Anti-apoptotic actions of the platelet activating factor acetylhydrolase I α2 catalytic subunit

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Summary

Platelet activating factor (PAF) is an important mediator of cell loss following diverse pathophysiological challenges but the manner in which PAF transduces death is not clear. Both PAF receptor-dependent and independent pathways are implicated. In this study, we show that extracellular PAF can be internalized through PAF receptor-independent mechanisms and can initiate caspase-3-dependent apoptosis when cytosolic concentrations are elevated by approximately 15 pM/cell for 60 min. Reducing cytosolic PAF to less than 10 pM/cell terminates apoptotic signaling. By pharmacological inhibition of PAF acetylhydrolase I and II (PAF-AH) activity and downregulation of PAF-AH I catalytic subunits by RNA interference, we show that the PAF receptor-independent death pathway is regulated by PAF-AH I and, to a lesser extent, by PAF-AH II. Moreover, the anti-apoptotic actions of PAF-AH I are subunit-specific. PAF-AH I \( \alpha_1 \) regulates intracellular PAF concentrations under normal physiological conditions but expression is not sufficient to reduce an acute rise in intracellular PAF levels. PAF-AH I \( \alpha_2 \) expression is induced when cells are deprived of serum or exposed to apoptogenic PAF concentrations limiting the duration of pathological cytosolic PAF accumulation. To block the PAF receptor-death pathway, we screened a panel of PAF antagonists (CV-3988, CV-6209, BN 52021, and FR-49175). BN 52021 and FR-49175 accelerated PAF hydrolysis and inhibited PAF-mediated caspase 3 activation. Both antagonists act indirectly to promote PAF-AH I \( \alpha_2 \) homodimer activity by reducing PAF-AH \( \alpha_1 \) expression. These findings identify PAF-AH I \( \alpha_2 \) as a potent anti-apoptotic protein and describe a new means of pharmacologically targeting PAF-AH I to inhibit PAF-mediated cell death.
Platelet-activating factor (PAF\(^1\): 1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine) is a key mediator of neuronal death in ischemia, encephalitis, epileptic seizure, meningitis, and human immunodeficiency virus-1 dementia in vivo and participates in etoposide-, prion-, and \(\beta\)-amyloid-induced cell death in vitro (1-7). In the periphery, pathological increases in PAF concentrations underlie cytotoxicity in chronic inflammatory dermatoses and lethality in systemic anaphylaxis (8,9). Although the majority of PAF effects are understood to be transduced by its G-protein coupled receptor (PAFR) (10), PAFR signaling has been shown to be both pro- and anti-apoptotic. Ectopic PAFR expression exacerbates cell death induced by etoposide and mitomycin C but protects cells from tumour necrosis factor \(\alpha\) (TNF\(\alpha\)), TRAIL, and extracellular PAF (6,7,11,12). These opposing effects likely depend upon the relative ratio of NF\(\kappa\)B-dependent pro- and anti-apoptotic gene products elicited in different cell types in response to the combination of an external apoptotic inducer and PAF (6).

Accumulating evidence points to additional PAF signaling pathways transduced independently of PAFR (11,13-17). PAFR-negative cells undergo apoptosis when extracellular PAF concentrations reach 100 nM and necrosis when PAF levels exceed the critical micelle concentration of 3 \(\mu\)M (11). Little is known about how PAF signals cell death in the absence of PAFR. PAF activates NF-\(\kappa\)B, glycogen synthase kinase 3\(\beta\), and caspase 3 and triggers mitochondrial release of cytochrome C (6,18-20). Whether

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\(^1\) Abbreviations: B-PAF, Bodipy-platelet activating factor; B-lyso-PAF, Bodipy lyso-PAF; BSA, bovine serum albumin; GAPDH, glyceraldehydes phosphate dehydrogenase; DFP, diisopropyl fluorophosphates; DTNB, 5,5’-dithiobis(2-nitrobenzoic acid); mc-PAF, methyl-carbamyl platelet activating factor; PAF, platelet activating factor; PAF-AH, PAF acetylhydrolase; PAFR, platelet activating factor receptor; PARP, poly ADP-ribose polymerase; PBS, phosphate buffered saline; siRNA, small interfering RNA; RT-PCR, reverse transcriptase-polymerase chain reaction; \(t_{1/2}\), time to reach half maximal concentrations; TLC, thin-layer chromatography; TNF\(\alpha\), tumour necrosis factor \(\alpha\); TUNEL, terminal deoxytransferease dUTP nick-end labeling.
or not these effects are dependent on PAFR activation is not clear.

One approach to intervening in both PAFR-dependent and independent cell death lies in reducing pathological increases in PAF. PAF is hydrolyzed by a unique family of serine esterases or PAF acetylhydrolases (PAF-AHs) that cleave the biologically sn-2 active side-chain generating lyso-PAF. Three PAF-AH enzymes have been identified. Cytosolic PAF-AH I cleaves the acetyl group at the sn-2 position of PAF and PAF-like lipids with other phosphate head groups (21). The enzymatic complex is a G-protein-like trimer composed of two 29 kDa $\alpha_1$ and $\alpha_2$ catalytic subunits. The $\alpha$ subunits form homodimers or heterodimers that complex with a non-catalytic 45 kDa regulatory $\beta$ subunit LIS1. Mutations in the LIS1 gene are the genetic determinant of Miller Dieker Syndrome, a developmental brain disorder defined by type 1 lissencephaly (22). PAF-AH II is a single 40 kDa polypeptide (23). This isoenzyme recognizes both PAF and acyl analogs of PAF with moderate length sn-2 chains as well as short-chain diacylglycerols, triacylglycerols, and acetylated alkanols (24). Ectopic expression reduces cell death triggered by oxidative stress (25,26). Plasma PAF-AH is a 45 kDa monomer secreted into circulation by endothelial and hematopoietic cells (27,28). The enzyme recognizes PAF and PAF analogs with short to medium sn-2 chains including oxidatively cleaved long-chain polyunsaturated acyl chains (24). In vitro, recombinant plasma PAF-AH or ectopic expression protects cells from excitotoxicity or hypercholesterolemia (29-31). In vivo, intravenous injection of human plasma PAF-AH reduces lethality in experimental models of anaphylactic shock (8). These findings provide compelling evidence that PAF-AH activity regulates PAF-mediated apoptosis. It remains to be determined whether these enzymes can, in fact,
be targeted to inhibit PAF-mediated degeneration and disease.

In this study, we used the PC12 cell model to investigate the anti-apoptotic actions of PAF-AH I and PAF-AH II in the PAFR-independent pathway. We show that PC12 cells express all three PAF-AH I proteins (\(\alpha_1\), \(\alpha_2\) and LIS1) as well as PAF-AH II but not plasma PAF-AH or PAFR. Expression of PAF-AH I \(\alpha_2\) but not PAF-AH I \(\alpha_1\) is induced when PC12 cells are deprived of serum. We found that this induction regulates the duration of apoptotic signaling initiated by PAF challenge. To enhance the endogenous anti-apoptotic activity of PAF-AH I \(\alpha_2\), we screened a panel of PAF antagonists and identified two compounds that blocked PAFR-independent death. Both compounds, the fungal derivate FR 49175 and the ginkgolide BN 52021, protected cells by accelerating PAF hydrolysis. Surprisingly, both inhibitors suppressed \(\alpha_1\) protein expression thereby promoting \(\alpha_2/\alpha_2\) homodimer activity following PAF treatment. These findings point to a novel anti-apoptotic function for the \(\alpha_2\) subunit of PAF-AH I and a potential means of pharmacologically targeting PAF-AH enzymes to reduce PAF-mediated cell death.

**Experimental Procedures**

*Cell culture:* PC12-AC cells, a clonal derivate of the PC12 pheochromocytoma cell line (American Tissue Culture Collection), were cultured in complete media composed of RPMI 1640 containing 10% horse serum and 5% newborn calf serum at 37°C in a 5% CO\(_2/95\%\) air atmosphere. Culture reagents were obtained from Invitrogen.
Reverse transcriptase polymerase chain reaction (RT-PCR) analyses: Rat brain RNA was prepared from Wistar rats approximately 3 months of age (Charles Rivers). Rodents were anesthetized with sodium pentobarbital and euthanized by decapitation. All manipulations were performed in compliance with approved institutional protocols and according to the strict ethical guidelines for animal experimentation established by the Canadian Council for Animal Care. Total RNA was isolated using Trizol reagent (Invitrogen) and treated with RQ1-DNase1 (Promega) to eliminate residual genomic DNA. First strand cDNA synthesis was performed using random hexamer primers (Promega) and Superscript II RT (Invitrogen). Control reactions for residual genomic contamination were carried out in the absence of Superscript II RT. cDNA synthesis was performed using 5 U of Taq DNA polymerase (Invitrogen) in the presence of 1 mM MgCl₂ and 10 pmol per primer for glyceraldehyde phosphate dehydrogenase (GAPDH), 20 pmol per primer for PAF-AH II#2, PAF-AH II#3, and PAF-AH II#4, 25 pmol per primer for PAF-AH I α₁, PAF-AH I α₂, PAF-AH I LIS1, PAF-AH II#1, plasma PAF-AH and PAFR. Sequences are provided in Table 1. Primers were synthesized at the Biochemistry Research Institute, University of Ottawa. Reactions were amplified in a GeneAmp PCR System 2400 (Applied Biosystems): 94°C for 5 minutes, 30-35 cycles of 94°C for 30 seconds, 55°C for 60 seconds, and 72°C for 2 minutes, followed by a final incubation at 72°C for 7 minutes.

Western analysis: Rat brain protein was prepared from Wistar rat pups on postnatal day 10. Rodents were anesthetized with sodium pentobarbital and euthanized by decapitation. Brains were removed and placed in a 10 cm dia. tissue
culture plate containing artificial cerebrospinal fluid (26 mM NaHCO₃, 124 mM NaCl, 5mM KCl, 2mM CaCl₂, 1.3 mM MgCl₂, 10 mM D-glucose, 100 units/ml penicillin, 100 µg/ml streptomycin, pH 7.3) and homogenized using a Tissue Tearor (Fisher). Protein was isolated using Trizol reagent (Invitrogen). Proteins from PC12 cells were isolated in RIPA buffer (10 mM PBS, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 30 µl/ml aprotinin, 10 mM Na orthovanadate, 100 µl/ml PMSF). Protein samples (30 µg) were separated by SDS-PAGE under reducing conditions. Antibodies were diluted in 1% heat-denatured casein in 10 mM phosphate buffered saline (PBS: 10 mM sodium phosphate, 2.7 mM KCl, 4.3 mM NaCl, pH 7.5). Western analyses were performed using polyclonal anti-LIS1 (1:500, Chemicon), monoclonal anti-α₁ (1:1000, kind gift of Dr H. Arai, University of Tokyo, Tokyo, Japan), monoclonal anti- α₂ (1:1000, Dr H. Arai), poly-ADP-ribose polymerase (PARP, 1:10 000, Clontech), and actin (1:1000, Sigma). Secondary antibodies were horseradish peroxidase-conjugated or biotin-conjugated anti-mouse IgG (1:2000, Jackson Immunolabs; 1:10 000, Sigma) or anti-rabbit IgG antibodies (1:5000, Jackson Immunolabs) and tertiary reagents were extravidin alkaline phosphatase (1:300 000, Sigma) as appropriate. Immunoreactive bands were visualized using SuperSignal West Pico (MJS BioLynx Inc) or NBT/BCIP (Sigma). Densitometry was performed using ImageJ analysis software (NIH) standardized to actin loading controls.

**PAF-AH activity:** PAF-AH activities in complete media, serum-free RPMI, PC12-AC cells, PC12-AC conditioned treatment media, and C57Bl/6 mouse brain approximately 3 months of age (positive control) were determined using a commercial
PAF-AH assay kit (Cayman Chemicals). Cells and tissue were homogenized in 250 mM sucrose, 10 mM Tris-HCl (pH 7.4), and 1 mM EDTA using a Tissue Tearor (Fisher). Samples were centrifuged at 600 X g for 10 min and at 100 000 X g for 60 min. Cytosolic supernatants were concentrated using an Amicon centrifuge concentrator with a molecular weight cut-off of 10 000 kD (Millipore). Protein (30-50 μg) was incubated with C_{16}-2-thio-PAF substrate for 30 minutes at room temperature. In some cases, lysates were pretreated for 15 or 30 min at room temperature with diisopropyl fluorophosphate (DFP) at the concentrations indicated in the text. Percent inhibition was calculated relative to lysates treated with vehicle (PBS) for the same period of time. Hydrolysis of the thioester bond at the sn-2 position was detected by conjugation with 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) at 405 nM. Control reactions included samples incubated without lysate or media and samples incubated without substrate.

**Internalization assay (live cell imaging):** PC12-AC cells (5x10^4 cells/well) were plated in complete media overnight in 24-well plates (VWR) coated with 0.1% gelatin. Cells were washed in 10 mM PBS and incubated with 1 μM Bodipy FL C_{11}-PAF (B-PAF) in RPMI 1640 containing 0.1% bovine serum albumin (BSA, Sigma). B-PAF was custom synthesized for our laboratory by Molecular Probes. At each time point (0,10, 20, 30, 40, 50, 60, 80, 90, 100, 110, 120 min), incubation media was removed, cells were washed with 10 mM PBS. Live cell imaging under phase and fluorescence of identical cell fields was performed using a DMR inverted microscope (Leica) equipped with a QICAM digital camera (Quorum Technologies) and captured using OpenLab software v3.17 (Improvision). Following time-lapse imaging, the incubation media was
replaced and internalization allowed to continue. Quantitation of fluorescence intensity of individual cells was performed using the Advanced Measurement Module of OpenLab v3.17.

Lipid extraction and thin layer chromatography (TLC): PC12-AC cells seeded at 1 x 10^5 cells onto 10 cm plates were maintained in complete media at 37°C in 5% CO₂ for 72 h. Cultures were incubated at 37°C with 1 μM B-PAF in RPMI 1640 containing 0.1% BSA, for 0, 5, 15, 30, 45, 60, and 75 minutes. At each time point, 4 plates/condition were removed from the incubator and were placed on ice. One ml of methanol acidified with 2% acetic acid was added to each plate and the extracellular fraction was collected. This fraction contained B-PAF in the culture media and uninternalized B-PAF bound to cell surface proteins or associated with the plasma membrane. The remaining monolayer of cells was collected in acidified methanol by scraping the plate with a cell lifter (Fisher). Lipids were extracted from the extracellular milieu and cytosolic fractions by the Bligh and Dyer method (32) and developed on TLC plates (20 x 20 cm Silica gel 60 (Fisher) in a solvent system of chloroform/methanol/acetic acid/water (50:30:8:5, v/v). B-PAF and B-lyso-PAF (Molecular Probes) were used as authentic markers. Fluorescent lipids were visualized under UV light using Alphalmager-1220 software (Alpha Innotech Corporation). Fluorescence intensity corresponding to lipid yield was determined by densitometry using the Advanced Measurement Module of OpenLab v3.17. Concentrations of cytosolic B-PAF were estimated in comparison to a B-PAF standard curve resolved in parallel. Data are expressed as pM/PAF-responsive cell following standardization to the
number of cells per culture.

**Cell death assays:** PC12-AC cells (8800 cells/cm²) were plated overnight in complete media in 6 cm dia. tissue culture plates (VWR). Cells were treated in serum-free RPMI media containing 0.025% BSA (treatment media) for 24-72 h with EtOH (0.1%), PAF (10 nM-1 μM, Biomol Research Laboratories), methyl-carbamyl PAF (mc-PAF; 100 nM-1 μM, Biomol Research Laboratories), or lyso-PAF (10 nM-1 μM, Biomol Research Laboratories). In some cases, cells were pretreated with BN 52021 (1-100 μM, Biomol Research Laboratories), CV-3988 (0.2-2 μM, Biomol Research Laboratories), CV-6209 (1-10 μM, Biomol Research Laboratories), FR-49175 (0.5-50 μM, Biomol Research Laboratories), DFP (0.1 mM or 1 mM, Sigma), DTNB (1 mM, Sigma) or combinations thereof for 15 min followed by addition of PAF (1 μM) for 24 h. Cell survival was assessed by hemocytometer cell counts of Trypan Blue-excluding cells. Metabolic activity was assessed based on the ability of mitochondrial dehydrogenases, active in viable cells, to reduce the formazan salt, WST-1 (measured at A₄₅₀-A₆₉₀ nm, Roche). DNA fragmentation was determined by terminal deoxynucleotidyltransferase dUTP nick-end labeling (TUNEL, Roche) of cultures fixed for 20 min in 4% paraformaldehyde in 10 mM as described in (33). Cells, processed for TUNEL, were double-labeled with Hoechst 33258 (0.2 μg/ml) for 20 min at room temperature for additional morphological evidence of apoptotic loss.

**RNA interference transfection:** To suppress expression of the PAF-AH I α₂ subunit, we designed a double-stranded short interfering RNAs (siRNA) to the α₂
sequence (AATAAACATGCTTGCACCTCCC/CTGTCTC) and a negative control scrambled sequence (AATGCATTGAGGAGGAGGC/CTGTCTC). Oligonucleotides were obtained from the Biotechnology Research Institute at the University of Ottawa. siRNA duplexes were generated using the Silencer siRNA Construction Kit (Ambion). Transfection of siRNA was performed with Lipofectamine 2000 (Invitrogen) and optimized to yield maximal transfection efficiency according to manufacturer’s protocol. Briefly, 2 µl of Lipofectamine 2000 was diluted in 198 µl of RPMI-1640 media for 5 minutes at room temperature. siRNA duplexes (PAF-AH α2 or scrambled) were suspended in 100 µl of RPMI-1640 media. PC12-AC cells grown in complete media to 30% confluence in 4-well gelatin-coated Labtek wells were treated with 100 µl of the Lipofectamine/siRNA complex and incubated for 72 hours at 37°C. Transfection efficiency was determined in separate cultures by counting EGFP-positive cells upon transfection with pEGFP-C1 as well as morphological changes using Ambion’s β-actin siRNA positive control. Because we were unable to obtain >20% transfection efficiency, we co-transfected PAF-AH α2 or scrambled siRNAs with 0.028 µg/µl of pEGFP-C1 to identify silenced cells. Cells were then exposed to 1 µM PAF in treatment media (RPMI + 0.025% BSA) for either 45 minutes or 24 hours. Data were standardized to the number of EGFP-positive cells in vehicle-treated cultures transfected with pEGFP-C1 only.

Statistical analysis: Data were analyzed using ANOVA tests or unpaired Student’s t-tests, as applicable. Following detection of a statistically significant difference in a given series of treatments, post hoc Dunnett’s t-tests or Tukey tests were
performed where appropriate. P values under 0.05 were considered statistically significant (shown as * or †); P values under 0.01 were considered highly statistically significant (shown as ** or ††).

RESULTS

*PAF can elicit apoptosis independently of its G-protein coupled receptor*

We have previously demonstrated that PC12-AC cells do not express PAFR but undergo apoptosis-associated DNA fragmentation 24 h after treatment with > 100 nM PAF and necrotic lysis when treated with > 3 μM PAF (11). In this study, we addressed the kinetics and underlying signaling mechanism of PAF-induced apoptosis in the absence of PAFR (Fig 1A, inset). To determine whether cell death is mediated by PAF or downstream PAF metabolites, PC12-AC cells were treated with PAF (1-O-hexadecyl-2-acetyl-sn-glycero-3-phosphocholine), mc-PAF (1-O-hexadecyl-2-N-methyl-carbamyl-sn-glycero-3-phosphocholine), the PAF-AH-resistant synthetic PAF analog (34), or lyso-PAF (1-O-hexadecyl-2-lyso-sn-glycero-3-phosphocholine), the immediate PAF metabolite. Both mc-PAF and PAF triggered comparable concentration-dependent cell death 24 h after treatment (Fig 1A). Lyso-PAF had no significant effect on cell viability (Fig 1A). To establish the kinetics of PAF-mediated cytotoxicity, cell survival was assessed at various periods of time after phospholipid administration. PAF (1 μM) elicited significant cell loss within 24 h of treatment; no further reductions in cell number were detected at 48 h or 72 h relative to vehicle controls (Fig 1B). mc-PAF (1 μM) elicited incremental cell loss for up to 72 after treatment (Fig 1B). Comparable kinetics were observed if PAF (1 μM) was replenished in fresh media at 24 h intervals with cells.
repeatedly treated with PAF exhibiting sustained impairment of cellular metabolic activity (Fig 1C) and an incremental reduction in cell survival (Fig 1D).

To determine whether PAF activates caspases in the absence of PAFR, we examined cleavage of the caspase 3 substrate, PARP. Caspase 3-mediated PARP cleavage from 116 kD to 85 kD was observed 45 min after a single PAF treatment (Fig 2A). Cleavage markedly increased 3 h after phospholipid administration and was detected for up to 24 h after treatment (Fig 2A) but not at later time points (data not shown). TUNEL-positive cells, evidence of terminal DNA fragmentation, were first observed 24 h after PAF exposure with no evidence additional death 48 h after exposure (Fig 2B).

Collectively, these findings indicate that a) PAF can activate caspase 3 independently of its G-protein coupled receptor within 45 min of treatment, b) this PAFR-independent apoptotic pathway culminates in terminal DNA fragmentation 24 h after treatment, c) cell death is not mediated by downstream metabolites, and d) incremental cell death depends upon repeated exposure to active ligand or treatment with PAF-AH resistant PAF analogs.

_Cytosolic PAF-AH enzymes limit the duration of PAF-induced apoptotic signaling_

These kinetics implicate PAF degradation in the control of apoptotic signaling. To test this hypothesis, we first identified _Lis1_, PAF-AH I α1, PAH-AH I α2, and PAF-AH II but not plasma PAF-AH transcripts in PC12 cells by RT-PCR (Fig 3A-F). Amplicon integrity of the PAF-AH I subunits was verified by sequencing on both strands. We then confirmed protein expression by Western analysis. α1 protein was constitutively...
expressed in the presence or absence of PAF (Fig 3G). \(\alpha_2\) protein was present at extremely low levels (Fig 3H,I) with marked induction following exposure to PAF (Fig 3H,I). Closer examination revealed that \(\alpha_2\) protein also increased when cells were cultured in serum-free treatment media suggesting a response to serum-withdrawal rather than PAF-specific induction (Fig 3I). LIS1 was constitutively expressed by PC12-AC cells (Fig 3J).

Lacking an antibody to PAF-AH II to verify protein expression, we sequenced the full-length transcript and assayed functional PAF-AH activity in the presence of cytosolic PAF-AH I and II inhibitors. Full-length transcript was amplified from Wistar rat brain and PC12-AC cells using a series of primer pairs homologous to conserved sequences in murine, bovine, and human genes (Table 1). A 1173 bp open reading frame was identified in both PC12-AC cells and Wistar rat brain with no base-pair mismatches between the sequences\(^2\). In functional assays, total cytosolic PAF-AH activity in cell lysates was comparable to enzymatic activity in adult mouse brain homogenates (Table 2). PAF hydrolytic activity was not detected in treatment media harvested from cells after 24 h of culture confirming the RT-PCR analysis that PC12-AC cells do not express the secreted PAF-AH isoenzyme (Table 2). To distinguish between PAF-AH I and PAF-AH II activity, cell lysates were treated with the active serine blocking agent DFP. 0.1 mM DFP inhibits PAF-AH I \(\alpha_1/\alpha_1\) or \(\alpha_1/\alpha_2\) dimer activity while 1 mM DFP inhibits PAF-AH I \(\alpha_1/\alpha_1\), PAF-AH I \(\alpha_1/\alpha_2\), and PAF-AH II activity. PAF- AH I \(\alpha_2/\alpha_2\) dimers are DFP-resistant (21,23,35). 68-70% of the total cytosolic PAF-AH activity was blocked by 0.1 mM DFP indicative of PAFAH I \(\alpha_1/\alpha_1\) or \(\alpha_1/\alpha_2\) activity (Table 2). Increasing the DFP

\(^2\) The rat PAF-AH II cDNA cloned from adult Wistar rat brain was deposited under GenBank accession number AY225592.
concentration to 1 mM blocked an additional 4-7% of PAF-AH activity attributed to PAF-
AH II. DFP-resistant PAF-AH I $\alpha_2/\alpha_2$ homodimer activity comprised 25% of cytosolic
PAF-AH activity in lysates extracted from cells cultured in complete media.

The kinetics of PAF hydrolysis by PAF-AH I and II were established using the
fluorescent PAF substrate, Bodipy FL C11-PAF (B-PAF). Cells were treated with 1 $\mu$M
B-PAF to initiate apoptotic signaling. B-PAF and its metabolites were extracted at
various time points using a modified Bligh and Dyer procedure, identified by TLC, and
fluorescent intensity quantified by comparison to resolved fluorescent standards.
Cytosolic and extracellular lipids were fractionated from B-PAF-treated cultures with an
acidified methanol wash separating phospholipids bound to proteins on plasma
membrane from internalized lipids (36). We found that 1 $\mu$M B-PAF was stable in cell-
free, serum-free treatment media at 37°C for at least 60 min; no degradation to B-lyso-
PAF was observed in the absence of cells (Fig 4A). B-PAF was rapidly internalized by
PC12-AC cells (Fig 4B, Extracellular) with concentrations reaching approximately 16-20
pM/cell within 5 min of incubation (Fig 4B, Cytosolic). Internalized B-PAF levels
remained constant for 60 min dropping to 6-8 pM/cell by 75 min (Fig 4B, Cytosolic). B-
PAF degradation was detected within 15 min of internalization (Fig 4C, Vehicle). A
linear rate of metabolism ($r^2=0.97$) was observed for up to 75 min (Fig 4C, Vehicle).
Closer examination of the fate of B-PAF metabolite by fractionation revealed that B-
lyso-PAF was released from cells in two stages (Fig 4D). Between 5 and 15 min of
incubation, 50% of internalized B-PAF was converted to B-lyso-PAF (Fig 4D, Cytosolic)
and secreted from cells (Fig 4D, Extracellular). Between 30 and 60 min, B-lyso-PAF
was not released and accumulated in the cell cytosol (Fig 4D, Cytosolic). A delayed
phase of secretion was observed between 60 and 75 min (Fig 4D, Extracellular).

To track the fate of secreted B-lyso-PAF, we performed quantitative time-lapse fluorescence microscopy (Fig 4E). B-PAF-containing media was removed and cells were washed with PBS containing 1% BSA at various time points to remove free lipids loosely associated with the plasma membrane but not phospholipids bound to membrane proteins. Cells were photographed and the B-PAF containing media was replaced. A linear increase in cell-associated fluorescence was observed over the first 70 min of exposure ($r^2=0.97$) followed by a plateau in cell-associated B-PAF (Fig 4E).

Taken together, these data indicate that PAF administered to cells is metabolized by PAF-AH I and II with a $t_{1/2}$ of 75 min. The PAF metabolite, lyso-PAF, is secreted from cells but remains bound, apparently to carrier proteins, on the extracellular surface of the plasma membrane.

Cytosolic PAF-AHs can be targeted pharmacologically to promote cell survival

The kinetics of PAF-induced apoptosis (Fig 1, 2) and PAF degradation (Fig 4) suggest that cytosolic PAF concentrations must remain elevated for at least 60 min to elicit apoptosis. This hypothesis predicts that compounds capable of inhibiting PAF internalization or increasing cytosolic PAF-AH activity will block PAF-mediated death triggered independently of PAFR. To test this hypothesis, we evaluated the anti-apoptotic actions of four PAF antagonists (CV-3988, CV-6209, BN 52021, and FR-49175). These compounds were chosen because of their affinities for different PAF binding sites identified pharmacologically. CV-3988 and CV-6209 are competitive PAF antagonists that preferentially interact with synaptosomal and microsomal PAF binding
sites (17). CV-3988 competes for PAF at the plasma membrane, likely PAFR, as well as interacting with intracellular microsomal binding sites, likely internalized PAFR (17). CV-6209 preferentially interacts with a single binding site in microsomal membranes (17). BN 52021 (also known as ginkgolide B) is a non-competitive PAF antagonist with affinity for three discrete PAF binding sites (17) as well as potent antioxidant activity (37). FR-49175 is a fungal metabolite derivative that inhibits PAF-induced biological activity through unknown mechanisms (38,39). Treatment of PC12-AC cells with CV-3988, BN 52021, or FR-49175 had no effect on cell survival (Fig 5A-C) however CV-6209 was toxic to cells at concentrations above 1 μM (Fig 5D). We observed significant concentration-dependent anti-apoptotic activity when cells were pre-incubated for 15 min with BN 52021 (1-100 μM) or FR-49175 (0.5-50 μM) and then exposed to PAF (Fig 5E). Both BN 52021 and FR 49175 inhibited PAF-mediated caspase 3 activation as assessed by PARP cleavage (Fig 5F). CV-3988 (0.1-10 μM) and CV-6209 (0.01-1 μM) did not exhibit anti-apoptotic activity (Fig 5E).

We next tracked B-PAF fate by lipid extraction and TLC in the presence or absence of BN 52021 and FR-49175 to establish whether these antagonists affect phospholipid internalization or PAF-AH activity. FR-49175 and BN 52021 did not alter the rate or extent of B-PAF internalization (Fig 6A,D, Extracellular). In both antagonist- and vehicle-treated cultures, maximal cytosolic B-PAF levels were attained within 5 min of B-PAF exposure (Fig 6A,D Cytosolic 5 min). We did not observe an increase in total B-PAF hydrolysis. Cytosolic B-PAF concentrations, estimated at 8 pM/cell, in BN 52021- and FR-49175-treated cultures were comparable to vehicle-treated cells by the end of the 75 min test period (Fig 6A,D Cytosolic, 75 min). Significantly, we found that
FR-49175 and BN 52021 accelerated both the kinetics of B-PAF degradation (Fig 6A,D, 45 min) and the release of B-lyso-PAF into the media (compare Fig 6B,E Cytosolic 45 min and Fig 6C,F Extracellular 45 min). By 45 min, 39% (FR-49175) or 41% (BN 52021) of exogenous B-PAF had been converted to B-lyso-PAF compared to 29% in vehicle-treated cells. This acceleration dropped cytosolic B-PAF levels more rapidly in antagonist-treated cultures from 16 pM/cell (5 min) to 10 pM/cell (45 min) in the presence of FR-49175 or 8 pM/cell (45 min) in the presence of BN 52021 (Fig 6B,E). Maximal release of B-lyso-PAF from cells was observed within 45 min in antagonist-treated cultures compared to 75 min in vehicle-treated cultures (Fig 6C,F). 50% of the released metabolite diffused or was transported back into the cells within 75 min in FR-49175 treated cultures; 32% of B-lyso-PAF re-entered cells in BN 52021-treated cultures (Fig 6B-F).

*PAF-AH I α2/α2 activity is anti-apoptotic*

To determine how BN 52021 and FR-49175 accelerate PAF-AH hydrolysis, we examined PAF-AH I α1 and α2 expression. Cells were preincubated in serum-free treatment media with vehicle (DMSO), BN52021, or FR-49175 for 15 min before addition of PAF (Fig 7). We found PAF-AH α1 expression remained constant whether cells were cultured in complete media, serum-free treatment media, pretreated with DMSO, or exposed to PAF (Fig 7A, PAF). Surprisingly, PAF-AH α1 protein levels decreased progressively over the first 30 min of PAF treatment and remained low until the end of the 90 min test period (Fig 7A, BN52021). Comparable results were observed in FR-49175-treated cultures (Fig 7A, FR 49175). α2 expression was not
altered by BN 52021 or FR-49175 in that protein expression increased dramatically during the 15 min preincubation period in serum-free treatment media regardless of antagonist or vehicle administration and remained elevated over the 90 min PAF test period (Fig 7B).

The finding that both compounds accelerate PAF degradation by reducing PAF-AH I subunit \( \alpha_1 \) expression was unexpected. Two alternative, but not mutually exclusive, explanations lie in the possibility that this reduction in \( \alpha_1 \) expression promotes formation of PAF-AH I \( \alpha_2/\alpha_2 \) homodimers and/or that PAF-AH II activity is increased. Following exposure to PAF in serum-free media, the catalytic composition of PAF-AH I would be expected to change from a predominance of \( \alpha_1/\alpha_1 \) homodimers in control cultures (Table 1) to \( \alpha_1/\alpha_2 \) heterodimers and \( \alpha_2/\alpha_2 \) homodimers given the 8-10 fold increase in \( \alpha_2 \) protein expression (Fig 3, 7). In cultures pre-treated with BN 52021 or FR-49175, suppression of \( \alpha_1 \) protein expression likely favours a more rapid transition from \( \alpha_1/\alpha_1 \) to \( \alpha_2/\alpha_2 \) homodimers following PAF exposure. This hypothesis is supported by the dramatic increase in the \( \alpha_2 \) to \( \alpha_1 \) ratio observed 30-90 min after PAF exposure in BN 52021 and FR-49175-treated cultures relative to vehicle (Fig 7C). Alternatively, in addition to effects on PAF-AH \( \alpha_1 \), BN 52021 and/or FR-49175 may alter PAF-AH II activity, an enzyme with proven anti-apoptotic properties (26).

To address these possibilities, we performed three loss of function studies. First, we treated cells with 0.1 mM DFP to inhibit PAF-AH I \( \alpha_1/\alpha_1 \) and \( \alpha_1/\alpha_2 \) catalytic activity without changing the relative ratio of \( \alpha_1 \) to \( \alpha_2 \) protein expression (21,23,35). In the unlikely event that the anti-apoptotic effects of BN 52021 and FR-49175 are mediated by a loss of \( \alpha_1 \) catalytic activity, then 0.1 mM DFP should be cytoprotective. Cells were
exposed for 15 min (Fig 4C, Fig 8A) before addition of B-PAF. DFP did not affect B-PAF internalization (data not shown) but reduced B-PAF degradation by 38% 75 min after B-PAF exposure (Fig 4C, 0.1 mM DFP, 75 min). To ensure intracellular concentrations of DFP reached 0.1 mM by the time cells were exposed to PAF, we extended the preincubation period from 15-30 min with no additional effect on the kinetics of PAF-AH inhibition (data not shown). Inhibition of PAF-AH I $\alpha_1/\alpha_1$ and $\alpha_1/\alpha_2$ catalytic activity did not alter survival of cells treated for 24 h with vehicle (Fig 8A, Vehicle (0.1% EtOH)) and did not intensify PAF-mediated cell death (Fig 8A, PAF). These data suggest that PAF-AH I $\alpha_1$ does not play a significant role in regulating PAF-induced apoptosis.

Second, to determine the role of PAF-AH II in control of PAF-mediated cell death, we treated cultures with PAF (1 $\mu$M) or PAF vehicle (0.1% EtOH) in the presence of DFP, the sulfhydryl blocking reagent (DTNB), or vehicle (PBS) (Fig 4C, 8A). Cells were pre-exposed to 1 mM DFP or 1 mM DTNB for 15 min (Fig 4C, 8A) or 30 min (data not shown) before treatment with PAF or vehicle. Individually DFP and DTNB were found to have no effect on the survival of vehicle-treated cells (Fig 8A, Vehicle (0.1% EtOH)). Increasing the DFP concentration from 0.1 mM to 1 mM inhibited 55% of B-PAF degradation within 75 min indicating that 17% of DFP-sensitive PAF-AH activity was PAF-AH II (Fig 4C, 1 mM DFP, 75 min). Despite this substantial inhibition of PAF hydrolysis (55%), PAF-mediated cell loss was intensified by only 8% (Fig 8A, 1 mM DFP). To confirm these results, we used a pharmacological agent that, unlike DFP, inhibits PAF-AH II but not PAF-AH I through different mechanisms. Exposure to 1 mM DTNB elicited the same results as 1 mM DFP (Fig 8A, DTNB). To ensure complete
inhibition of PAF-AH II, we exposed cells to both DFP and DTNB. Combination treatment reportedly abolishes activity of purified PAF-AH II (35). Preincubation in DFP and DNTB followed by 24 h exposure to EtOH decreased vehicle-treated cell survival by 12% (Fig 8A, Vehicle (0.1% EtOH)). Combination treatment reduced PAF-treated cell survival by 20% likely the cumulative results of inhibitor toxicity (12%) and PAF-AH II inhibition (8%) (Fig 8A, PAF). These findings suggest that a) PAF-AH I \( \alpha_1 \) activity is not anti-apoptotic and b) that while PAF-AH II activity is cytoprotective, PAF-AH II plays a secondary role to PAF-AH I \( \alpha_2 \) in regulating PAF-mediated apoptosis.

The data point to PAF-AH I \( \alpha_2 \) as a potential therapeutic target to reduce apoptogenic PAF concentrations under diverse pathophysiological conditions. To directly confirm this role, we acutely suppressed \( \alpha_2 \) expression using a RNAi strategy. PAF-AH I \( \alpha_2 \)-specific RNAi but not the scrambled control or transfection reagent alone reduced \( \alpha_2 \) protein expression (Fig 8B). Because we were unable to achieve > 20% transfection efficiency, we could not knockdown PAF-AH I \( \alpha_2 \) expression in all cells. To identify transfected cells, we co-transfected cultures with EGFP in combination with \( \alpha_2 \)-specific or scrambled siRNAs (Fig 8C). To control for possible cytotoxic effects of siRNAs, parallel experiments were performed in which cultures were transfected with EGFP alone and survival was expressed relative to EGFP-positive vehicle-treated cells. Importantly, PAF-AH I \( \alpha_2 \) RNAi downregulation significantly enhanced PAF-mediated cytotoxicity (Fig 8D).

**DISCUSSION**

PAF-like lipids have been implicated as key apoptotic second messengers
induced by a variety of pathological stressors (6,19,20,25,26,29,30,33,40). Converging evidence points to PAFR activation of the conserved mitochondrial death pathway (6,12,18-20,41). Our previous work has demonstrated that PAF can also transduce cell death independently of its G-protein coupled receptor (11) but it is not known how cell death is regulated in the absence of PAFR. The importance of this apoptotic cascade is underlined by the fact that neurons express low levels to no PAFR (33,42) and yet PAF is a principle mediator of neuronal loss in ischemia, encephalitis, epileptic seizure, meningitis, and human immunodeficiency virus-1 dementia (1-5). To provide mechanistic insight into this PAFR-independent pathway, we show that: 1) PAF can initiate caspase-dependent cell death in the absence of it G-protein coupled receptor; 2) The duration of PAF apoptogenic signaling is regulated by the \( \alpha_2 \) subunit of PAF-AH I and, to a lesser extent, by PAF-AH II; 3) PAFR-independent cell death can be inhibited by two PAF antagonists: the gingkolide BN 52021 and the fungal derivate FR-49175, but not by CV-3988 or CV-6209 that share PAF's glycerophospholipid; 4) Both BN 52021 and FR-49175 protect cells from PAF-induced cell death by reducing PAF-AH I \( \alpha_1 \) protein levels indirectly promoting PAF-AH I \( \alpha_2 \) homodimer activity.

**Apoptotic PAF signal transduction in the absence of PAFR**

To provide mechanistic insight into how PAF transduces cell death in the absence of PAFR, we followed the internalization and metabolic fate of Bodipy fluorophore-conjugated PAF in PAF-sensitive cells (11,43). PC12 cells express all of the components of both cytosolic PAF-AH enzymes (I and II) but not plasma PAF-AH or PAFR. Extracellular PAF was internalized by PC12 cells with a \( t_{1/2} \) of approximately 5
min. Downstream activation of caspase 3 was initiated when cytosolic PAF concentrations were elevated by approximately 15-20 pM/cell. These findings complement previous work demonstrating that exposure to oxidative stressors triggers apoptogenic remodeling of membrane phospholipids into PAF-like lipids (26) and provides strong evidence that cytosolic accumulation of PAF and PAF-like lipids can trigger apoptotic death independently of PAFR. In fact, internalization of PAF mediated by PAFR endocytosis (36) may protect cells from this cell death cascade. Endocytosis of the PAF/PAFR complex triggers release of plasma PAF-AH from macrophages thereby reducing extracellular ligand concentrations (36).

While we have yet to identify the effector proteins responsible for transducing increases in intracellular PAF concentration into downstream caspase activation, our data indicate that the temporal kinetics of PAF accumulation regulate the duration of apoptotic signaling and that PAFR-independent cell death is triggered by PAF and not by its immediate metabolite lyso-PAF. We found that intracellular PAF levels must remain elevated by approximately for 60 min to elicit significant apoptotic death. Decreasing cytosolic concentrations to less than 10 pM/cell of exogenous PAF stops the cell death signalling. Lyso-PAF, the immediate PAF metabolite, does not transduce downstream apoptotic induction given that equimolar concentrations of lyso-PAF are not cytotoxic and that the endogenous metabolite remains cell-associated without detectable effect. Accelerating the kinetics of PAF degradation with BN 52021 or FR-49175 reduces PAF concentration to sub-apoptotic levels (~ > 8 pM/cell) within 45 min and prevents PAF-induced caspase activation. The PAFR-specific antagonists CV-3988 and CV-6209 have no effect on PAF-mediated PAFR-independent cell death
when administered at concentrations up to 20 times that of their reported IC 50 antagonist activities (44-46). Interestingly, the anti-cell death activities of the antagonists tested in this study (FR-49175 > BN 52021 > CV-3988 or CV-6209) are in direct opposition to their PAF antagonist potency in other biological assays (CV-6209 > CV-3988 > BN 52021 > FR 49175) (38,44-47) suggesting that their PAFR-independent anti-apoptotic actions are inversely proportional to their affinity for PAFR.

We do not know how PAF is internalized by PC12 cells in the absence of its G-protein coupled receptor. In addition to endocytosis as a PAF/PAFR complex, active trafficking of PAF across the plasma membrane is accomplished by a PAF-specific transglutaminase and by interaction with low affinity binding sites yet to be identified at the molecular level (14,48). Passive PAF internalization is regulated by transbilayer movement (flipping) across the plasma membrane occurring as a result of physicochemical changes in membrane properties accompanying cellular activation (15). It has been suggested that PAF internalization in the absence of PAFR is not rapid enough to elicit acute biological activity in hematopoietic cells (36,49). In this study, we present evidence that PAFR-independent PAF internalization by non-hematopoietic cells is indeed sufficient to trigger apoptotic cell loss.

**PAF-AH α2 activity is anti-apoptotic**

Administration of recombinant PAF-AH II or plasma PAF-AH and ectopic overexpression protects cells from death elicited by low-density lipoprotein, glutamate, or oxidative stress (25,26,29-31). In this study, we show that PAF-AH I exerts similar cytoprotective effects with three important distinctions. First, the anti-apoptotic actions
of PAF-AH I are subunit specific. Under normal culture conditions, we found PAF hydrolysis in PC12 cells to be primarily mediated by the PAF-AH I $\alpha_1$ subunit and to a lesser extent by PAF-AH II. Following serum deprivation and exposure to pathological PAF concentrations, PAF-AH I $\alpha_2$, but not PAF-AH I $\alpha_1$, protein expression is acutely upregulated. By pharmacological inhibition using DFP and DTNB and by RNA interference, we found that PAF-AH I $\alpha_2$ but not PAF-AH I $\alpha_1$ activity reduces the duration of PAF-mediated apoptotic signaling. When cells were cultured under normal conditions, we found that cytosolic PAF-AH activity is PAF-AH I $\alpha_1$/1 and $\alpha_1$/2 (~70%) > PAF-AH I $\alpha_2$/2 (~25%) > PAF-AH II (5%). When cells were deprived of serum and challenged with PAF, this activity profile shifted in favor of PAF-AH I $\alpha_2$/2 (45%) > PAF-AH $\alpha_1$/1 or $\alpha_1$/2 (38%) > PAF-AH II (17%). Surprisingly, pharmacological inhibition of $\alpha_1$ enzymatic activity had no effect on PAF-mediated cell death while suppression of $\alpha_2$ induction by RNA interference significantly enhanced cell loss providing strong evidence for anti-apoptotic subunit specificity.

Second, PAF-AH I $\alpha_2$ is mobilized as part of an endogenous cell survival response. These findings complement studies documenting the anti-apoptotic actions of plasma PAF-AH and PAF-AH II (8,25,26,29-31). Moreover, we find that PAF-AH II is not able to compensate for a loss of PAF-AH I $\alpha_2$ function. In fact, we were surprised to find that a 55% reduction in PAF hydrolysis observed 75 min after PAF challenge following DFP treatment only moderately enhanced PAF-mediated cell death. The kinetics of PAF-AH activation may explain the lack of significant PAF-AH II protection. PAF-AH $\alpha_2$ is mobilized acutely in response to serum withdrawal but an increase in DFP- or DTNB-sensitive PAF-AH II activity is only observed after 30-60 min of PAF
exposure. Only PAF-AH I \(\alpha_2\) was found to reduce apoptogenic concentrations of cytosolic PAF within the first 30 min of exposure. PAF-AH II is mobilized as part of a delayed cell survival program possibly to ensure that “sub-apoptotic” PAF concentrations are maintained.

Third and perhaps most importantly, the anti-apoptotic response afforded by shifting PAF-AH I \(\alpha_1\) subunit expression in favour of \(\alpha_2\) can be enhanced by PAF antagonists. Pharmacological suppression of \(\alpha_1\) by BN 52021 or FR-49175 accelerates the kinetics of PAF deacetylation to lyso-PAF and protects cells from PAF-mediated apoptosis. While it would at first appear counterintuitive that a reduction in PAF-AH subunit expression enhances PAF hydrolysis, these data are consistent with previous reports that BN 52021 inhibits PAF degradation under non-apoptotic culture conditions (48,50). PAF-AH I \(\alpha_2/\alpha_2\) is known to hydrolyze PAF more efficiently than \(\alpha_1/\alpha_1\) homodimers and it is likely that reducing PAF-AH \(\alpha_1\) coincident with PAF-AH \(\alpha_2\) induction would promote more rapid formation of \(\alpha_2/\alpha_2\) homodimers, enhance PAF hydrolysis, and increase cell survival. Interestingly, this phenomenon models the graded reduction in \(\alpha_1\) observed over the course of cerebral development changing the predominant PAF-AH I catalytic composition from \(\alpha_1/\alpha_2\) heterodimers in embryonic central nervous system to \(\alpha_2/\alpha_2\) homodimers in adult brain (51). Our data suggest that this shift may render adult brain more resistant to PAF challenge than embryonic brain.

**Summary**

In summary, this study provides mechanistic evidence that sustained exposure to elevated intracellular PAF concentrations is sufficient to elicit apoptosis through a
PAFR-independent cell death pathway. Previous studies have demonstrated that oxidative modification of membrane lipids during atherosclerotic injury, treatment with chemotherapeutic agents, ultraviolet B exposure, or excitotoxicity is sufficient to produce PAF-like lipids (6,19,30,31,40). Our data suggest that these effects can occur in the absence of PAFR. To intervene in PAF-mediated death, we describe a novel anti-apoptotic function for PAF-AH I $\alpha_2$ and II and identify two PAF antagonists that accelerate cytosolic PAF-AH I activity to inhibit PAF-mediated apoptosis.

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Table 1. Primer pairs used to detect cytosolic and plasma PAF-AH transcript by RT-PCR.

| Gene       | Strand | Sequence (5’-3’) | Amplicon size (bp) |
|------------|--------|------------------|--------------------|
| GAPDH      | Sense  | TGGTGCTGAGTATGTCGTGGAGT | 292                |
|            | Antisense | AGTCTTCTGAGTGCGAGGAGTGG |                 |
| PAF-AH I α1| Sense  | GACGGACGGCTGGATGCTCT | 587                |
|            | Antisense | AGACGAAGCAGCAAGGAGTG |                 |
| PAF-AH I α2| Sense  | TGCAAGTACGGAGGATATGG | 418                |
|            | Antisense | AACATGTCGTGGGAGAGAT |                 |
| PAF-AH I LIS1 | Sense | CTGCTTCAGAGGATGCTACA | 373                |
|            | Antisense | ATCAGAGTGGCGTGCTGAT |                 |
| PAF-AH II #1 | Sense | GGATGTGATGGAGGTC | 1017               |
|            | Antisense | TGCTTCTCAGGAAGCGGCAA |            |
| PAF-AH II #2 | Sense | CGCCTGTGATGAGGATGG | 224                |
|            | Antisense | GCCGTGTACTGCAAGGT |                 |
| PAF-AH II #3 | Sense | TGAGCGGAGGCTGGATGATG | 472                |
|            | Antisense | CGCCTGATGAGGATGTC |                 |
| PAF-AH II #4 | Sense | CAGCTGTGATGAGGATGG | 1250               |
|            | Antisense | CAACTCAGAGGCTGGAGGA |            |
| Plasma PAF-AH | Sense | GGGGCATTTCTTTGAGGAG | 413                |
|            | Antisense | GACAGTCCACTGATCAAACGT |            |
| PAFR       | Sense  | CACTTTAAACCCTTACAGGACAG | 381                |
|            | Antisense | AAGACAGTGGCAGACCACACAG |            |
Table 2. PAF-AH activity in PC12-AC cells, mouse brain lysate, and tissue culture media

|                      | Mouse brain lysate | PC12-AC Lysates | Cell-free media |
|----------------------|--------------------|-----------------|-----------------|
|                      | Positive control   | Untreated Lysates | % inhibition$^2$ | Complete Media | Serum-Free Treatment Media | PC12-AC conditioned Treatment Media |
|                      |                    |                 | Time 0.1 mM DFP | 1 mM DFP | | |
| nmol/min/µg or % inhibition | 15 min             | 68% ± 0.19 n=6 | 75% ± 0.10 n=2 | 3.36 ± 0.01 n=2 | 0.00 ± 0.00 n=3 |
|                      | 30 min             | 70% ± 0.19 n=3 | 74% ± 0.10 n=3 | 0.00 ± 0.00 n=2 |

$^1$Data represent mean ± SEM. N denotes the number of replicates conducted over 1-4 experiments.

$^2$Lysates were pre-treated for 15 or 30 min at room temperature with vehicle (10 mM PBS) or the indicated concentrations of DFP. Activity in vehicle-treated cultures was equivalent to that detected in untreated cultures. % inhibition was determined as described in Materials and Methods.
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FIGURE CAPTIONS

Figure 1. The kinetics of PAF-mediated apoptosis initiated independently of PAFR depend upon sustained exposure to active ligand. (A) PC12-AC cells were treated for 24 h with PAF (0.01-1 μM), mc-PAF (0.01-1 μM), or lyso-PAF (0.01-1 μM) in serum free treatment media. A dose-dependent decrease in cell number relative vehicle (0.1% ETOH)-treated cells was observed after 24 h of treatment with 1 μM PAF or mc-PAF (**p<0.01). RT-PCR analysis of PC12-AC cultures (inset) confirmed that both PC12-AC cells and PC12-AC cells differentiated to a neuronal phenotype for 7 days with nerve growth factor (PC12+NGF) do not express PAFR mRNA. The positive control was RNA isolated from PC12 cells stably transfected with PAFR. cDNA template integrity was confirmed using GAPDH as an internal standard (GAPDH). (B) PAF (1 μM) treatment resulted in significant cell loss within 24 h of treatment (*p<0.05) after which no additional reductions in cell number were observed. mc-PAF (1 μM) elicited incremental cell loss for up to 48 h after exposure (*p<0.05, **p<0.01). Culture in serum-free treatment media in the presence of vehicle (EtOH, 0.1%) did not affect cell viability until 48 h after treatment. Arrows indicates the time of PAF administration. (C) Repeated PAF (1 μM) administration incrementally decreased the metabolic activity of cells for up to 72 h as defined by the ability of mitochondrial dehydrogenases to reduce the formazan salt WST (**p<0.01). Arrows indicate the when PAF (1 μM) or vehicle (0.1% EtOH) was replenished in fresh media. (D) Incremental cell loss was observed for up to 48 h following chronic PAF (1 μM) treatment. (*p<0.05, **p<0.01, ANOVA, post-hoc Dunnett’s t test). As in (C), media was replaced and PAF (1 μM) or vehicle (0.1% EtOH) was added every 24 h (arrows). In (A), data are expressed as
percent survival of vehicle-treated cultures and, in (B,C,D) data are standardized to untreated cells cultured in treatment media to present the effect of vehicle treatment on cell survival. Results are reported as mean± SEM of n=5-26 cultures per data point.

Figure 2: **PAF triggers a caspase-dependent apoptotic cascade within 45 minutes of exposure to active ligand.** (A) PC12-AC cells were exposed to PAF (1 µM) for 24 h and assayed for caspase 3 activity. PARP cleavage to 85 kD was first detected 45 min after PAF treatment. Blots were re-probed with actin as a loading control. (B) TUNEL analysis detected apoptosis-associated DNA cleavage (arrows, upper panel) and DNA condensation (arrows, lower panel) 24 h after PAF treatment. No additional cell loss was observed 48 h after treatment. Scale bar, 10 µm.

Figure 3. **Expression patterns of PAF-AH I, PAF-AH II, and plasma PAF-AH in PC12-AC cells.** RT-PCR was performed for (A) LIS1, (B) PAF-AH I α1, (C) PAF-AH I α2, and (D) PAF-AH II. Plasma PAF-AH mRNA was not detected (E). The absence of genomic DNA contamination or reagent contamination was confirmed by the control reactions: no RT during the RT reaction, no template during the PCR reaction, and no primers during the PCR reaction. Rat brain RNA was reverse-transcribed as a same-species positive control (Rat Brain lane). Template integrity of the random-primed PC12-AC RT product was verified using GAPDH (F). Equal amounts of protein (30 µg) from cells cultured in complete media (0) or cells treated for 60 min with 1 µM PAF in treatment media (60) were separated under reducing conditions and immunoblotted for α1 (G), α2 (H,I), or LIS1 (J). Protein lysates from neonatal rat brain were used as a
same-species positive control. Representative immunoblots of replicate experiments are depicted. PAF-AH $\alpha_1$ was constitutively expressed (G). Significant PAF-AH $\alpha_2$ protein was not detected until cells were treated with PAF (1 µM) (H). Longer exposure times (overnight) indicated that PC12-AC cells expressed low levels of protein and that serum deprivation was sufficient to induce $\alpha_2$ protein expression (I). LIS1 was constitutively expressed (J). The major species was a 45 kD protein although multiple bands were detected in untreated PC12-AC cells consistent with phosphorylated LIS1 (arrows). An additional non-specific high molecular weight band (~70 kDa) was also detected (asterisk). Blots were probed for actin as a loading control (G-J).

**Figure 4. Internalization, kinetic analysis, and metabolic fate of B-PAF and B-lyso-PAF in PC12-AC cells.** PC12 cells were incubated with 1 µM B-PAF at 37°C for 0, 5, 15, 30, 45, 60, and 75 minutes. At each time point, lipids were extracted from the extracellular and cytosolic fractions, and were separated by TLC. (A-C) Data represent relative fluorescence of B-PAF in each fraction expressed as a percentage of the total fluorescent lipids recovered. Data are the mean of 2-4 independent experiments conducted in replicate. (A) B-PAF was not degraded by treatment media in the absence of PC12 cells. (B) In cell cultures, B-PAF was rapidly internalized within 5 min (Extracellular fraction) after which, levels remained stable until 60 min and dropped by 75 min (Cytosolic fraction). (C) Conversion of B-PAF to B-lyso-PAF was monitored to determine the degree of PAF hydrolysis in the presence or absence of the PAF-AH $\alpha_1$ and PAF-AH II inhibitor DFP. Cells were pre-treated for 15 min in treatment media with Vehicle (PBS) or DFP before addition of B-PAF. (D) The fate of the B-PAF metabolite
B-lyso-PAF was followed by fractionation. (E) To determine whether B-lyso-PAF secreted from cells remains loosely associated with the lipid bilayer or bound to membrane proteins, cell-associated fluorescence was tracked by live-cell imaging. The B-PAF containing media was removed every 10 min and cells were washed with PBS + 1% BSA to remove phospholipids at the extracellular face of the plasma membrane but not lipids associated with membrane proteins. Data are reported as the mean Increase in fluorescence intensity standardized to background fluorescence in untreated cells ± SEM (n= 16-25 per data point). Cell-associated fluorescence increased 3-fold over the first 75 min of exposure and remained constant between 75 and 120 min despite repeated washes indicating that the secreted material removed by the acidified methanol wash in (D, Extracellular) was likely bound to proteins at the plasma membrane (E). Scale bars, 5 μm.

Figure 5. Anti-apoptotic actions of the PAF antagonists BN 52021 and FR-49175 but not CV 6209 or CV 3988 in PAFR-negative cells. PC12-AC cells were pre-treated for 15 min with 0.1% DMSO (Vehicle) or different concentrations of PAF antagonists and exposed to 0.1% EtOH (A-D) or PAF 1 μM (E,F) for 24 h. In A-E, data are expressed as percent survival of vehicle-treated cultures. Results are reported as Mean± SEM of n=5-47 cultures per data point. BN 52021, FR-49175, and CV 3988 had no effect on the viability of vehicle-treated cultures. CV 6209 (10 μM) was toxic (*p<0.05). BN 52021 and FR-49175 but not CV 3988 or CV 6209 dose-dependently inhibited PAF-mediated death († p<0.05, †† p<0.01). (F) BN 52021 and FR-49175 inhibited PAF-mediated caspase 3 activation as assessed by PARP cleavage.
Figure 6. FR-49175 and BN 52021 accelerate the kinetics of PAF-AH activity.
PC12-AC cells were pre-treated with FR-49175 (10 μM), BN 52021 (50 μM), or PBS (vehicle) for 15 minutes and then incubated with 1 μM B-PAF at 37°C. Lipids were extracted from the extracellular and cytosolic fractions and separated by TLC. The relative fluorescence of B-PAF or B-lyso-PAF in each fraction is expressed as the percentage of the total fluorescent lipids recovered. Data represent the mean ± SEM of n=4 (FR-49175) or n=8 (BN 52021) independent experiments conducted in replicate. (A) FR-49175 and (D) BN 52021 had no effect on B-PAF internalization (Extracellular) but cytosolic B-PAF levels were reduced within 45 min of exposure (Cytosolic). (B,E) The kinetics of B-PAF degradation and (C,F) release were accelerated in FR-49175- (B,C) and BN 52021- (E,F) treated cells.

Figure 7. BN 52021 and FR-49175 increase the relative ratio of PAF-AH Iα₂ to Iα₁ expression following PAF challenge. PC12 cells were treated with PAF (1 μM) following a 15 min pre-incubation in the presence or absence of BN 52021 (50 μM), FR-49175 (50 μM), or vehicle (0.1% DMSO). Immunoblotting was performed for Iα₁ (A) or Iα₂ (B). BN 52021 and FR-49175 reduced Iα₁ expression (A) without affecting the Iα₂ induction (B). Blots were reprobed for actin as a loading controls (A,B). Iα₁ (A) and Iα₂ (B) protein levels were standardized to actin and expressed as a percent of the basal expression in cells cultured in complete media. (C) The relative ratio of Iα₂ to Iα₁ protein following BN-52021 or FR-49175 revealed a stochiometric increase in the levels of Iα₂ compared to Iα₁ relative to cells exposed to PAF in the absence of antagonist.
Figure 8. PAF-AH α2 activity is anti-apoptotic. (A) PC12 cells were treated with PAF (1 μM) or ethanol (0.1%) at 37°C for 24 h following 15 min pre-incubation with DFP (0.1 mM), DFP (1 mM), DTNB (1mM), or DFP (1 mM) + DTNB (1 mM) to abolish PAF-AH α1 and PAF-AH II activity. Cell survival was assessed as described in Fig 5. Inhibition of PAF-AH II but not PAF-AH α1 activity moderately intensified PAF-mediated death. (ANOVA, post hoc Tukey tests, * p<0.05, **p<0.01). (B) Short interfering RNA (siRNA) oligonucleotides were employed to silence PAF-AH α2. Western analysis for α2 was performed at various time points after transfection following a 45 min treatment with PAF (1 μM) in serum-free treatment media. A partial knockdown in α2 protein levels, consistent with the maximal 20% transfection efficiency, was detected 72 h after transfection (α2 RNAi) compared to non-transfected cells (Control) or cells that had been transfected with nonspecific scrambled RNAi (Scrambled). β-actin blot was performed as a loading control. (C) PC12 cells were transfected with pEGFP-C1 (Control) or cotransfected with pEGFP-C1 and α2 RNAi (α2 RNAi) or scrambled RNAi (Scrambled). 72 h after transfection, cells were treated with PAF (1 μM) or vehicle (0.1% EtOH) for 24 h. Data are expressed as % survival of EGFP-positive cells standardized to vehicle-treated controls. Survival was reduced to 28% in cells transfected with α2 RNAi compared to 70% in cells transfected with EGFP alone able to upregulate PAF-AH I α2 (Fig 1). Asterisks indicate statistically significant differences by Student’s t test.
Figure 1
Figure 2

A

| Time | 0 | 5 | 30 | 45 | 90 | 3 | 6 | 24 |
|------|---|---|----|----|----|---|---|----|
| PAF  | - | + | +  | +  | +  | + | + | +  |

- PARP (85 kD)
- Actin (42 kD)

B

TUNEL

Hoechst 33258

0 h | 6 h | 24 h | 48 h

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Figure 4
Figure 5
Figure 6
