

**Inhibition of PAF acetylhydrolase by methyl arachidonyl fluorophosphonate
potentiates PAF synthesis in thrombin-stimulated human coronary artery
endothelial cells.**

Pamela J. Kell, Michael H. Creer, Kimberley N. Crown, Karin Wirsig and Jane McHowat.

Department of Pathology, St. Louis University School of Medicine, St. Louis, MO 63104

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Address Correspondence to: Dr. Jane McHowat, Department of Pathology St. Louis University
School of Medicine, 1402 S. Grand Blvd., St. Louis, MO 63104

Phone: (314) 577-8302 Fax: (314) 268-5649 E-mail: mchowatj@slucare1.sluh.edu

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ABBREVIATIONS: Alkyl acyl glycerophosphocholine, PakCho; alkyl acyl glycerophosphoethanolamine, PakEtn; bromoenol lactone, BEL; dimethylaminopyridine, DMAP; human coronary artery endothelial cells, HCAEC; human umbilical artery endothelial cells, HUAEC; lysoplasmeylcholine, LysoPlsCho; lysoplasmeylethanolamine, LysoPlsEtn; methyl arachidonyl fluorophosphonate, MAFP; phosphatidylcholine, PtdCho; phosphatidylethanolamine, PtdEtn; phospholipase A₂, PLA₂; plasmeylcholine, PlsCho; plasmeylethanolamine, PlsEtn; platelet activating factor, PAF; vascular endothelial cell growth factor, VEGF

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ABSTRACT

We have previously demonstrated that thrombin stimulation of endothelial cells results in increased membrane-associated, Ca^{2+} -independent phospholipase A_2 (iPLA $_2$) activity, accelerated hydrolysis of membrane plasmalogen phospholipids and production of several biologically active phospholipid metabolites, including prostacyclin and platelet-activating factor (PAF) that is abolished by pretreatment with the iPLA $_2$ -selective inhibitor bromoenol lactone (BEL). This study was designed to further investigate the role of alternative PLA $_2$ inhibitors, including methyl arachidonyl fluorophosphonate (MAFP, an inhibitor of cytosolic PLA $_2$ isoforms) on phospholipid turnover and PAF production from thrombin-stimulated human coronary artery endothelial cells (HCAEC). Paradoxically, pretreatment of HCAEC with MAFP (5 to 25 μM) resulted in a significant increase in PAF production in both unstimulated and thrombin-stimulated cells that was found to be a direct result of inhibition of PAF acetylhydrolase (PAF-AH) activity. Pretreatment with MAFP did not significantly inhibit HCAEC PLA $_2$ activity, possibly due to the localization of PLA $_2$ activity in the membrane fraction rather than the cytosol. Bromoenol lactone did not inhibit PAF-AH activity, even at concentrations as high as 20 μM . We conclude that MAFP augments thrombin-stimulated PAF production by inhibition of PAF catabolism without affecting membrane-associated iPLA $_2$ activity.

We reported previously that thrombin stimulation of human umbilical artery endothelial cells (HUAEC) activates a membrane-associated, Ca^{2+} -independent PLA_2 (i PLA_2) that selectively hydrolyzed membrane plasmalogen phospholipids leading to increased production of lysoplasmalogens and arachidonic acid (McHowat et al., 2001). We confirmed the involvement of i PLA_2 activation in platelet activating factor (PAF) generation by pretreating the cells with bromoenol lactone (BEL, a selective inhibitor of i PLA_2 (Hazen et al., 1991)). Incubation of HUAEC with BEL resulted in complete inhibition of thrombin-stimulated i PLA_2 activity and PAF production (McHowat et al., 2001). We proposed that i PLA_2 -catalyzed ethanolamine plasmalogen hydrolysis and increased lysoplasmalethanolamine production resulted in increased PAF synthesis via the remodeling pathway (McHowat et al., 2001). Endothelial cell PAF production contributes importantly to the recruitment of leukocytes and monocytes to inflamed tissue by promoting adhesion to the endothelium and thus can play a major role in the progression of inflammatory diseases such as atherosclerosis and asthma (Montrucchio et al., 2000).

In a recent study, Bernatchez and co-workers (2001) demonstrated that PAF production in endothelial cells in response to stimulation with vascular endothelial cell growth factor (VEGF) was dependent upon both PLA_2 and lyso-PAF acetyltransferase activities. Using several pharmacological inhibitors designed to be selective for different PLA_2 isoforms, the authors concluded that secretory PLA_2 (s PLA_2) activity was responsible for the increased PAF production and that there was minimal contribution from the intracellular cytosolic, Ca^{2+} -activated PLA_2 (c PLA_2) or i PLA_2 . The discrepancy between the results found in the two studies could arise from the different agents used for stimulation or the use of endothelial cells from different vascular sources. Since these authors had used methyl arachidonyl fluorophosphonate (MAFP), in addition to BEL, to examine inhibition of endothelial PAF production, we evaluated the role of MAFP in PAF production in thrombin-stimulated human coronary artery endothelial cells (HCAEC).

MAFP was originally developed as a specific inhibitor for c PLA_2 , but was subsequently found to inhibit cytosolic i PLA_2 at similar concentrations (Lio et al., 1996; Balsinde and Dennis, 1996a). MAFP

has an arachidonyl tail coupled to a fluorophosphonate group that reacts with activated serine groups. The compound competes with endogenous phospholipid molecules for the active catalytic site on the PLA₂ enzyme. MAFP has been shown to irreversibly inhibit soluble cytosolic cPLA₂ and iPLA₂, possibly by phosphorylation of the active site serine residue (Ghomashchi et al., 1999). Although the relatively polar MAFP has direct access to the catalytic site of soluble PLA₂ isoforms, the active site serine residue of membrane-associated iPLA₂ may be “protected” from this inhibitor. In addition to PLA₂ inhibition, MAFP has been shown to inhibit anandamide amidase, the enzyme responsible for the hydrolysis of arachidonyl ethanolamide (Deutsch et al., 1997) and has been shown to be an irreversible cannabinoid receptor antagonist (Deutsch et al., 1997; Fernando and Pertwee, 1997).

In this study, we demonstrate that pretreatment of HCAEC with MAFP prior to thrombin stimulation did not inhibit membrane-associated endothelial cell PLA₂ activity and instead resulted in a significant augmentation of PAF production as a result of inhibition of PAF-AH. Phospholipase A₂ inhibitors have been proposed to be potential anti-inflammatory agents since PLA₂-catalyzed hydrolysis of membrane phospholipids is the rate-limiting step for the generation of inflammatory phospholipid metabolites such as eicosanoids and PAF. Our data demonstrate the importance of identifying the PLA₂ isoforms involved in phospholipid metabolite production to guide the development of specific PLA₂ inhibitors that could be used therapeutically.

METHODS

Materials. Human coronary artery endothelial cells (HCAEC) were obtained from Cambrex Bio Science (Walkersville, MD). Bromoenol lactone (BEL) was a gift from Hoffmann-La Roche (Nutley, NJ). Methyl arachidonyl fluorophosphonate (MAFP) was obtained from Cayman Chemical Co. (Ann Arbor, MI). [¹⁴C]lysophosphatidylcholine and [¹⁴C] acetic anhydride were purchased from Amersham (Arlington Heights, IL). [³H]PAF, [³H]acetic anhydride and [³H]arachidonic acid were obtained from Perkin Elmer (Boston, MA). P-selectin antibody was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) and horse radish peroxidase-conjugated anti-goat IgG antibody was obtained from Amersham Biosciences UK, Ltd (Little Chalfont, England). Other reagents were purchased from Sigma Chemical Co. (St. Louis, MO).

Endothelial cell cultures. Endothelial cells were grown to confluence in MCDB-131 medium with 5% fetal calf serum, 10 ng/ml epidermal growth factor, 1 µg/ml 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 achieving confluence, cells were passaged in a 1:3 dilution and cells from passages 3-4 used for experiments.

Phospholipase A₂ activity. Confluent HCAEC cultures were stimulated with thrombin with or without MAFP or BEL for the allocated time intervals. At the end of the stimulation period, iPLA₂ activation was arrested by the removal of the surrounding buffer and immediate replacement with ice-cold buffer containing (mmol/liter): Sucrose 250, KCl 10, Imidazole 10, EDTA 5, DTT 2 with 10% glycerol, pH = 7.8. Cells were removed from the tissue culture well using a cell scraper and the suspension was sonicated on ice for 6 bursts of 10 seconds each. Following sonication, the suspension was centrifuged at 14,000 x g @ 4°C for 20 minutes and the resultant supernatant was centrifuged at 100,000 x g to separate the cytosolic (supernatant) and membrane (pellet) fractions. Phospholipase A₂ activity was assessed by

incubating 50 μg of cytosolic protein or 8 μg of membrane protein with 100 μM (16:0, [^3H]18:1) plasmenylcholine in assay buffer containing 100 mM Tris, 4 mM EGTA, 10% glycerol, pH = 7.0 at 37°C for 5 mins in a total volume of 200 μl . Reactions were initiated by adding the radiolabeled phospholipid substrate as a concentrated stock solution in ethanol. Specific enzyme activity was expressed as the rate of radiolabeled fatty acid production determined following separation from the labeled phospholipid substrate using thin layer chromatography and liquid scintillation spectrometry with activity normalized to protein content as described previously (McHowat and Creer, 1997; 1998a; 1998b).

Separation and quantification of individual choline and ethanolamine glycerophospholipid molecular species. Cellular phospholipids were extracted from HCAEC by the method of Bligh and Dyer (1959). The chloroform layer was dried under N_2 and the lipid residue resuspended in 1 ml chloroform: methanol 1:1 v/v. Phospholipids were separated into different classes by HPLC using gradient elution with a mobile phase comprised of hexane/isopropanol/water as described previously (McHowat et al., 2001). Individual choline and ethanolamine glycerophospholipid molecular species were separated by reverse-phase HPLC using a gradient elution system with a mobile phase comprised of acetonitrile/methanol/water with 20 mM choline chloride (McHowat et al., 2001). Quantification of individual phospholipid molecular species was achieved by determination of lipid phosphorus in reverse phase HPLC column effluents (McHowat et al., 2001).

PAF production. Confluent HCAEC monolayers were washed twice with Hanks' balanced salts solution containing NaCl 135 mM, MgSO_4 0.8 mM, HEPES (pH=7.4) 10 mM, CaCl_2 1.2 mM, KCl 5.4 mM, KH_2PO_4 0.4 mM, Na_2HPO_4 0.3 mM and glucose 6.6 mM and incubated with 50 μCi [^3H] acetic acid for 20 mins. After thrombin stimulation for the selected time interval, lipids were extracted from the cells by the method of Bligh and Dyer (1959). The chloroform layer was concentrated by evaporation under N_2 , applied to a silica gel 60 TLC plate, and developed in chloroform/methanol/acetic acid/water (50/25/8/4

vol/vol). The region corresponding 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 dimethylaminopyridine (DMAP) as a catalyst (McHowat et al., 2001) and was purified by HPLC.

Acid-catalyzed hydrolysis of [^3H]PAF. [^3H]PAF produced in thrombin-stimulated HCAEC with or without MAFP pretreatment were divided into two equal samples and one sample was incubated with 0.1M HCl for 20 mins at 37°C to hydrolyze the vinyl ether linkage at the *sn*-1 position of PAF species derived from plasmalogen lysophospholipids (McHowat et al., 2001). Following acid-catalyzed hydrolysis, both samples were extracted using the Bligh and Dyer method (1959) and [^3H]PAF separated by HPLC using a mobile phase comprised of hexane/isopropanol/water (465/465/70). Radioactivity in the eluate was collected at one minute intervals and counted by liquid scintillation spectrometry.

PAF-acetylhydrolase activity. HCAEC were removed from the tissue culture plate in 1.2 mM Ca^{2+} HEPES buffer and sonicated on ice. 25 μg cellular protein was incubated with 0.1 mM [acetyl- ^3H] PAF (10 mCi/mmol) for 30 min at 37°C. The reaction was stopped by the addition of acetic acid and released [^3H] acetic acid was separated from the [^3H] PAF substrate by passing the reaction mixture through a C_{18} gel cartridge (Baker Chemical Co., Phillipsburg, NJ). Released [^3H]acetic acid was quantified by liquid scintillation spectrometry.

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

RESULTS

We have demonstrated previously that thrombin-stimulated PAF production in HUAEC involves iPLA₂-catalyzed hydrolysis of membrane plasmalogen phospholipids. Pretreatment of HUAEC with the iPLA₂-selective inhibitor BEL resulted in complete inhibition of thrombin-stimulated iPLA₂ activity and increased PAF production. Since MAFP inhibits the cytosolic isoforms of both cPLA₂ and iPLA₂, we measured PAF production in HCAEC pretreated with MAFP prior to thrombin stimulation. Contrary to the results we expected, we observed that, at concentrations of 5 μ M or greater, MAFP pretreatment resulted in significant increases in PAF production in response to thrombin stimulation (Figure 1). In addition, incubation of HCAEC with 25 μ M MAFP without thrombin stimulation resulted in a significant increase in PAF production (Figure 1). Thus, our initial data demonstrate that MAFP pretreatment augments, rather than inhibits, thrombin stimulated PAF production in HCAEC.

The thrombin-stimulated acceleration of membrane plasmalogen phospholipid hydrolysis results in increased production of both lysoplasmethylcholine (LysoPlsCho) and lysoplasmethylethanolamine (LysoPlsEtn). However, we subsequently determined that there was little, if any, PAF produced by the subsequent acetylation of LysoPlsCho. We hypothesized that the generation of LysoPlsEtn promotes PAF synthesis through a transacylation pathway by acting as an acyl group acceptor (Ref. 1, Figure 9) and proposed that LysoPlsEtn production by iPLA₂ was the rate-controlling step in PAF production rather than the activation of PAF acetyltransferase. To demonstrate the validity of this hypothesis, we incubated HCAEC with LysoPlsEtn and measured PAF production. Incubation of HCAEC with 5 μ M LysoPlsEtn for 10 min resulted in a significant increase in PAF production that was comparable to that observed in thrombin-stimulated cells (Figure 2, filled bars), demonstrating that basal PAF acetyltransferase activity is sufficient to support PAF synthesis and that availability of LysoPlsEtn is rate-limiting. Pretreatment with MAFP (25 μ M, 10 min) resulted in a significant increase in PAF production in unstimulated-, thrombin-stimulated and LysoPlsEtn-stimulated HCAEC (Figure 2, open bars). In further studies, we determined that incubation of HCAEC with 5 μ M lyso-PAF for 10 min also resulted in a 3.3-fold increase in PAF

production that was comparable to the increase in thrombin-stimulated cells (data not shown). Thus, incubation of HCAEC with either LysoPlsEtn or lyso-PAF results in increased PAF production. In contrast, increased PAF production was not observed when HCAEC were incubated with LysoPlsCho or lysophosphatidylcholine (LPC) (data not shown). Taken together, these data support our hypothesis that thrombin-stimulated PAF production occurs primarily via the remodeling pathway. However, to ensure that the increase in PAF production by MAFP proceeded through the transacylase pathway and was not due to increased acetylation of lysoplasmalogens produced in response to thrombin stimulation, we incubated the PAF extracted from thrombin-stimulated and MAFP-pretreated HCAEC with 0.1 M HCl prior to separation by HPLC (Figure 3). Acid pretreatment of HCAEC-derived PAF did not result in a significant loss of radioactivity in PAF production in thrombin-stimulated HCAEC with or without MAFP pretreatment, demonstrating that MAFP does not increase acetylation of plasmalogen lysophospholipids generated by thrombin-stimulated PLA₂. Accordingly, MAFP augments PAF production by a mechanism that does not result from alterations in PLA₂ activity or increased acetylation of acid-labile (plasmalogen) lysophospholipids.

Previous studies in P388D₁ macrophages have indicated that multiple PLA₂ isoforms in cells may interact with each other by generating products (eg. eicosanoids) that modulate PLA₂ activity by specific receptor-mediated events and also possibly by competing for the same substrate pools. Although we have determined that the majority of endothelial cell PLA₂ activity is inhibited by BEL, is selective for plasmalogen substrates and is maximal in the absence of Ca²⁺ and thus represents iPLA₂, we cannot rule out the possibility that MAFP inhibits a PLA₂ isoform in HCAEC that potentially regulates iPLA₂ activity. To determine whether MAFP alters HCAEC iPLA₂ activity, we pretreated cells with MAFP prior to thrombin stimulation and measured cellular PLA₂ activity in the absence of Ca²⁺ using (16:0, [³H]18:1) plasmenylcholine as substrate (Figure 4). Pretreatment with MAFP did not significantly alter iPLA₂ activity in unstimulated HCAEC (Figure 4, open squares and dotted lines) or inhibit thrombin-stimulated iPLA₂ activity (Figure 4, open squares, solid lines) when compared to activity measured in

cells without MAFP. In contrast, pretreatment with BEL resulted in a significant decrease in iPLA₂ activity measured in both unstimulated (Figure 4, filled circles, dotted lines) and thrombin-stimulated HCAEC (Figure 4, filled circles, solid lines). Thus, MAFP does not affect the activity of thrombin-stimulated, membrane-associated iPLA₂ despite previous reports demonstrating MAFP inhibition of soluble iPLA₂ isoforms. Mass measurements of individual molecular species in choline and ethanolamine phospholipids demonstrate that MAFP pretreatment does not significantly alter accelerated membrane phospholipid hydrolysis in response to thrombin stimulation (Table 1). This supports our hypothesis that MAFP pretreatment does not augment PAF production via alterations in membrane phospholipid hydrolysis resulting in increased lysophospholipid production.

Endothelial cell PAF synthesis is tightly controlled by rapid, inducible synthesis initiated by iPLA₂ and equally efficient degradation mediated by PAF-AH. PAF-AH is a unique, soluble, cytosolic PLA₂ isoform that does not have a Ca²⁺ requirement for activity and possesses a preference for substrates containing short acyl chains at the *sn*-2 position. We proposed that since MAFP has been demonstrated to inhibit other PLA₂ isoforms, it may also inhibit endothelial cell PAF-AH activity. Inhibition of PAF-AH activity would augment PAF generation in HCAEC as a result of inhibition of PAF catabolism rather than increased PAF production.

Although PAF-AH activity has been characterized previously in human umbilical vein endothelial cells (Blank et al., 1986) it has not been characterized in HCAEC. Consequently, we performed initial studies to ensure our assay conditions would result in linear reaction velocities with respect to time and protein content. Incubation of HCAEC protein with [³H] PAF resulted in linear reaction velocities for PAF-AH activity for up to 120 min, after which time the rate of acetate production decreased (Figure 5). In previously published assay systems, investigators have routinely used an incubation time of 15 min, so we chose to use the same time interval for subsequent experiments. Varying the amount of cellular protein used, linear reaction velocities were observed with protein content between 10 μg and 100 μg, thus we chose to use 25 μg protein in subsequent assays. Maximal reaction

velocities were consistently achieved with [³H] PAF substrate concentrations greater than 50 μM, thus we used 100 μM substrate concentration for our assays.

To examine whether the PLA₂ inhibitors BEL and MAFP inhibited PAF-AH, we pretreated HCAEC with either BEL or MAFP and measured activity under our linear maximal rate conditions (Figure 6). PAF-AH activity was significantly inhibited by MAFP concentrations greater than 1 μM, but was unaffected by BEL concentrations as high as 20 μM (Figure 6). Incubation of HCAEC cellular protein with increasing concentrations of MAFP demonstrated that the inhibition of PAF-AH by MAFP was found to be rapid, and both concentration- and time-dependent (Figure 7).

Taken together, these data indicate that the increase in thrombin-stimulated PAF production by pretreatment of HCAEC with MAFP is due to inhibition of PAF catabolism by PAF-AH. PAF-AH activity in HCAEC was found to be approximately equal for 1-O-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine (3.4 ± 0.4 nmol/mg protein/min, n=4), 1-O-alkenyl-2-acetyl-*sn*-glycero-3-phosphocholine (2.9 ± 0.7 nmol/mg protein/min, n=4) and 1-O-acyl-2-acetyl-*sn*-glycero-3-phosphocholine (2.8 ± 0.6 nmol/mg protein/min, n=4). Pretreatment of HCAEC with 5 μM MAFP resulted in complete inhibition of PAF-AH activity using each of the three classes of PAF species as substrate. Conversely, BEL pretreatment did not inhibit PAF-AH activity with any substrate used.

DISCUSSION

Inhibitors of PLA₂ activity have been proposed to be anti-inflammatory agents for many years since PLA₂-catalyzed hydrolysis of membrane phospholipids is the rate-limiting event in the generation of proinflammatory lipid mediators such as prostaglandins, leukotrienes and PAF. However, following their development, almost all selective PLA₂ inhibitors have been subsequently demonstrated to inhibit other enzymes that are involved in phospholipid hydrolysis or remodeling. For example, arachidonyl trifluoromethyl ketone (AACOCF₃), a selective cytosolic PLA₂ inhibitor, has been shown to inhibit both 5-lipoxygenase and CoA-independent transacylase (Fonteh, 2002) and BEL has been shown to inhibit phosphatidate phosphohydrolase (Balsinde and Dennis, 1996b). MAFP has been shown previously to induce cyclo-oxygenase-2 (COX-2) expression in murine macrophages, leading to an increase in prostaglandin E₂ release (Lin and Chen, 1999). Thus, the use of selective PLA₂ inhibitors in the context as potential anti-inflammatory agents has to be treated with caution.

The inflammatory actions of PAF include activation of polymorphonuclear leukocytes and increased vascular permeability (Prescott et al., 2002). The concentration of PAF in plasma and tissues is tightly regulated by the balance of synthesis and degradation (Snyder, 1995). PAF-AH, the enzyme that catalyzes the hydrolysis of biologically active PAF into biologically inactive lyso-PAF, is a member of the PLA₂ family of enzymes that selectively hydrolyze phospholipids with short acyl chains at the *sn*-2 position (Snyder, 1995; Stafforini et al., 1997). Thus, a PLA₂ inhibitor that inactivates PAF-AH could act as a pro-inflammatory agent, prolonging the inflammatory response and increasing the recruitment of inflammatory cells to areas of injury (Prescott et al., 2002).

PAF acetylhydrolases are maximally active in the basal state and do not require Ca²⁺ for activity, thus providing an immediate mechanism for inactivation of PAF (Stafforini et al., 1996; 1997). Several studies have determined that the level of PAF accumulation is determined by the PAF-AH activity (Elstad et al., 1989; Suzuki et al., 1988; Touqui et al., 1985;). In addition, the magnitude of the inflammatory response to PAF may function in an autoregulatory fashion since increased production of PAF stimulates

the expression of PAF-AH (Sato et al., 1991). Thus, it follows that inhibition of PAF-AH would propagate the PAF-mediated inflammatory response. Indeed, a decrease in plasma PAF-AH activity has been observed in asthma (Miwa, 1988; Stafforini et al., 1999), systemic lupus erythematosus (Tetta et al., 1990) and septic shock (Graham et al., 1994).

The intracellular cPLA₂ and iPLA₂ isoforms do not require Ca²⁺ for catalysis, use a central Ser for catalysis and operate via the formation of an acyl-enzyme intermediate (Six and Dennis, 2000). Since their catalytic features are similar and MAFP acts at the catalytic site of the enzyme, it is not surprising that MAFP inhibits both iPLA₂ and cPLA₂ isoforms. PAF-AH resembles many neutral lipases, since its active site is composed of a Ser-Asp-His catalytic triad (Tjoelker et al., 1995), thus it might be expected that MAFP would inhibit this enzyme. However, the selectivity of this enzyme for short chain *sn*-2 acyl groups suggests that a non-hydrolyzable analog of arachidonic acid may not be recognized and targeted for hydrolysis by PAF-AH. This study is the first to demonstrate inhibition of PAF-AH by MAFP suggesting that MAFP may be a non-selective inhibitor of multiple serine-dependent lipases. The failure of MAFP to inhibit PAF production would suggest that the serine-dependent intracellular PLA₂ isoforms inhibited by MAFP do not play a role. This finding may explain the reason that a role for endothelial PAF production by intracellular PLA₂ isoforms was ruled out previously (Bernatchez et al., 2001). However, in addition to inhibition of PAF-AH activity, we measured PLA₂ activity in HCAEC and found that MAFP had little effect on membrane-associated, BEL-inhibitible PLA₂ activity measured in the absence of Ca²⁺. These results are consistent with the conclusion that the majority of thrombin-stimulated iPLA₂ activity in HCAEC is membrane-associated rather than cytosolic and that the MAFP inhibitor cannot gain access to the enzyme when it is present as an integral membrane protein. In a previous study, we have demonstrated that inhibition of membrane-associated iPLA₂ activity with BEL results in inhibition of PAF production in thrombin-stimulated endothelial cells (McHowat et al., 2001). In this study, we show that BEL does not inhibit PAF-AH, even though this enzyme is a serine-dependent intracellular PLA₂. Thus, BEL is a selective, active site-directed inhibitor that can inhibit both soluble, cytosolic and

membrane-associated iPLA₂ isoforms. In a separate study, we found that BEL pretreatment would inhibit the MAFP-induced increase in PAF production in response to thrombin stimulation, however, this required total inhibition of PLA₂ activity and a significant reduction in lysophospholipid content before it could be achieved (data not shown).

Bernatchez and co-workers have reported previously that MAFP and BEL pretreatment of bovine aortic endothelial cells and human umbilical artery endothelial cells resulted in a potentiation of VEGF-stimulated PAF production (Bernatchez et al., 2001). In their study, inhibition of endothelial cell PLA₂ activity by MAFP or BEL was only inferred from measurement of PAF accumulation and the authors did not measure either endothelial cell PLA₂ or PAF-AH activities directly. Thus, from the data obtained, the authors concluded that iPLA₂ activity did not play a role in PAF production. We have found that BEL pretreatment results in specific inhibition of thrombin-stimulated, membrane-associated iPLA₂ activity, with no inhibition of PAF-AH. Conversely, we find that although MAFP does not inhibit thrombin-stimulated, membrane-associated endothelial cell PLA₂ activity, MAFP is a potent inhibitor of PAF-AH, and results in a net potentiation of thrombin-stimulated PAF production. In human umbilical vein endothelial cells, Bernatchez et al (2001) observed a 6-fold increase in VEGF-stimulated PAF production by MAFP and a 2-fold increase in VEGF-stimulated PAF production by BEL. Thus, our results using MAFP are similar, whereas our finding with BEL are different and may be due to different endothelial cells or the stimulus used.

Inhibition of PAF-AH would suggest that MAFP pretreatment would potentiate PAF accumulation in endothelial cells regardless of the initiating event. We proposed previously that the majority of PAF produced in thrombin-stimulated human umbilical artery endothelial cells (HUAEC) was synthesized via the remodeling pathway, involving the production of LysoPlsEtn that acts as an acceptor for the *sn*-2 fatty acid from alkylacyl glycerophosphocholine, resulting in the production of lyso-PAF that can be acetylated subsequently (McHowat et al., 2001). To further support this hypothesis, we incubated HUAEC with LysoPlsEtn, but were not able to demonstrate an increase in PAF production (McHowat et

al, 2001). However, in the present study we incubated HCAEC with LysoPLsEtn and demonstrated increased PAF production that was similar to that observed in thrombin-stimulated cells. It is not clear why increased PAF production is observed in HCAEC, but not HUAEC, when incubated with LysoPLsEtn, however it may be that the uptake of LysoPLsEtn into HCAEC is greater or that the catabolism of LysoPLsEtn in HCAEC is slower than in HUAEC. The increase in PAF production following incubation with LysoPLsEtn demonstrates the involvement of the remodeling pathway for PAF synthesis in HCAEC. As would be expected, the inhibition of PAF-AH with MAFP resulted in increased PAF production in response to both thrombin and LysoPLsEtn incubation.

In summary, we have demonstrated that pretreatment of human endothelial cells with MAFP did not inhibit thrombin-stimulated, membrane-associated iPLA₂ activity, but paradoxically enhanced thrombin-stimulated PAF production by virtue of MAFP-induced inhibition of endothelial cell PAF-AH. Accordingly, MAFP may provide a useful reagent to further study the role of PAF-AH inhibition on atherosclerotic disease progression.

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FOOTNOTE

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Choline phospholipids

<u>Identity</u>	<u>Control</u>	<u>Thrombin</u>	<u>MAFP</u> <u>& thrombin</u>
(18:2, 20:4) PtdCho	0.9±0.1	1.0±0.2	0.9±0.1
(16:0, 20:4) PtdCho	6.2±0.6	5.6±0.2	5.9±0.4
(16:0, 20:4) PlsCho	2.1±0.2	0.9±0.2	1.2±0.2
(16:0, 18:2) PlsCho &	1.8±0.3	1.3±0.2	1.4±0.2
(18:1, 20:4) PlsCho			
(18:0, 20:4) PtdCho	5.4±0.8	5.2±0.3	5.0±0.3
(18:0, 20:4) PlsCho	3.2±0.2	1.7±0.1	1.8±0.2
(16:0, 18:3) PtdCho	1.1±0.1	1.3±0.1	1.5±0.1
(18:2, 18:2) PtdCho	0.8±0.1	1.1±0.3	1.2±0.2
(16:0, 18:2) PtdCho	4.4±0.5	3.9±0.3	4.0±0.2
(18:1, 18:2) PtdCho	2.2±0.3	2.5±0.1	2.4±0.2
(16:0, 18:2) PakCho	7.8±1.3	8.8±0.5	8.5±0.8
(18:0, 18:2) PtdCho	5.1±0.8	4.7±0.2	4.8±0.2
(16:0, 18:1) PtdCho	5.6±0.7	5.5±0.4	5.4±0.3
(18:0, 18:1) PtdCho	3.3±0.8	2.4±0.6	2.3±0.3

Ethanolamine phospholipids

<u>Identity</u>	<u>Control</u>	<u>Thrombin</u>	<u>MAFP</u> <u>& thrombin</u>
(18:2, 20:4) PtdEtn	1.4±0.6	0.8±0.3	1.1±0.3
(16:0, 20:4) PtdEtn	1.0±0.2	0.7±0.2	1.5±0.1
(16:0, 20:4) PlsEtn	3.5±0.5	2.2±0.3	2.3±0.3
(18:1, 20:4) PlsEtn &	4.0±0.3	2.8±0.4	2.7±0.5
(16:0, 18:2) PlsEtn			
(18:0, 20:4) PtdEtn	3.8±0.4	5.4±0.9	4.5±0.7
(18:0, 20:4) PlsEtn	6.7±0.9	3.2±0.7	1.9±0.6
(18:1, 18:3) PtdEtn	1.5±0.3	0.7±0.1	0.7±0.2
(16:0, 18:3) PlsEtn	1.7±0.4	0.5±0.1	0.6±0.1
(18:2, 18:2) PtdEtn	0.7±0.2	0.8±0.3	0.8±0.2
(16:0, 18:2) PtdEtn	1.4±0.3	1.3±0.2	1.8±0.2
(18:1, 18:2) PtdEtn	1.7±0.3	1.6±0.2	1.8±0.2
(18:1, 18:2) PlsEtn	1.3±0.2	4.7±0.7	3.8±0.1

(18:0, 18:2) PlsEtn	5.5±0.3	9.7±0.4	8.5±0.4
(16:0, 18:1) PtdEtn	2.8±0.7	2.8±0.8	2.1±0.3
(18:1, 18:1) PtdEtn	2.5±0.5	2.1±0.6	1.6±0.3
(16:0, 18:1) PakEtn	3.5±0.9	2.0±0.3	2.5±1.2
(18:0, 18:1) PtdEtn	3.8±0.6	3.1±0.7	3.8±0.3
(18:0, 18:1) PlsEtn	2.7±0.5	4.1±0.3	4.9±0.3

Table 1. Quantitation of phospholipid molecular species (nanomoles of PO₄ per milligram of protein) in control, thrombin-stimulated (0.1 IU/ml, 10 mins) or MAFP pretreated (5 μM, 10 mins) HCAEC. Phospholipids in bold are significantly different (p<0.05) between control and thrombin-stimulated samples. Values are means ± SEM for five different cell cultures.

FIGURE LEGENDS

FIGURE 1. Effect of pretreatment with increasing concentrations of methyl arachidonyl fluorophosphonate (MAFP, 10 min incubation) on control (X) and thrombin-stimulated (0.1 IU/ml, 10 min, □) PAF production in human coronary artery endothelial cells (HCAEC). * $p < 0.05$ between unstimulated cells in the presence and absence of MAFP. ** $p < 0.01$ when comparing control to thrombin-stimulated values in the presence and absence of each concentration of MAFP. Values shown represent the mean \pm SEM for results derived from 6 different cell cultures.

FIGURE 2. Incubation of HCAEC with thrombin (0.1 IU/ml, 10 min) and lysoplasmylethanolamine (LysoPlsEtn, 5 μ M, 10 min) results in a significant increase in PAF production (filled bars, * $p < 0.05$ when comparing stimulated and unstimulated PAF production). Pretreatment with MAFP (5 μ M, 10 min) significantly potentiated both thrombin- and LysoPlsEtn-induced PAF production (open bars, ** $p < 0.01$ when compared to corresponding values in the absence of MAFP). Values shown represent the mean \pm SEM for results derived from 3 different cell cultures.

FIGURE 3. HPLC separation of PAF produced in thrombin-stimulated HCAEC (left panel, 0.1 IU/ml, 10 mins and in HCAEC pretreated with MAFP (5 μ M, 10 min) and stimulated with thrombin (right panel, 0.1 IU/ml, 10 min). Cells were stimulated and PAF extracted as described. Aliquots of the extract 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) did not significantly alter the radioactivity present in the peak corresponding to the PAF produced in thrombin-stimulated HCAEC with or without MAFP pretreatment, thus little, if any, PAF is derived directly from plasmalogen lysophospholipid precursors.

FIGURE 4. Effect of pretreatment with MAFP (open squares, 25 μ M, 10 min) or bromoenol lactone (filled circles, 10 μ M, 10 min) on membrane-associated phospholipase A₂ (PLA₂) activity in HCAEC in the absence (dotted lines) or presence (solid lines) of thrombin (0.1 IU/ml). X represents control PLA₂ activity measurements made in the absence of either PLA₂ inhibitor. PLA₂ activity was measured using 100 μ M (16:0, [³H]18:1) plasmenylcholine substrate in the absence of Ca²⁺ (4 mM EGTA). *p<0.05, **p<0.01 when compared to untreated HCAEC. Values shown represent the mean \pm SEM for results derived from 4 different cell cultures.

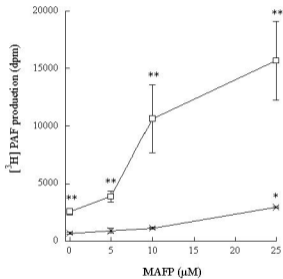
FIGURE 5. Release of [³H] acetic acid from 0.1 mM [³H] PAF substrate after incubation with 25 μ g HCAEC protein for increasing time intervals at 37°C. The rate of release of free acetic acid was found to be linear for up to 120 minutes.

FIGURE 6. Inhibition of PAF acetylhydrolase in HCAEC incubated with increasing concentrations of the PLA₂ inhibitors MAFP (open squares) and BEL (filled circles) for 10 min. **p<0.01 when compared to untreated activity. Values shown represent the mean \pm SEM for results derived from 8 different cell cultures.

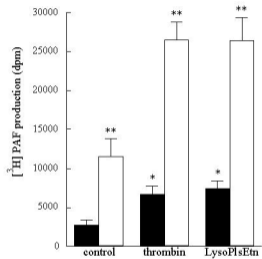
FIGURE 7. Effect of inhibition of PAF acetylhydrolase activity with increasing

concentrations of MAFP over a 60 min time course. Inhibition of PAF acetylhydrolase by MAFP is both time- and concentration-dependent. Aliquots of HCAEC suspensions were incubated with the indicated concentration of MAFP for increasing time intervals. PAF-acetylhydrolase activity was then measured by the addition of 0.1 mM [acetyl-³H] PAF for 30 min at 37°C and subsequent quantitation of released [³H] acetic acid. Values shown are for a single cell culture.

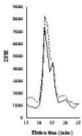
FIGURE 8. Activation of calcium-independent phospholipase A₂ (iPLA₂) by thrombin stimulation of human coronary artery endothelial cells results in selective hydrolysis of membrane plasmalogen phospholipids. Lysoplasmeneylethanolamine (lysoPlsEtn) acts as an acceptor for the *sn*-2 fatty acid from alkylacyl glycerophosphocholine (PakCho), resulting in production of lyso platelet activating factor (lysoPAF) which is then acetylated with lyso-PAF acetyltransferase using acetylCoA to form biologically active PAF. Inhibition of iPLA₂ activity with bromoenol lactone (BEL) results in inhibition of PAF production as a result of decreased lysoPlsEtn production. Pretreatment with methyl arachidonyl fluorophosphonate (MAFP) augments PAF production by inhibiting PAF hydrolysis by PAF acetylhydrolase.

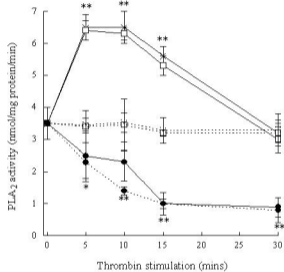


MAFP potentiation of PAF production

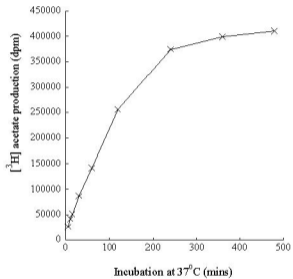


MAFP pretreatment of HCAEC

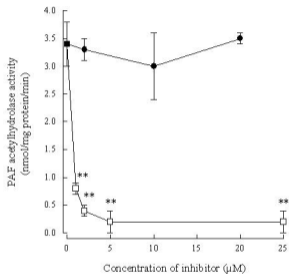




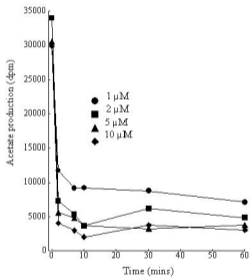
PLA₂ activity with BEL and MAFP



PAF-AH time course



Inhibition of PAF-AH by BEL & MAFP



Effect of MAFF concentration on PAF-AH activity

