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

Running title: PAF acetylhydrolase inhibition by MAFP

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

16 text pages

1 table

8 figures

34 references

Abstract 188 words

Introduction 561 words

Discussion 1231 words

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; lysoplasmenylcholine, LysoPlsCho; lysoplasmenylethanolamine, LysoPlsEtn; methyl arachidonyl fluorophosphonate, MAFP; phosphatidylcholine, PtdCho; phosphatidylethanolamine, PtdEtn; phospholipase A_2, PLA_2; plasmenylcholine, PlsCho; plasmenylethanolamine, PlsEtn; platelet activating factor, PAF; vascular endothelial cell growth factor, VEGF

SECTION ASSIGNMENT: Cardiovascular

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 ($iPLA_2$) that selectively hydrolyzed membrane plasmalogen phospholipids leading to increased production of lysoplasmalogens and arachidonic acid (McHowat et al., 2001). We confirmed the involvement of $iPLA_2$ activation in platelet activating factor (PAF) generation by pretreating the cells with bromoenol lactone (BEL, a selective inhibitor of $iPLA_2$ (Hazen et al., 1991)). Incubation of HUAEC with BEL resulted in complete inhibition of thrombin-stimulated $iPLA_2$ activity and PAF production (McHowat et al., 2001). We proposed that $iPLA_2$-catalyzed ethanolamine plasmalogen hydrolysis and increased lysoplasmenylethanolamine 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 ($sPLA_2$) activity was responsible for the increased PAF production and that there was minimal contribution from the intracellular cytosolic, Ca^{2+}-activated PLA_2 ($cPLA_2$) or $iPLA_2$. 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 $cPLA_2$, but was subsequently found to inhibit cytosolic $iPLA_2$ 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

PLA_2 enzyme. MAFP has been shown to irreversibly inhibit soluble cytosolic $cPLA_2$ and $iPLA_2$, 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 PLA_2 isoforms, the active site serine residue

of membrane-associated $iPLA_2$ may be "protected" from this inhibitor. In addition to PLA_2 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 PLA_2 activity and instead resulted in a

significant augmentation of PAF production as a result of inhibition of PAF-AH. Phospholipase A_2

inhibitors have been proposed to be potential anti-inflammatory agents since PLA_2-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 PLA_2

isoforms involved in phospholipid metabolite production to guide the development of specific PLA_2

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). [^{14}C]lysophosphatidylcholine and [^{14}C] acetic anhydride were purchased from Amersham (Arlington Heights, IL). [^{3}H]PAF, [^{3}H]acetic anhydride and [^{3}H]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/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 achieving confluence, cells were passaged in a 1:3 dilution and cells from passages 3-4 used for experiments.

Phospholipase A_2 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, iPLA$_2$ activation was arrested by the removal of the surrounding buffer and immediate replacment 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 A_2 activity was assessed by

incubating 50 µg of cytosolic protein or 8 µg of membrane protein with 100 µM (16:0, [^{3}H]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$_2$PO$_4$ 0.4 mM, Na$_2$HPO$_4$ 0.3 mM and glucose 6.6 mM and incubated with 50 µCi [^{3}H] 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 [^{3}H]PAF. [^{3}H]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 [^{3}H]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- ^{3}H] PAF (10 mCi/mmol) for 30 min at 37°C. The reaction was stopped by the addition of acetic acid and released [^{3}H] acetic acid was separated from the [^{3}H] PAF substrate by passing the reaction mixture through a C$_{18}$ gel cartridge (Baker Chemical Co., Phillipsburg, NJ). Released [^{3}H]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

$iPLA_2$-catalyzed hydrolysis of membrane plasmalogen phospholipids. Pretreatment of HUAEC with the

$iPLA_2$-selective inhibitor BEL resulted in complete inhibition of thrombin-stimulated $iPLA_2$ activity and

increased PAF production. Since MAFP inhibits the cytosolic isoforms of both $cPLA_2$ and $iPLA_2$, 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 µM 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 µM 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 lysoplasmenylcholine (LysoPlsCho) and lysoplasmenylethanolamine

(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 $iPLA_2$ 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 µM 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 µM, 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 µM 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 PLA_2. Accordingly, MAFP augments PAF

production by a mechanism that does not result from alterations in PLA_2 activity or increased acetylation

of acid-labile (plasmalogen) lysophospholipids.

Previous studies in $P388D_1$ macrophages have indicated that multiple PLA_2 isoforms in cells may

interact with each other by generating products (eg. eicosanoids) that modulate PLA_2 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 PLA_2 activity is inhibited by BEL, is selective for

plasmalogen substrates and is maximal in the absence of Ca^{2+} and thus represents $iPLA_2$, we cannot rule

out the possibility that MAFP inhibits a PLA_2 isoform in HCAEC that potentially regulates $iPLA_2$

activity. To determine whether MAFP alters HCAEC $iPLA_2$ activity, we pretreated cells with MAFP

prior to thrombin stimulation and measured cellular PLA_2 activity in the absence of Ca^{2+} using (16:0,

$[^3H]18:1$) plasmenylcholine as substrate (Figure 4). Pretreatment with MAFP did not significantly alter

$iPLA_2$ activity in unstimulated HCAEC (Figure 4, open squares and dotted lines) or inhibit thrombin-

stimulated $iPLA_2$ 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 $iPLA_2$

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 $iPLA_2$ despite previous reports demonstrating MAFP inhibition of

soluble $iPLA_2$ 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

$iPLA_2$ and equally efficient degradation mediated by PAF-AH. PAF-AH is a unique, soluble, cytosolic

PLA_2 isoform that does not have a Ca^{2+} 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 PLA_2 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 PAFcatabolism 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 [^{3}H] PAF substrate concentrations greater than 50 µM, thus we used 100 µM substrate concentration for our assays.

To examine whether the PLA$_2$ 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 $\pm$ 0.4 nmol/mg protein/min, n=4), 1-O-alkenyl-2-acetyl-*sn*-glycero-3-phosphocholine (2.9 $\pm$ 0.7 nmol/mg protein/min, n=4) and 1-O-acyl-2-acetyl-*sn*-glycero-3-phosphocholine (2.8 $\pm$ 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 PLA_2 activity have been proposed to be anti-inflammatory agents for many years since PLA_2-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 PLA_2 inhibitors have been subsequently demonstrated to inhibit other enzymes that are involved in phospholipid hydrolysis or remodeling. For example, arachidonyl trifluoromethyl ketone ($AACOCF_3$), a selective cytosolic PLA_2 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 E_2 release (Lin and Chen, 1999). Thus, the use of selective PLA_2 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 PLA_2 family of enzymes that selectively hydrolyze phospholipids with short acyl chains at the *sn*-2 position (Snyder, 1995; Stafforini et al., 1997). Thus, a PLA_2 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 Ca^{2+} 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 (Satoh 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 $cPLA_2$ and $iPLA_2$ isoforms do not require Ca^{2+} 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 $iPLA_2$ and $cPLA_2$ 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 PLA_2 isoforms

inhibited by MAFP do not play a role. This finding may explain the reason that a role for endothelial PAF

production by intracellular PLA_2 isoforms was ruled out previously (Bernatchez et al., 2001). However,

in addition to inhibition of PAF-AH activity, we measured PLA_2 activity in HCAEC and found that

MAFP had little effect on membrane-associated, BEL-inhibitable PLA_2 activity measured in the absence

of Ca^{2+}. These results are consistent with the conclusion that the majority of thrombin-stimulated $iPLA_2$

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 $iPLA_2$ 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 PLA_2.

Thus, BEL is a selective, active site-directed inhibitor that can inhibit both soluble, cytosolic and

membrane-associated iPLA$_2$ 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 PLA$_2$ 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 PLA$_2$

activity by MAFP or BEL was only inferred from measurement of PAF accumulation and the authors did

not measure either endothelial cell PLA$_2$ or PAF-AH activities directly. Thus, from the data obtained, the

authors concluded that iPLA$_2$ activity did not play a role in PAF production. We have found that BEL

pretreatment results in specific inhibition of thrombin-stimulated, membrane-associated iPLA$_2$ activity,

with no inhibition of PAF-AH. Conversely, we find that although MAFP does not inhibit thrombin-

stimulated, membrane-associated endothelial cell PLA$_2$ 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 $iPLA_2$ 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.

REFERENCES

Balsinde J and Dennis EA (1996a) Distinct roles in signal transduction for each of the Phospholipase A_2 enzymes present in P388D$_1$ macrophages. *J. Biol. Chem.* **271:** 6758-6765

Balsinde J and Dennis EA (1996b) Bromoenol lactone inhibits magnesium-dependent Phosphatidate phosphohydrolase and blocks triacylglycerol biosynthesis in mouse P388D1 macrophages. *J Biol Chem* **271:** 31937-31941

Bernatchez PN, Winstead MV, Dennis EA and Sirois MG (2001) Regulation of VEGF-induced endothelial cell PAF synthesis: role of p42/44 MAPK, p38 MAPK and PI3K pathways.*Br. J. Pharmacol* **134:** 1253-1262

Blank ML, Spector AA, Kaduce TL, Lee TC and Snyder F (1986) Metabolism of platelet-activating factor (1-alkyl-2-acetyl-sn-glycero-3-phosphocholine) and 1-alkyl-2-acetyl-sn-glycerol by human endothelial cells *Biochim. Biophys. Acta.* **876:** 373-378

Bligh EG and Dyer WJ (1959) A rapid method of total lipid extraction and purification *Can J Physiol* **37:**911-917

Deutsch DG, Omeir R, Arreaza G, Salehani D, Prestwich GD, Huang Z and Howlett A (1997) Methyl arachidonyl fluorophosphonate: a potent irreversible inhibitor of anandamide amidase*. Biochem Pharmacol* **53:** 255-260

Elstad MR, Stafforini DM, McIntyre TM, Prescott SM, and Zimmerman GA (1989) Platelet-activating factor acetylhydrolase increases during macrophage differentiation. A novel mechanism that regulates accumulation of platelet-activating factor. *J Biol Chem* **264:** 8467-8470

Fernando SR Pertwee RG (1997) Evidence that methyl arachidonyl fluorophosphonate is an

irreversible cannabinoid receptor antagonist. *Br J Pharmacol* **121:** 1716-1720

Fonteh AN (2002) Differential effects of arachidonoyl tripfluoromethyl ketone on arachidonic

acid release and lipid mediator biosynthesis by human neutrophils. Evidence for different

arachidonate pools. *Eur J Biochem* **269:** 3760-3770

Ghomashchi F, Loo R, Balsinde J, Bartoli F, Apitz-Castro R, Clark JD, Dennis EA and Gelb MH

(1999) Trifluoromethyl ketones and methyl fluorophosphonates as inhibitors of group IV and VI

phospholipases A$_2$: structure-function studies with vesicle, micelle, and membrane assays.

Biochim Biophys Acta **1420:** 45-56

Graham RM, Stephens CJ, Silvester W, Leong LLL, Sturm MJ and Taylor RR (1994) Plasma

degradation of platelet-activating factor in severely ill patients with clinical sepsis. *Critical Care

Med* **22**: 204-212

Hazen SL, Zupan LA, Weiss RH, Getman DP, and Gross RW (1991) Suicide inhibition of canine

myocardial cystolic calcium-independent phospholipase A2. Mechanism-based discrimination

between calcium-dependent and -independent phospholipases A2. *J Biol Chem* **266:** 7227-7232

Heery JM, Kozak M, Stafforini DM, Jones DA, Zimmerman GA, McIntyre TM and Prescott

SM (1995) Oxidatively modified LDL contains phospholipids with platelet-activating factor-like

Activity and stimulates the growth of smooth muscle cells. *J Clin Invest* **96**: 2322-2330

Lin WW and Chen BC (1999) Induction of cyclo-oxygenase-2 expression by methyl arachidonyl

fluorophosphonate in murine J774 macrophages: roles of protein kinase C, ERKs and p38

MAPK. *Br J Pharmacol* **126:** 1419-1425

Lio YC, Reynolds LJ, Balsinde J, and Dennis EA (1996) Irreversible inhibition of Ca^{2+}-

Independent phospholipase A_2 by methyl arachidonyl fluorophosphonate *Biochim Biophys Acta*

1302: 55-60

McHowat J, Kell PJ, O'Neill HB and Creer MH (2001) Endothelial cell PAF synthesis following

thrombin stimulation utilizes Ca^{2+}-independent phospholipase A_2 *Biochemistry* **40:** 14921-14931

McHowat J and Creer MH (1997) Lysophosphatidylcholine accumulation in cardiomyocytes

Requires thrombin activation of Ca^{2+}-independent PLA_2. *Am J Physiol*. **272:** H1972-1980

McHowat J and Creer MH (1998a) Thrombin activates a membrane-associated calcium-

independent PLA_2 in ventricular myocytes. *Am J Physiol* **274**, C447-454

McHowat J and Creer MH (1998b) Calcium-independent phospholipase A_2 in isolated rabbit

Ventricular myocytes. *Lipids.* **33:** 1203-1212

Miwa M, Miyake T, Yamanaka T, Sugatani J, Suzuk, Y, Sakata S, Araki Y and Matsumoto M

(1988) Characterization of serum platelet-activating factor (PAF) acetylhydrolase. Correlation

between deficiency of serum PAF acetylhydrolase and respiratory symptoms in asthmatic

children. *J Clin Invest.* **82:** 1983-1991

Montrucchio, G, Alloatti G and Camussi G (2000) Role of platelet-activating factor in

cardiovascular pathophysiology. *Physiol Rev* **80**: 1669-1699

Patel K.D, Zimmerman GA, Prescott SM, McEver RP and McIntyre TM (1991*)* Oxygen radicals Induce human endothelial cells to express GMP-140 and bind neutrophils. *J Cell Biol* **112**: 749-759

Prescott SM, McIntyre, TM, Zimmerman GA and Stafforini DM (2002) Sol Sherry lecture in thrombosis: molecular events in acute inflammation. *Arterioscler Thromb Vasc Biol* **22:** 727-733

Satoh K, Imaizumi TA, Kawamura Y, Yoshida H, Hiramoto M, Takamatsu S and Takamatsu M (1991) Platelet-activating factor (PAF) stimulates the production of PAF acetylhydrolase by the human hepatoma cell line, HepG2. *J Clin Invest* **87:** 476-481

Six DA and Dennis EA (2000) The expanding superfamily of phospholipase A_2 enzymes: Classification and characterization. *Biochim Biophys Acta* **1488:** 1-19

Snyder F (1995) Platelet-activating factor: the biosynthetic and catabolic enzymes. *Biochem J* **305:** 689-705

Stafforini DM, Prescott SM, Zimmerman GA and McIntyre TM (1996) Mammalian platelet-Activating factor acetylhydrolases *Biochim Biophys Acta* **1301**: 161-173

Stafforini DM, McIntyre TM, Zimmerman GA and Prescott SM (1997) Platelet-activating factor acetylhydrolases. *J Biol Chem.* **272**: 17895-17898

Stafforini DM, Numao T, Tsodikov A, Vaitkus D, Fukuda T, Watanabe N, Fueki N, McIntyre TM, Zimmerman GA, Makino S and Prescott SM (1999) Deficiency of platelet-activating factor

acetylhydrolase is a severity factor for asthma. *J Clin Invest* **103**: 989-997

Stremler KE, Stafforini DM, Prescott SM, Zimmerman GA and McIntyre TM (1989) An oxidized

derivative of phosphatidylcholine is a substrate for the platelet-activating factor acetylhydrolase

from human plasma. *J Biol Chem* **264:** 5331-5334

Suzuki, Y, Miwa M, Harada M and Matsumoto M (1988) Release of acetylhydrolase from

platelets on aggregation with platelet-activating factor. *Eur J Biochem* **172:** 117-120

Tetta C, Bussolino F, Modena V, Montrucchio G, Segoloni G, Pescarmona G and Camussi G

Release of platelet-activating factor in systemic lupus erythematosus. (1990) *Int Arch Allergy*

Appl Immunol **91**: 244-256

Tjoelker LW, Wilder C, Eberhardt C, Stafforini DM, Dietsch G, Schimpf B, Hooper S, Le

Trong H, Cousens LS and Zimmerman GA (1995) Plasma platelet-activating factor

acetylhydrolase is a secreted phospholipase A2 with a catalytic triad. *J Biol Chem* **270:** 25481-

25487

Touqui L, Hatmi M and Vargaftig B (1985) Human platelets stimulated by thrombin produce

platelet-activating factor (1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine) when the degrading

enzyme acetyl hydrolase is blocked. *Biochem J* **229:** 811-816

FOOTNOTE

Choline phospholipids

Identity	Control	Thrombin	MAFP & thrombin
(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

Identity	Control	Thrombin	MAFP & thrombin
(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 PO_4 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 $\pm$ 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

lysoplasmenylethanolamine (LysoPlsEtn, 5 µM, 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 µM, 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 µM, 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 µM, 10 min) or bromoenol

lactone (filled circles, 10 µM, 10 min) on membrane-associated phospholipase A_2

(PLA_2) activity in HCAEC in the absence (dotted lines) or presence (solid lines)

of thrombin (0.1 IU/ml). X represents control PLA_2 activity measurements made

in the absence of either PLA_2 inhibitor. PLA_2 activity was measured using 100

µM (16:0, [^{3}H]18:1) plasmenylcholine substrate in the absence of Ca^{2+} (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 [^{3}H] acetic acid from 0.1 mM [^{3}H] PAF substrate after incubation with

25 µg 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 PLA_2 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-^{3}H] PAF for 30 min at 37°C and

subsequent quantitation of released [^{3}H] acetic acid. Values shown are for a

single cell culture.

FIGURE 8.   Activation of calcium-independent phospholipase A_2 (iPLA$_2$) by thrombin

stimulation of human coronary artery endothelial cells results in selective

hydrolysis of membrane plasmalogen phospholipids.

Lysoplasmenylethanolamine (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

iPLA$_2$ 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
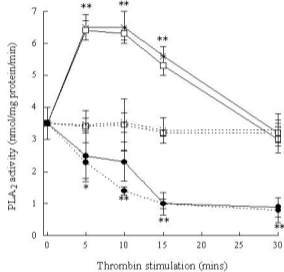
inhibiting PAF hydrolysis by PAF acetylhydrolase.
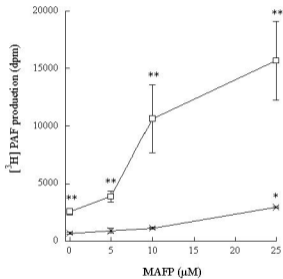
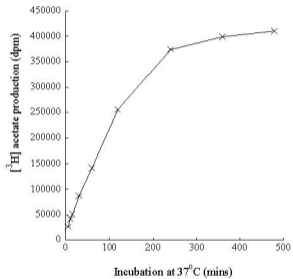MAFP potentiation of PAF production
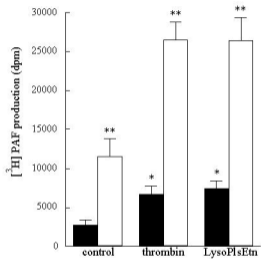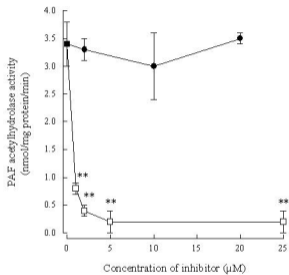
MAFP pretreatment of HCAEC
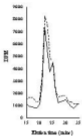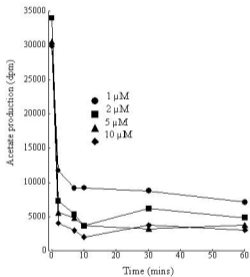
PLA$_2$ activity with BEL and MAFP

PAF-AH time course

Inhibition of PAF-AH by BEL & MAFP

Effect of MAFP concentration on PAF-AH activity

lyso-PAF

MAFP ·········▶ *PAF*
acetylhydrolase

PAF

lyso-PAF
acetyltransferase

Thrombin

PlsEtn ◀   ▶ lyso-PAF

PlsEtn $\xrightarrow{iPLA_2}$ lysoPlsEtn   PakCho

BEL