ by using an arachidonylated phospholipid substrate ($cPLA_2$ demonstrates selectivity for arachidonylated phospholipids) and using 10 mM Ca^{2+} in the assay buffer ($cPLA_2$ requires Ca^{2+} for association with its phospholipid substrate). However, using the conditions employed in system II, it is possible that all three types of PLA_2 could contribute to the total PLA_2 activity. We have extended these two assay systems to include the measurement of the activity of PLA_2 with each substrate measured in both the presence and absence of Ca^{2+}. System III has been used previously by us and other groups to measure PLA_2 activity in both the presence and absence of Ca^{2+} and using all three types of phospholipids (diacyl, plasmalogen, and alkylacyl) found in membranes. In all three assay systems, PLA_2 activity measured in the absence of Ca^{2+} (presence of 4 mM EGTA) is presumed to be due to $iPLA_2$; however, PLA_2 activity measured in the presence of Ca^{2+} could be due to any PLA_2

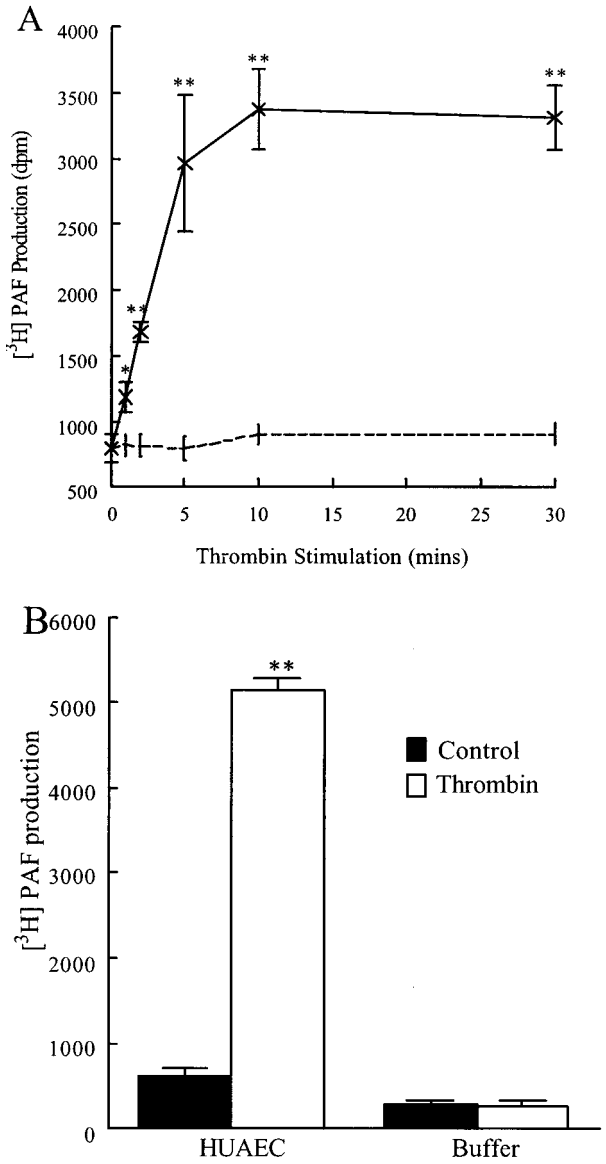


FIGURE 1: (A) Time course of PAF production in HUAEC following stimulation with thrombin (×, 0.1 IU/mL). PAF production in unstimulated HUAEC is represented by the dashed line. (B) The increased PAF production remains associated with cells after HUAEC are stimulated with thrombin (0.1 IU/mL, 10 min). When experimental values are compared to unstimulated values, one asterisk denotes $p < 0.05$ and two asterisks denote $p < 0.01$. Values shown represent the means ± SEM for results derived from eight (A) or four (B) different cell cultures.

isoform present in endothelial cells. In all the assay systems that were used, the PLA_2 specific activity in the membrane fraction was at least 10-fold greater than that measured in the cytosol (Table 1). After correction for the total amount of protein in each subcellular fraction, total membrane-associated PLA_2 activity was at least 5-fold higher than that in the cytosol (data not shown). Both cystolic and membrane-associated PLA_2 activity was maximal in the absence of Ca^{2+} with all the phospholipid substrates that were tested (Table 1). Thus, the majority of HUAEC PLA_2 does not require Ca^{2+} for substrate hydrolysis and is thus Ca^{2+}-independent ($iPLA_2$).

Stimulation of the HUAEC monolayer with thrombin (0.1 IU/mL) resulted in a time-dependent increase in membrane-associated $iPLA_2$ activity measured using (16:0,[^{3}H]18:1)-plasmenylcholine in the absence of Ca^{2+} (system III) that was maximal at 2 min and returned to basal levels after stimulation for 10 min (Figure 2). Basal and thrombin-activated $iPLA_2$ demonstrated a distinct preference for substrates with a vinyl ether linkage at the *sn*-1 position and arachidonic acid esterified at the *sn*-2 position (i.e., arachidonylated plasmalogens). Although there was a consistent ~2-fold increase in membrane-associated $iPLA_2$ activity in response to thrombin using phosphatidylcholine and alkylacyl glycerophosphocholine substrates, this did not reach statistical significance (Figure 2). Measurement of PLA_2 activity in thrombin-stimulated (0.1 IU/mL, 1 min) HUAEC using different PLA_2 assay systems demonstrated a significant increase in activity with plasmenylcholine substrates only (Table 1). A significant increase in thrombin-stimulated $iPLA_2$ activity was observed when using the plasmenylcholine substrate in assay systems I and III. Although there was a consistent increase in thrombin-stimulated $iPLA_2$ activity using system II, this did not reach statistical significance. Thrombin-stimulated $iPLA_2$ thus displays catalytic features that are distinct from those previously described for $cPLA_2$ (*10*) and for the ATP-activated $iPLA_2$ from $P388D_1$ macrophages whose activity is maximal with disaturated substrates in the presence of Triton X-100 (*13*).

Following thrombin treatment, there was no corresponding decrease in cystolic PLA_2 activity, suggesting that minimal translocation of PLA_2 activity from the cystolic to the membrane fraction occurred in response to thrombin stimulation. Accordingly, thrombin stimulation of HUAEC likely activates latent $iPLA_2$ activity in the HUAEC membrane fraction.

Pretreatment of HUAEC with the $iPLA_2$ selective inhibitor BEL (5 μM, 10 min) significantly decreased basal membrane-associated $iPLA_2$ activity [from 6.0 ± 1.1 to 0.7 ± 0.7 nmol (mg of protein)$^{-1}$ min^{-1}, $n = 6$, $p < 0.01$] and inhibited completely the thrombin-stimulated increase (from 22.9 ± 1.7 to 1.3 ± 0.6 nmol/mg of protein, $n = 6$, $p < 0.01$) in membrane-associated $iPLA_2$ activity measured using the (16:0,[^{3}H]18:1)plasmenylcholine substrate in the presence of 4 mM EGTA.

To determine which membrane phospholipids may serve as endogenous substrates for thrombin-stimulated HUAEC $iPLA_2$, detailed characterization and mass measurements of individual molecular species in choline and ethanolamine phospholipids were performed. For these studies, individual phospholipid molecular species were isolated by gradient-elution, reverse-phase HPLC. The identity of individual species was confirmed by GC analysis of the volatile FAME and DMA derivatives prepared from the corresponding column eluates, by coelution with synthetic phospholipids of known composition, by demonstration of the acid lability of species containing *sn*-1 vinyl ether linkages (for plasmalogen species), and by demonstration of radiolabel incorporation after incubation with [^{3}H]arachidonic acid (for arachidonylated species). For each of the peaks identified in Figures 3 and 4, the indicated molecular species comprised >80% of the total phospholipid mass in the corresponding column eluates.

Choline glycerophospholipids were comprised of 70% diacyl species, 14% plasmalogens, and 16% alkyl ether glycerophospholipids. Although less than 15% of choline glycerophospholipids are plasmalogens, the vast majority of

Table 1: Phospholipase A_2 Specific Activity (picomoles per milligram of protein per minute) in Human Umbilical Artery Endothelial Cells without or with Thrombin Stimulation (0.1 IU/mL, 1 min)[a]

	cytosol		membrane	
assay substrate with or without calcium	control	thrombin	control	thrombin
System I. Phospholipid Substrate (100 μM), 30 min Incubation at 40 °C				
(16:0,[^{14}C]16:0) PtdCho/EGTA	28 ± 3	34 ± 4	1153 ± 354	1219 ± 278
(16:0,[^{14}C]16:0) PtdCho/Ca	28 ± 2	26 ± 3	973 ± 145	1000 ± 234
(16:0,[^{3}H]18:1)PlsCho/EGTA	39 ± 6	34 ± 5	2337 ± 230	6712 ± 276[b]
(16:0,[^{3}H]18:1)PlsCho/Ca	48 ± 9	39 ± 4	1978 ± 221	3189 ± 302[b]
System II. Phospholipid Substrate (30 μM), 30 min Incubation at 40 °C				
(18:0,[^{3}H]20:4)PtdCho/EGTA	120 ± 23	96 ± 18	3564 ± 349	4137 ± 385
(18:0,[^{3}H]20:4)PtdCho/Ca	71 ± 9	101 ± 11	3971 ± 297	4350 ± 328
(16:0,[^{3}H] 18:1)PlsCho/EGTA	37 ± 5	33 ± 5	733 ± 110	1078 ± 128
(16:0,[^{3}H] 18:1)PlsCho/Ca	35 ± 3	30 ± 5	645 ± 63	898 ± 85
System III. Phospholipid Substrate (100 μM), 5 min Incubation at 37 °C				
(16:0,[^{3}H]18:1)PtdCho/EGTA	491 ± 121	402 ± 99	3156 ± 338	4267 ± 402
(16:0,[3H]18:1)PtdCho/Ca	394 ± 94	431 ± 102	2676 ± 419	3439 ± 381
(16:0,[^{3}H]18:1)PlsCho/EGTA	337 ± 86	384 ± 45	6512 ± 496	18146 ± 2655[b]
(16:0,[^{3}H]18:1)PlsCho/Ca	196 ± 78	244 ± 95	4336 ± 252	15442 ± 2067[b]
(16:0,[^{3}H]18:1)PakCho/EGTA	233 ± 65	265 ± 59	2896 ± 388	4334 ± 645
(16:0,[^{3}H]18:1)PakCho/Ca	207 ± 83	232 ± 85	2332 ± 347	3887 ± 736
(16:0,[^{3}H]20:4)PtdCho/EGTA	1074 ± 233	693 ± 167	7236 ± 576	6674 ± 1244
(16:0,[^{3}H]20:4)PtdCho/Ca	1016 ± 305	634 ± 178	7265 ± 1074	7598 ± 835
(16:0,[^{3}H]20:4)PlsCho/EGTA	1280 ± 263	1213 ± 264	13217 ± 1542	31458 ± 1785[b]
(16:0,[^{3}H]20:4)PlsCho/Ca	1076 ± 199	1123 ± 284	11812 ± 1370	27054 ± 2097[b]
(16:0,[^{3}H]20:4)PakCho/EGTA	1032 ± 256	1007 ± 276	6186 ± 1045	6689 ± 1274
(16:0,[^{3}H]20:4)PakCho/Ca	967 ± 131	894 ± 219	5888 ± 1221	6038 ± 1881

[a] Membrane (8 μg) or cytosolic (200 μg) protein was incubated at 37 °C in the presence (1 mM Ca) or absence (4 mM EGTA) of calcium with (16:0,[^{14}C]16:0)phosphatidylcholine (PtdCho) or (16:0,[^{3}H]18:1)PlsCho for 30 min (system I), with (18:0,[^{3}H]20:4)PtdCho or (16:0,[^{3}H]18:1)PlsCho for 30 min (system II), or with (16:0,[^{3}H]18:1)- or (16:0,[^{3}H]20:4)PtdCho, plasmenylcholine (PlsCho), or alkylacyl glycerophosphocholine (PakCho) for 5 min (system III). Results shown represent means ± SEM for results obtained from four different cell populations. [b] $p < 0.01$ compared with the control value.

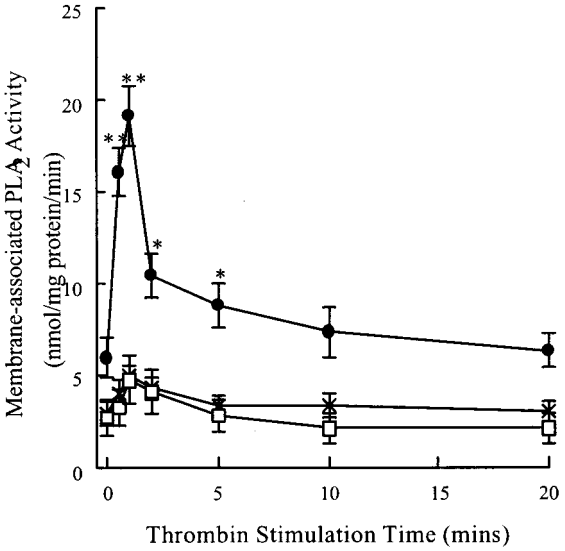
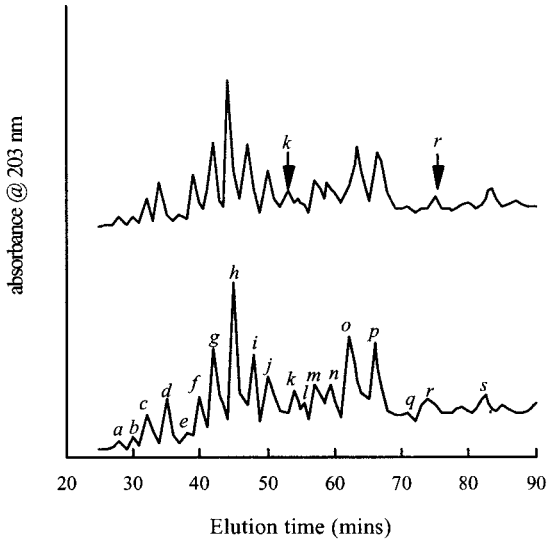


FIGURE 2: Time course of activation of membrane-associated PLA_2 activity in thrombin-stimulated (0.1 IU/mL) HUAEC. PLA_2 activity was measured by incubating the membrane protein (10 μg) with 100 μM (16:0,[^{3}H]18:1)plasmenylcholine (●), phosphatidylcholine (□), or alkylacyl glycerophosphocholine (×) in the absence of Ca^{2+} (4 mM EGTA) for 5 min at 37 °C. When experimental values are compared to the corresponding PLA_2 activity in unstimulated cells, one asterisk denotes $p < 0.05$ and two asterisks denote $p < 0.01$. Values shown represent the means ± SEM for results derived from four different cell cultures.

plasmenylcholine species in HUAEC are arachidonylated at the *sn*-2 position (Figure 3). Thrombin stimulation of HUAEC resulted in a significant decrease in both (16:0, 20:4)- and (18:0, 20:4)plasmenylcholine masses. Pretreatment with BEL prior to thrombin stimulation abolished completely the thrombin-induced decrease in the arachidonylated plasmenylcholine mass (Figure 3), demonstrating that arachidonylated plasmenycholine hydrolysis is mediated by $iPLA_2$ and is not the result of phospholipid hydrolysis through alternate, competing pathways.

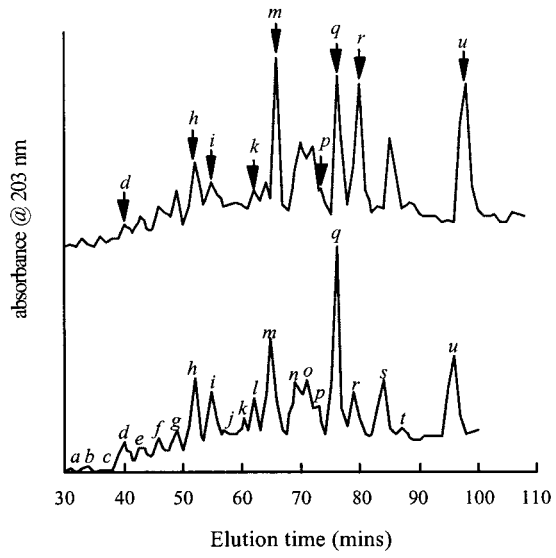
Ethanolamine glycerophospholipids in HUAEC are comprised predominantly of plasmalogen molecular species with smaller amounts of diacylglycerophospholipids and trace quantities of alkyl ether phospholipid species (Figure 4). Collectively, more than 85% of the total esterified arachidonate in ethanolamine glycerophospholipids is found in plasmalogen molecular species. Thrombin treatment was accompanied by a significant reduction in the masses of all arachidonylated plasmenyethanolamine species and other plasmalogen species containing polyunsaturated (18:3) fatty acid esterified at the *sn*-2 position. Significant decreases were observed in the mass of (18:1,18:3)phosphatidylethanolamine and of the (16:0,18:1)alkylacyl species. Thus, thrombin-stimulated ethanolamine phospholipid hydrolysis is not specific for plasmalogens or for arachidonylated species as was observed for hydrolysis of choline glycerophospholipids.

Interestingly, following thrombin treatment, significant increases were noted in the masses of ethanolamine plasmalogen species containing the 18:2 or 18:1 fatty acid esterified at the *sn*-2 position and of the ethanolamine diacylglycerophospholipid containing stearate (18:0) at the *sn*-1 position and arachidonate at the *sn*-2 position. These alterations may reflect the rapid acyl-CoA-dependent reacylation of ethanolamine lysophospholipids produced by thrombin-stimulated $iPLA_2$-catalyzed ethanolamine phospholipid hydrolysis, or alternatively, they may reflect the redistribution of esterified *sn*-2 fatty acids mediated by transacylation



Peak	Identity	Control	Thrombin	BEL & thrombin
b	(18:2, 20:4) PtdCho	0.9±0.1	1.0±0.2	0.9±0.3
h	(16:0, 20:4) PtdCho	6.2±0.6	5.6±0.2	4.7±0.9
k	**(16:0, 20:4) PlsCho**	**2.1±0.2**	**0.9±0.2**	**2.5±0.2**
l	(16:0, 18:2) PlsCho & (18:1, 20:4) PlsCho	1.8±0.3	1.3±0.2	1.9±0.3
o	(18:0, 20:4) PtdCho	5.4±0.8	5.2±0.3	4.4±0.4
r	**(18:0, 20:4) PlsCho**	**3.2±0.2**	**1.7±0.1**	**3.4±0.2**
e	(16:0, 18:3) PtdCho	1.1±0.1	1.3±0.1	1.3±0.2
c	(18:2, 18:2) PtdCho	0.8±0.1	1.1±0.3	1.1±0.1
i	(16:0, 18:2) PtdCho	4.4±0.5	3.9±0.3	4.2±0.3
j	(18:1, 18:2) PtdCho	2.2±0.3	2.5±0.1	2.3±0.4
n	(16:0, 18:2) PakCho	7.8±1.3	8.8±0.5	8.0±1.1
p	(18:0, 18:2) PtdCho	5.1±0.8	4.7±0.2	4.8±0.2
m	(16:0, 18:1) PtdCho	5.6±0.7	5.5±0.4	5.0±0.4
s	(18:0, 18:1) PtdCho	3.3±0.8	2.4±0.6	2.4±0.2

FIGURE 3: HPLC separation of choline phospholipids in control (bottom) and thrombin-stimulated [0.01 IU/mL, 10 min (top)] HUAEC and quantitation of each peak with a microphosphate assay demonstrates a thrombin-stimulated selective decrease in the level of arachidonylated plasmenylcholine (peaks *k* and *r*, denoted with arrows). Phospholipids in bold are significantly different ($p < 0.05$) between control and thrombin-stimulated samples. Values are expressed as nanomoles of PO_4 per milligram of protein and represent means ± SEM for independent results from eight different cell cultures. The composition of individual phospholipid molecular species is described by the shorthand notation (*a*:*b*,*c*:*d*), where *a* and *c* represent chain lengths and *b* and *d* represent the number of C=C bonds for the aliphatic groups at the *sn*-1 and *sn*-2 positions, respectively. PlsCho, plasmenylcholine; PtdCho, phosphatidylcholine; PlsEtn, plasmenylethanolamine; PtdEtn, phosphatidylethanolamine; PakCho, alkylacyl glycerophosphocholine; PakEtn, alkylacyl glycerophosphoethanolamine.



Peak	Identity	Control	Thrombin	BEL & thrombin
a	(18:2, 20:4) PtdEtn	1.4±0.6	0.8±0.3	1.4±0.3
e	(16:0, 20:4) PtdEtn	1.0±0.2	0.7±0.2	1.1±0.2
h	**(16:0, 20:4) PlsEtn**	**3.5±0.5**	**2.2±0.3**	**2.8±0.3**
i	**(18:1, 20:4) PlsEtn & (16:0, 18:2) PlsEtn**	**4.0±0.3**	**2.8±0.4**	**3.9±0.6**
m	**(18:0, 20:4) PtdEtn**	**3.8±0.4**	**5.4±0.9**	**4.4±0.3**
q	**(18:0, 20:4) PlsEtn**	**6.7±0.9**	**3.2±0.7**	**5.4±0.5**
c	**(18:1, 18:3) PtdEtn**	**1.5±0.3**	**0.7±0.1**	**1.2±0.3**
d	**(16:0, 18:3) PlsEtn**	**1.7±0.4**	**0.5±0.1**	**1.5±0.5**
b	(18:2, 18:2) PtdEtn	0.7±0.2	0.8±0.3	0.9±0.2
f	(16:0, 18:2) PtdEtn	1.4±0.3	1.3±0.2	1.4±0.2
g	(18:1, 18:2) PtdEtn	1.7±0.3	1.6±0.2	1.6±0.4
k	**(18:1, 18:2) PlsEtn**	**1.3±0.2**	**4.7±0.7**	**2.1±0.5**
r	**(18;0, 18:2) PlsEtn**	**5.5±0.3**	**9.7±0.4**	**6.8±0.4**
j	(16:0, 18:1) PtdEtn	2.8±0.7	2.8±0.8	2.6±0.6
l	(18:1, 18:1) PtdEtn	2.5±0.5	2.1±0.6	2.3±0.2
p	**(16:0, 18:1) PakEtn**	**3.5±0.9**	**2.0±0.3**	**3.0±0.5**
s	(18:0, 18:1) PtdEtn	3.8±0.6	3.1±0.7	3.8±0.3
u	**(18:0, 18:1) PlsEtn**	**2.7±0.5**	**4.1±0.3**	**3.2±0.3**

FIGURE 4: HPLC separation of ethanolamine phospholipids in control (bottom) and thrombin-stimulated [0.1 IU/mL, 10 min (top)] human HUAEC and quantitation of each peak with a microphosphate assay (nanomoles per milligram of protein). Phospholipids in bold are significantly different ($p < 0.05$) between control and thrombin-stimulated samples. Values are expressed as nanomoles of PO_4 per milligram of protein and represent means ± SEM for independent results from eight different cell cultures.

utilizing ethanolamine lysophospholipids as the fatty acyl group acceptor and another diradylglycerophospholipid as the acyl donor.

Following thrombin stimulation, we observed an overall decrease of 7.3 nmol/mg of protein in the masses of ethanolamine plasmalogen and diacyl glycerophospholipid species containing 18:3 and 20:4 fatty acids esterified at the *sn*-2 position accompanied by an increase of 9.0 nmol/mg of protein in the masses of plasmalogen species with 18:1 and 18:2 *sn*-2 fatty acids. There was no significant change in the total ethanolamine glycerophospholipid mass following thrombin stimulation, indicating there was no significant accumulation of ethanolamine lysophospholipids. BEL treatment before thrombin stimulation abrogated the observed alterations in composition of glycerophospholipid molecular species, demonstrating that the redistribution of *sn*-2 fatty acids among ethanolamine phospholipids was initiated by thrombin activation of $iPLA_2$.

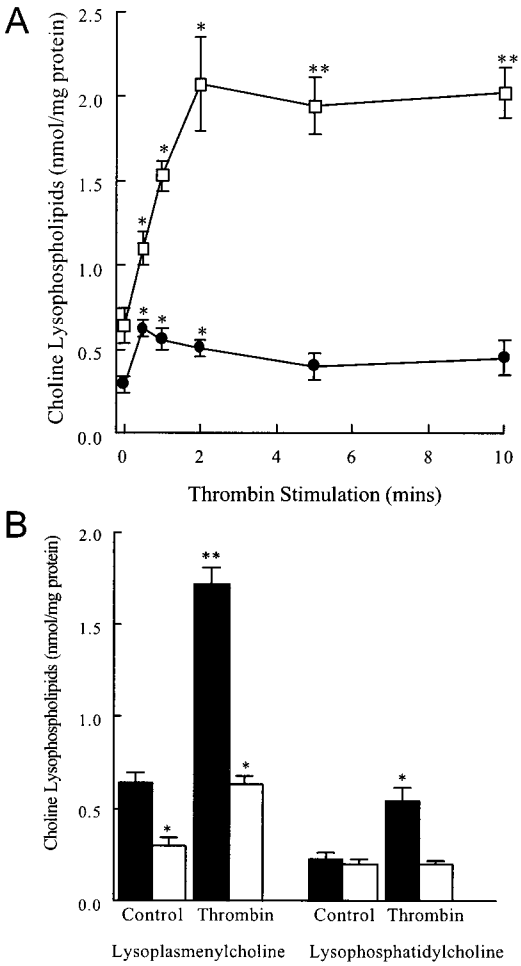


FIGURE 5: (A) Time course of lysoplasmenylcholine (□) and lysophosphatidylcholine (●) production in thrombin-stimulated (0.1 IU/mL) HUAEC. (B) Pretreatment of HUAEC with BEL (5 μM, 10 min, white bars) decreased basal lysoplasmenylcholine content (black bars) but did not affect basal lysophosphatidylcholine (black bars). Thrombin-stimulated increases in the levels of choline lysophospholipids (black bars) were inhibited completely by BEL pretreatment (white bars). When experimental values are compared to control values, one asterisk denotes $p < 0.05$ and two asterisks denote $p < 0.01$. Values shown represent the means ± SEM for results from four separate cell cultures.

Although thrombin treatment did not alter the total ethanolamine glycerophospholipid mass, there was a significant reduction in the level of choline glycerophospholipids in response to thrombin stimulation, suggesting the possibility of the net accumulation of choline lysophospholipids. Accordingly, we quantified the choline lysophospholipid mass following thrombin stimulation. Since thrombin-induced $iPLA_2$ activity was greatest with plasmenylcholine substrates (Table 1) and resulted in preferential hydrolysis of choline plasmalogens, we measured the amount of lysoPlsCho and lysoPtdCho separately in thrombin-stimulated HUAEC. Thrombin stimulation of HUAEC resulted in an increase in both lysoPlsCho and lysoPtdCho content that reached statistical significance after thrombin stimulation for 30 s (Figure 5A). The basal content and magnitude of the thrombin-stimulated increase were significantly lower for lysoPtdCho than for lysoPlsCho. The predominance of lysoPlsCho production over lysoPtdCho generation is consistent with the observed plasmalogen substrate specificity of thrombin-stimulated $iPLA_2$ in HUAEC. The lysoPtdCho content had returned to basal values after 2 min, whereas the lysoPlsCho content remained elevated over the course of 10 min (Figure 5A). Pretreatment with BEL resulted in a significant reduction in basal lysoPlsCho content and reduced thrombin-stimulated lysoPlsCho content to control values (Figure 5B). Pretreatment with BEL had no effect on the control lysoPtdCho content of HUAEC, but completely inhibited the increase in lysoPtdCho content in response to thrombin stimulation (Figure 5B). The reduction in the basal content of choline lysophospholipids following $iPLA_2$ inhibition with BEL is consistent with previous reports of the effects of BEL on basal lysoPtdCho mass in $P3888D_1$ macrophages (*13*) and pancreatic islets (*23*, *24*). Our finding that this reduction is due primarily to a decrease in the lysoPlsCho mass in HUAEC is also consistent with the observed plasmalogen substrate specificity of $iPLA_2$ in HUAEC.

Since thrombin treatment resulted in accelerated hydrolysis of arachidonylated plasmalogen species, we also examined arachidonic acid production and associated prostacyclin generation. Thrombin stimulation of arachidonic acid prelabeled diradylglycerophospholipids in HUAEC resulted in a significant increase in the level of arachidonic acid release after 1 min that was sustained for 15 min (Figure 6A). The coupled oxidation of free arachidonic acid to prostacyclin in HUAEC was evidenced by the significant increase in the level of release of 6-keto-$PGF_{1\alpha}$ (a stable metabolite of prostacyclin) following thrombin stimulation (Figure 6B). The increase in the level of 6-keto-$PGF_{1\alpha}$ in response to thrombin stimulation occurred after 2 min, was maximal at 5 min, and remained elevated over the course of 20 min (Figure 6B). Inhibition of $iPLA_2$ with BEL inhibited completely any thrombin-stimulated increase in the level of arachidonic acid and prostacyclin (6-keto-$PGF_{1\alpha}$) production (Figure 6).

Since pretreatment with BEL inhibited $iPLA_2$ activity and accompanying hydrolysis of plasmalogen phospholipids to lysophospholipids and free fatty acid, we determined whether BEL pretreatment inhibited PAF production in thrombin-stimulated HUAEC. Complete inhibition of PAF production in response to thrombin was observed at concentrations of BEL of ≥ 5 μM (Figure 7), corresponding to those concentrations of BEL that completely inhibit $iPLA_2$-catalyzed plasmalogen phospholipid hydrolysis.

To determine whether synthesis of PAF in HUAEC included the production of 1-*O*-alkenyl-2-acetyl PAF species, we performed acid-catalyzed hydrolysis on the [^{3}H]PAF produced in thrombin-stimulated HUAEC and separated PAF from other phospholipids by HPLC. Incubation with 0.1 M HCl resulted in the complete loss of synthetic 1-*O*-alkenyl-2-[^{3}H]acetyl-*sn*-glycero-3-phosphocholine (Figure 8B), but no significant decrease in the level of synthetic 1-acyl-2-[^{3}H]acetyl-*sn*-glycero-3-phosphocholine (Figure 8A) or synthetic 1-*O*-alkyl-2-[^{3}H]acetyl-*sn*-glycero-3-phosphocholine (Figure 8C), indicating that the acid treatment specifically hydrolyzed the vinyl ether linkage at the *sn*-1 position of PAF species derived from lysoplasmalogens. Acid treatment of the [^{3}H]PAF produced in thrombin-stimulated HUAEC resulted in no significant loss in the amount of radioactivity present in the peak corresponding to PAF (Figure 8D), indicating that little, if any, PAF production was derived from the acetylation of plasmalogen lysophospholipids.

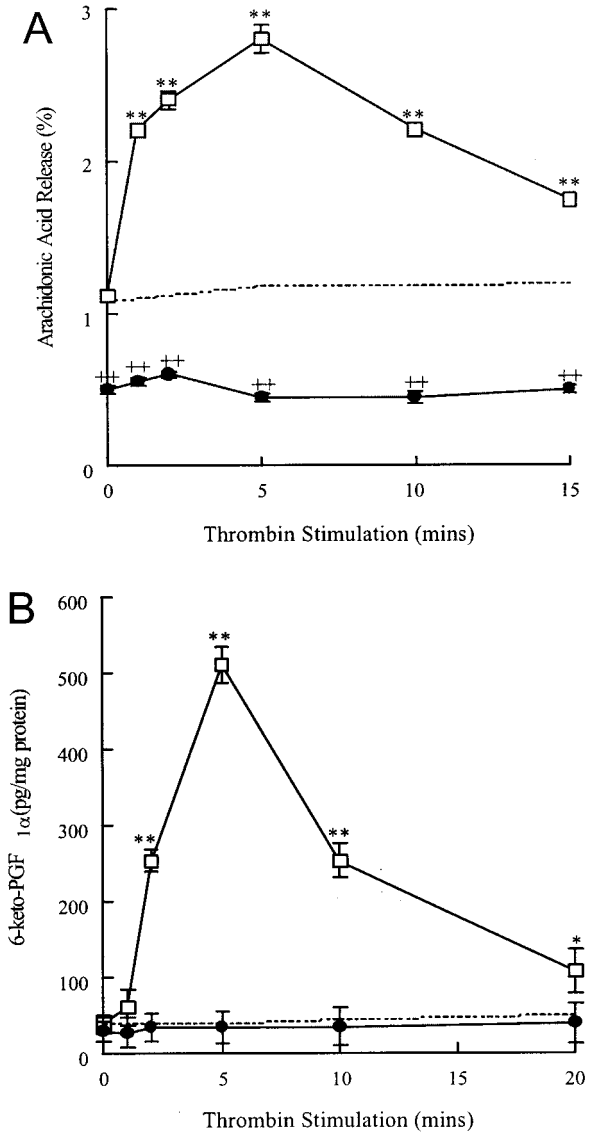


FIGURE 6: Effect of pretreatment with BEL [5 μM, 10 min (●)] on thrombin-stimulated [0.1 IU/mL (□)] arachidonic acid (A) and prostacyclin release [measured as its stable metabolite 6-keto-$PGF_{1\alpha}$ (B)] from HUAEC. Two asterisks denote $p < 0.01$ when experimental values are compared to those of untreated HUAEC. Two pluses denote $p < 0.01$ when experimental values are compared to those of both thrombin-stimulated and untreated HUAEC. Values for untreated HUAEC are represented by the dotted lines. Values shown represent the means ± SEM for results from six separate cell cultures.

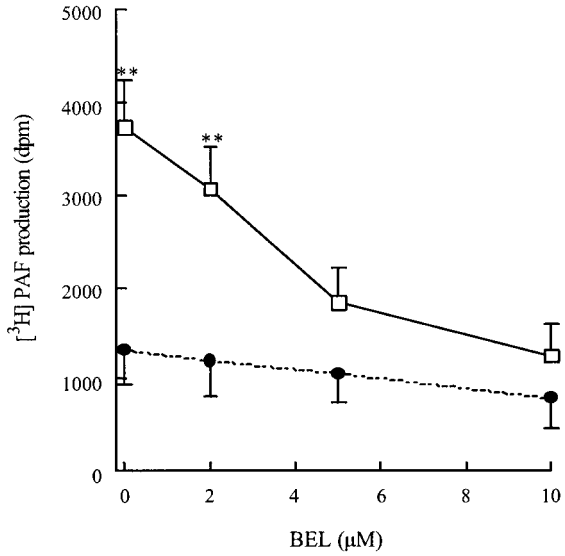


FIGURE 7: Concentration-dependent effect of pretreatment with BEL (10 min) on basal (●) and thrombin-stimulated [0.1 IU/mL (□)] PAF production in HUAEC. Two asterisks denote $p < 0.01$ when experimental values are compared to those of untreated HUAEC. Values shown represent the means ± SEM for results from six separate cell cultures.

Taken together, these data demonstrate that hydrolysis of plasmalogen phospholipids by $iPLA_2$ accompanied by an increased level of production of lysoplasmalogens is required for PAF synthesis in thrombin-stimulated HUAEC.

DISCUSSION

The final step in PAF synthesis is the acetylation of 1-*O*-alkyl-2-lyso-*sn*-glycero-3-phosphocholine (lyso-PAF) catalyzed by acetyl-CoA:lyso-PAF acetyltransferase, a key regulatory enzyme whose activity is modulated by phosphorylation catalyzed by p38 MAP kinase in response to agonist stimulation (*25*). PAF synthesis is also controlled by the availability of the lyso-PAF substrate which may be generated by direct PLA_2-catalyzed hydrolysis of 1-*O*-alkyl-2-acyl-*sn*-glycero-3-phosphocholine (alkylacyl-Gro*P*Cho) or by the sequential PLA_2-catalyzed hydrolysis of ethanolamine plasmalogen phospholipid and activity of CoA-independent transacyclase which utilizes ethanolamine lysoplasmalogen (lysoPlsEtn) as an acyl group acceptor and alkylacyl-Gro*P*Cho as an acyl group donor to produce lyso-PAF and a "remodeled" plasmalogen (i.e., plasmalogen species with a different *O*-acyl ester group at the *sn*-2 position) as products. In either case, the initial step in PAF synthesis is the PLA_2-catalyzed hydrolysis of the *sn*-2 fatty acid from a membrane phospholipid. Several methods have been employed to determine PLA_2 activities in cells or tissues. Most commonly, PLA_2 activity is measured indirectly by the release of arachidonic acid in cells prelabeled with [^{3}H]-arachidonic acid or directly by measuring the level of release of the *sn*-2 fatty acid from a synthetic phospholipid substrate. In this study, to help us identify the specific PLA_2 isoforms involved in PAF synthesis, we employed several previously published assay systems (*10*−*17*) to measure PLA_2 activity directly in HUAEC. The different assay systems were designed to optimize detection of intracellular PLA_2 isoforms and demonstrate striking differences in the values of PLA_2 specific activity, particularly when measuring activity in the cystolic fraction. System III (Table 1) was developed to optimize detection of $iPLA_2$ in cardiac myocytes and endothelial cells (*8*, *15*−*19*). In this system, enzyme activity is stabilized by dithiothreitol, the substrate is presented to the enzyme in the form of multilamellar liposomes, and the incubation period is kept short to maintain linear reaction rates with respect to time and protein concentration. Substrate concentration is utilized to ensure that maximal reaction rates are measured with no significant isotope dilution of substrate by endogenous phospholipid in membrane fractions (*15*−*17*). System II was developed to quantify $cPLA_2$ activity utilizing arachidonylated diacylglycerophopholipid as a lamellar substrate with a prolonged incubation period (30 min) and elevated temperature (40 °C) [$cPLA_2$ is selective for arachidonylated substrates (*15*)]. System III was developed to quantify group VI $iPLA_2$ activity from $P388D_1$

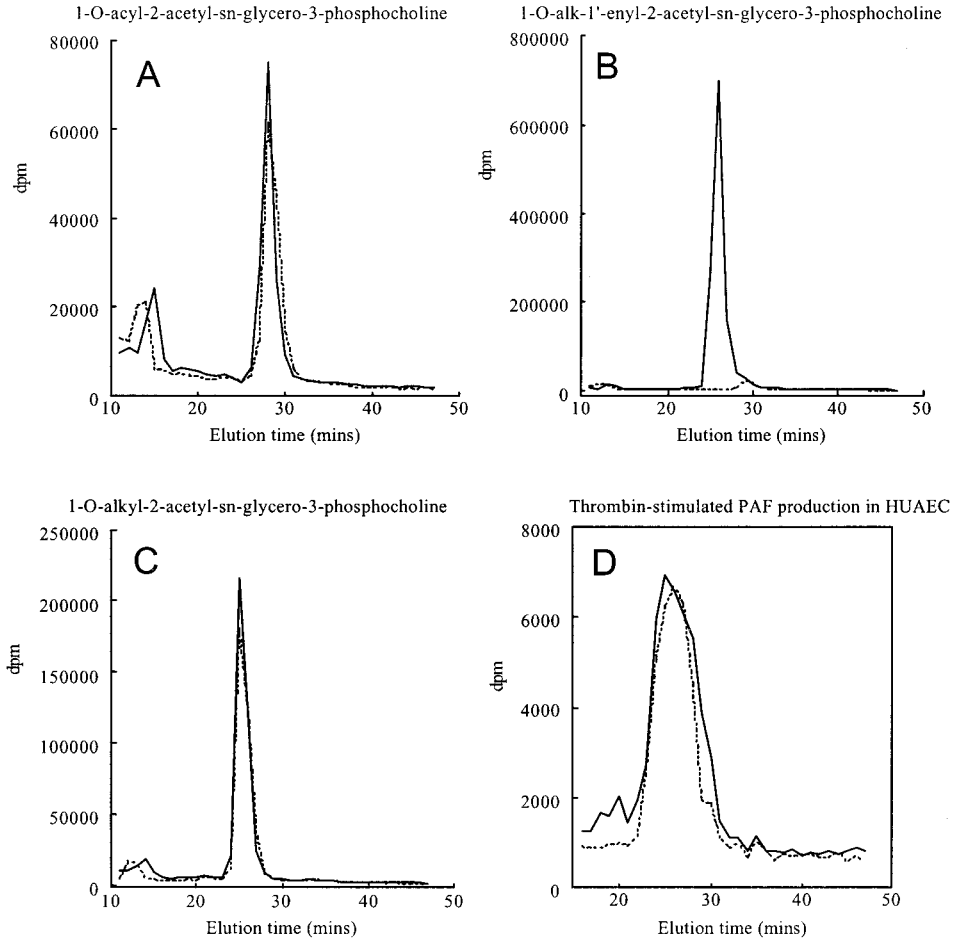


FIGURE 8: HPLC separation of 1-*O*-acyl-2-[^{3}H]acetyl-*sn*-glycero-3-phosphocholine (A), 1-*O*-alk-1′-enyl-2-[^{3}H]acetyl-*sn*-glycero-3-phosphocholine (B), 1-*O*-alkyl-2-[^{3}H]acetyl-*sn*-glycero-3-phosphocholine (C), and PAF produced in thrombin-stimulated HUAEC (D). Aliquots of each PAF species were equally divided, and one aliquot was incubated with 0.1 M HCl for 20 min at 37 °C to hydrolyze the vinyl ether linkage at the *sn*-1 position. Acid-catalyzed hydrolysis (dotted lines) eliminated the radioactivity present in the peak corresponding to PAF derived from plasmalogen lysophospholipid (solid line, B) but did not significantly alter the radioactivity in PAF derived from monoacyl lysophospholipid (solid line, A) or lyso-PAF (solid line, C). PAF production in thrombin-stimulated HUAEC (solid line, D) is resistant to acid-catalyzed hydrolysis (dotted line, D), indicating that little, if any, PAF is derived from plasmalogen lysophospholipid precursors.

macrophages (*16*) and is the only system which utilizes mixed micelles of disaturated choline phospholipid substrate and Triton X-100 detergent, a prolonged incubation (30 min), an elevated temperature (40 °C), and addition of ATP (the $iPLA_2$ isoform from $P388D_1$ macrophages is activated by ATP).

As shown in Table 1, we find that thrombin-stimulated PLA_2 in HUAEC is a membrane-localized $iPLA_2$ which displays a distinct preference for arachidonylated plasmalogen phospholipid substrates. The enzyme is not activated by ATP and is inhibited by the selective $iPLA_2$ inhibitor BEL. The catalytic features of thrombin-stimulated $iPLA_2$ are similar to, but distinct from, the characteristics of previously cloned cytosolic group VI $iPLA_2$ isoforms from $P388D_1$ (*13*), CHO (*26*), and pancreatic islets (*23*, *24*). Additional studies are required to specifically identify the thrombin-activated $iPLA_2$ isoform in HUAEC. No significant increase in $iPLA_2$ activity was observed with phosphatidylcholine or alkylacyl glycerophosphocholine substrates, irrespective of the assay system that was used. These studies highlight the importance of using multiple endogenous phospholipid substrates to assess alterations in PLA_2 activity in response to a particular stimulus. Although absolute values for PLA_2 in each system may differ due to differences in incubation times, the phospholipid substrates that are used, etc., the use of multiple assay systems illustrates the fact that characteristics such as calcium dependency and alterations in activity are comparable. Incubation of HUAEC with BEL, a selective inhibitor of $iPLA_2$ (*8*), resulted in 90% inhibition of membrane-associated $iPLA_2$ activity, further supporting the idea that $iPLA_2$ contributes to the majority PLA_2 activity in HUAEC.

Although plasmalogens account for less than 15% of the total choline phospholipids in HUAEC, this study demonstrates that they represent a metabolically active pool of membrane phospholipids that are preferentially hydrolyzed in response to thrombin stimulation. In addition, the majority of plasmenylcholine is arachidonylated, and thus, hydrolysis of plasmenylcholine may play a significant role in eicosanoid generation. Although activation of multiple phospholipases may account for the decrease in the level of plasmenylcholine, accumulation of lysoPlsCho is the direct result of activation of PLA_2. Since the pretreatment of HUAEC with BEL prior to thrombin stimulation inhibits plasmenylcholine hydrolysis, lysoPlsCho accumulation, and PAF production, it is likely that PAF synthesis in thrombin-stimulated HUAEC occurs via the remodeling pathway. Since PAF synthesis involves a tightly coupled reaction with PLA_2 and lyso-PAF acetyltransferase and PAF is produced in extremely

small amounts, we cannot rule out the involvement of direct PLA_2-catalyzed hydrolysis of alkylacyl glycerophosphocholine contributing to PAF production. However, in this study, we did not measure significant increases in PLA_2 activity using the alkylacyl glycerophosphocholine synthetic substrate, and we measured a small increase in the activity of membrane alkylacyl glycerophosphocholine, suggesting that the remodeling pathway is the predominant pathway for PAF synthesis.

Thrombin stimulation of HUAEC results in a decrease in the arachidonylated plasmenylethanolamine mass of 5 nmol/mg of protein. The increase in the mass of plasmenylethanolamine species with *sn*-2 oleic (18:1) or linoleic (18:2) acids was 5−6 nmol/mg of protein, suggesting that any lysoPlsEtn produced was rapidly reacylated possibly by CoA-independent transacylase enzymes. This suggests that plasmenylethanolamine hydrolysis may be involved in the production of PAF through the remodeling pathway. To demonstrate the potential involvement of the remodeling pathway in thrombin-stimulated PAF production, we pretreated HUAEC with lysoPlsEtn prior to thrombin stimulation. In these preliminary experiments with intact cells, we were unable to demonstrate any significant increase in PAF production following thrombin stimulation in the presence or absence of BEL in LPlasE-treated cells. The failure to augment PAF production in thrombin-stimulated, BEL-treated endothelial cells in the presence of LPlasE could be due to a variety of causes, including failure of LPlasE uptake by endothelial cells under the conditions of the experiment, rapid reacylation or hydrolysis of LPlasE by endothelial cells, or the failure of exogenous LPlasE to be "delivered" to the appropriate intracellular membrane site for PAF production. The basis of our conclusion that the remodeling pathway may contribute to PAF synthesis by thrombin-activated $iPLA_2$ includes the following. (1) Thrombin-stimulated $iPLA_2$ preferentially hydrolyzes PC plasmalogen substrates, but the majority of the PAF produced in response to thrombin contains acid-stable 1-*O*-alkyl and 1-*O*-acyl groups at the *sn*-1 position, consistent with the idea that the majority of the PAF is synthesized from lyso-PAF produced by acyl group transfer from diacyl and alkyl ether PC to a lysoPlsEtn acceptor by the transacylation pathway. (2) Plasmenylethanolamine hydrolysis by thrombin-stimulated $iPLA_2$ is accompanied by remodeling of the *sn*-2 fatty acid composition of plasmenylethanolamine (i.e., decrease in the level of plasmenylethanolamine species containing 18:3 and 20:4 and increase in the level of species containing 18:1 and 18:2 fatty acids at the *sn*-2 position). (3) Thrombin stimulation results in the hydrolysis of plasmenylethanolamine substrates without any net loss of phosphatidylethanolamine mass, indicating that lysoPlsEtn generated by $iPLA_2$ may be rapidly utilized for transacylation. In the absence of lysoPlsEtn augmentation of PAF synthesis in thrombin-stimulated BEL-treated cells, our data do not conclusively prove the involvement of the remodeling pathway in PAF synthesis by thrombin-stimulated $iPLA_2$.

Thrombin stimulation of HUAEC results in simultaneous production of PAF and PGI_2, and this has been demonstrated previously in endothelial cells stimulated with several agonists (*27*). In many cells and tissues, PAF causes production of arachidonic acid and its metabolites via activation of PLA_2 following ligation of the PAF receptor

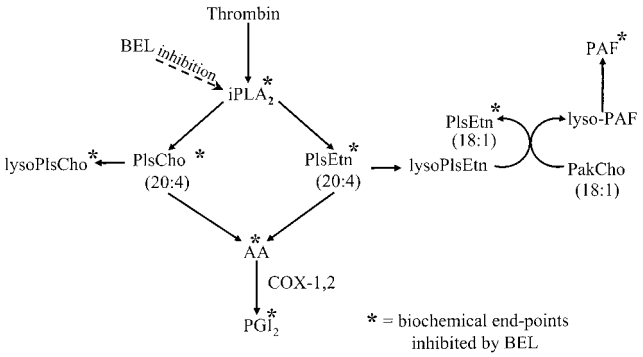


FIGURE 9: Proposed pathway for synthesis of PAF in thrombin-stimulated HUAEC. Our data indicate that activation of $iPLA_2$ by thrombin results in selective hydrolysis of membrane plasmalogen phospholipids, leading to the production of lysoplasmalogens that act as an acceptor for the *sn*-2 fatty acid from alkylacyl glycerophosphocholine, resulting in production of lyso-PAF which is then acetylated to form biologically active PAF.

(*28*). Additionally, eicosanoid production may be associated with autocrine stimulation of PLA_2 activity (*29*). Accordingly, the possibility exists that PGI_2 generation in thrombin-treated EC may itself augment PAF production or vice versa by autocrine pathways that include activation of other PLA_2 isoforms. The concomitant inhibition of PAF and PGI_2 synthesis by BEL following thrombin treatment demonstrates that $iPLA_2$ activation is essential for production of both of these biologically active products; however, BEL inhibition does not exclude a potential role for other PLA_2 isoforms in these alternative autocrine pathways.

We have demonstrated that increased PAF production in response to thrombin stimulation remains associated with HUAEC. These findings agree with several previous studies which showed that PAF stays associated with endothelial cells (*30*−*32*) and plays an important role in interactions with numerous cells circulating in the blood. PAF produced in activated endothelial cells serves as a signal for neutrophils to bind to the endothelium (*32*) and may serve as a signal to bind other cells such as eosinophils (*33*) and basophils (*34*). This enhanced endothelium−cell interaction may play an important role in many disease processes, such as inflammation, thrombosis, and atherosclerosis (*1*−*3*). In addition, PAF has several effects on endothelial cells such as increased permeability, faster phosphoinositide turnover, increased protein kinase C activity, and an increase in the intracellular Ca^{2+} level. Interestingly, the steady-state level of PAF in endothelial cells is tightly controlled by rapid, inducible synthesis initiated by intracellular PLA_2 and equally efficient degradation mediated by PAF acetylhydrolase, a unique PLA_2 with a preference for substrates containing short acyl chains at the *sn*-2 position. The PLA_2 enzymes which control this steady-state PAF concentration may exhibit specificity for individual PAF species possibly contributing to "fine-tuning" of the cellular response to increased PAF production.

In summary, this study provides evidence that in thrombin-stimulated HUAEC, the predominant pathway for PAF synthesis involves $iPLA_2$-catalyzed hydrolysis of membrane plasmalogen phospholipids. The $iPLA_2$-catalyzed hydrolysis of plasmenylcholine is accompanied by accumulation of lysoPlsCho and arachidonic acid, whereas the hydrolysis of plasmenylethanolamine, in addition to arachidonic acid production, generates lysoPlsEtn that may function to

promote PAF synthesis through the remodeling pathway (Figure 9).

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