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Platelet activating factor-induced apoptosis is inhibited by ectopic expression of the platelet activating factor G-protein coupled receptor

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Abstract

The pro-inflammatory lipid mediator platelet activating factor (PAF: 1-*O*-alkyl-2-acetyl-sn-glycero-3-phosphocholine) accumulates in ischemia, epilepsy, and human immunodeficiency virus-1-associated dementia and is implicated in neuronal loss. The present study was undertaken to establish a role for its G-protein coupled receptor in regulating neurotoxicity. PC12 cells do not express PAF receptor mRNA as demonstrated by northern analysis and RT-PCR. In the absence of the G-protein coupled receptor, PAF (0.1–1 μM) triggered chromatin condensation, DNA strand breaks, oligonucleosomal fragmentation, and nuclear disintegration characteristic of apoptosis. *Lyso*-PAF (0.001–1 μM), the immediate metabolite of PAF, did not elicit apoptotic death. Concentrations of PAF or *lyso*-PAF that exceeded critical micelle concentration had physicochemical effects on plasma membrane resulting in necrosis. Apoptosis but not necrosis was inhibited by the PAF antagonist BN52021 (1–100 μM) but not CV3988 (0.2–20 μM). Ectopic PAF receptor expression protected PC12 transfectants from ligand-induced apoptosis. PAF receptor-mediated protection was inhibited by CV3988 (1 μM). These data provide empirical evidence that: (i) PAF can initiate apoptosis independently of its G-protein coupled receptor; (ii) PAF signaling initiated by its G-protein coupled receptor is cytoprotective to PC12 cells; (iii) the pro- and anti-apoptotic effects of PAF on PC12 cells can be pharmacologically distinguished using two different PAF antagonists.

Keywords: apoptosis, CV3988, ginkgolide B, G-protein coupled receptor, necrosis, platelet activating factor.

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Platelet activating factor (PAF: 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) is a biologically active phospholipid with potent pro-inflammatory and neuromodulatory effects (Bazan 1998). *In vivo*, PAF can act as a retrograde transmitter participating in long-term potentiation and enhancing behavioural criteria of learning and memory (Wieraszko *et al*. 1993; Kato *et al*. 1994; Izquierdo *et al*. 1995; Teather *et al*. 1998; Kato 1999; Chen *et al*. 2001; Grassi *et al*. 2001). Transient PAF stimulation mobilizes intracellular calcium, inhibits ionotropic GABA-receptor activation, and elicits glutamate synthesis in neuronal cultures (Bito *et al*. 1992; Clark *et al*. 1994; Serou *et al*. 1999) while chronic exposure to elevated concentrations of PAF have repeatedly been shown to be neurotoxic (DeCoster *et al*. 1998; Pulliam *et al*. 1998; Mukherjee *et al*. 1999; Tong *et al*. 2001). *In vivo*, accumulation of PAF, synthesized and released by activated microglia, is thought to be a principle initiator of neuronal dysfunction and death in human immunodeficiency virus (HIV)-dementia and a secondary mediator of neuronal loss in ischemia and epilepsy (Bazan *et al*. 1995; Bazan 1998; Perry

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Abbreviations used: DIG, digoxigenin; FCS, fetal calf serum; HS, horse serum; NF-κB, nuclear factor-κB; PAF, platelet activating factor; PAFR, platelet activating factor G-coupled receptor, TdT, terminal deoxytransferase; TNFα, tumor necrosis factor-α; TUNEL, dUTP nick-end labeling.

et al. 1998). However, the underlying mechanisms of PAF-mediated cell death remain elusive.

The majority of physiologically relevant PAF effects are initiated by a PAF-specific G-protein coupled receptor (PAFR) (Ishii and Shimizu 2000). In the central nervous system, PAFR is expressed abundantly by activated microglia and at lower levels by discrete neuronal populations (Mori *et al*. 1996; Bennett *et al*. 1998). The ability of PAF antagonists to inhibit neurotoxicity has been studied extensively in various cell types and has implicated PAFR in initiation of PAF-mediated neuronal loss (Mori *et al*. 1996; Bennett *et al*. 1998; Perry *et al*. 1998). However, therapeutic application of PAFR antagonists has met with limited clinical success and it is not clear whether PAFR initiates or protects against PAF-induced toxicity. Over expression of PAFR inhibits tumor necrosis factor-α (TNFα) and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis (Southall *et al*. 2001). Furthermore, PAF degradation is enhanced significantly by receptor-dependent ligand internalization, indicating that receptor-dependent mechanisms are important for rapid removal of PAF from extracellular space and subsequent inactivation by intracellular PAF acetylhydrolases (Ohsima *et al*. 2002).

In the present study, we sought to determine whether PAF-mediated cell death can be elicited in cells lacking PAFR and to assess the role of receptor expression in regulating cytotoxicity. Using the PC12 cell model, we demonstrate that PAF dose-dependently triggers apoptosis at physiologically relevant concentrations and necrosis at artificially elevated concentrations of ligand in cells lacking PAFR mRNA or protein. Ectopic expression of PAFR protects PC12 cells from PAF-induced apoptotic loss but not from PAF-mediated necrosis. Together, these data suggest: (i) that PAF can elicit apoptosis at physiological concentrations independently of PAFR; (ii) that PAF-mediated cell lysis occurs when the concentrations of PAF or its metabolite *lyso*-PAF exceed critical micelle concentration; (iii) that PAFR expression is cytoprotective.

Materials and methods

Cell culture

The PC12 rat pheochromocytoma cell line, kindly provided by Dr Gerry Melino (University Tor Vergata, Italy), was cultured in Roswell Park Memorial Institute (RPMI) containing 10% horse serum (HS) and 5% newborn calf serum (NCS) at 37°C in a 5% CO_2/95% air atmosphere. As a negative control for PAFR expression, the promyelocytic human HL60 leukemia line (American Type Culture Collection (ATCC), Manassas, VA, USA) was cultured in RPMI containing 10% fetal calf serum (FCS). As a positive control for PAFR expression, HL60s were cultured in RPMI containing 10% FCS in the presence of 1.25% dimethyl sulfoxide (DMSO; Sigma, St Louis, MO, USA) for 5 days. Culture reagents were obtained from Invitrogen (Burlington, ON, Canada) except where indicated.

Transfection of PC12 cells with human PAFR-GPCR or empty vector

Cells were transfected with empty vector pcDNA3.1/GS or pcDNA3.1/GS containing the 1029 bp human PAFR (HPAFR) open reading frame tagged at the C-terminus with six histidine residues and the V5 epitope (Invitrogen). Transfections were performed using Transfast reagent (Promega, Madison, WI, USA). Stably-transfected clones were selected with 400 μg/mL zeocin (Invitrogen). Expanded transfectants were maintained in complete media containing 200 μg/mL zeocin.

RNA isolation

American Type Culture CollectionTotal RNA was isolated using Trizol reagent (Invitrogen). As a positive control for rat PAFR, whole brain RNA was prepared from Sprague Dawley rats, approximately 3 months of age (Charles Rivers, St Constant, QC, Canada). Rats were deeply anesthetized with sodium pentobarbitol and killed by decapitation. All animal manipulations were performed in compliance with approved institutional protocols and according to the strict ethical guidelines for animal experimentation established by the Canadian Institute of Health Research.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

Five micrograms of total RNA isolated was treated with RQ1-DNAse (Promega) and reverse transcribed with 10 pmol random hexamer primer (Promega) using Superscript II RT. PCR reactions were carried out using 25 pmol/primer (PAFR) or 10 pmol/primer (glyceraldehyde phosphate dehydrogenase, GAPDH). Reagents were from Invitrogen except where indicated. Primers used were: (i) 5′-CACTTATAACCGCTACCAGGCAG-3′ (sense) and 5′-AAGACAGTGCAGACCATCCACAG-3′ (antisense), defining a 381-bp amplicon of rat and human PAFR; (ii) 5′-GCATCCTACTTCCTCATCCT-3′ (sense) and 5′-ACTTCAGTGACCGTATCCGT-3′ (antisense), defining a 538-bp amplicon of human PAFR; (iii) 5′-TGGTGCTGAGTATGTCGTGGAGT-3′ (sense) and 5′-AGTCTTCTGAGTGGCAGTGATGG-3′ (antisense), defining a 292-bp amplicon of rat GAPDH. Controls included RT reactions carried out in the absence of enzyme to detect genomic DNA contamination in the RNA template and PCR reactions performed with no template or no primers to detect contamination and false priming, respectively. PCR products were size-fractionated by electrophoresis on a 1.2% (w/v) agarose gel, stained with ethidium bromide, and digitized under UV light using AlphaImager-1220 software (Alpha Innotech Corporation, San Leandre, CA, USA).

Northern analysis

Total RNA (30 μg) was formaldehyde-denatured, electrophoretically separated on a 1.2% agarose gel containing 0.2 M formaldehyde, and transferred to Hybond-N nylon membrane (Amersham/Pharmacia, Baie d'Urfé, QC, Canada) as described in (Bennett *et al*. 1994). Blots were hybridized at 50°C for 16 h in digoxigenin (DIG) Easy Hyb solution (Roche Molecular Biochemicals, Mississanga, ON, USA) to a 538-bp DIG-labeled cDNA of the human leukocyte PAFR gene in pCDM8 (kindly provided by Dr N. Gerard, Harvard Medical School) or a 292-bp DIG-labeled cDNA amplicon of the GAPDH gene in pGAPDH (Piechaczyk *et al*. 1984) (kindly provided by Dr H. C. Birnboim, University of Ottawa). Blots were washed 2 × 5 min at 65°C in 2 × SSC/0.1% sodium doedecyl

sulfate (SDS), 2 × 15 min at 65°C in 0.1 × SSC/0.1% SDS, 1 × 5 min at room temperature (22°C) in 1 × DIG washing solution (Roche), and 1 × 1 h in 1 × DIG blocking solution (Roche). Blots were incubated in anti-DIG-horse radish peroxidase (HRP) antibody (1 : 5000; Roche) diluted in 1 × DIG blocking solution for 1 h at room temperature, visualized using CDP-Star (Roche), and exposed to Biomax X-ray film (Kodak).

For radioactive northern analyses, polyA$^+$ mRNA was isolated from PC12 cell lysates and rat brain by binding to oligo(dT) columns using a Quick-Prep Kit (Amersham/Pharmacia). Northern analysis of 3 μg mRNA was carried out as described in (Bennett *et al*. 1994) using a [^{32}P]-labeled 1 kb *Not*I/*Hind*III fragment of the human leukocyte PAFR gene in pCDM8/PAFR or a [^{32}P]-labeled 1.5 kb *Pst*I fragment of the rat GAPDH gene in pGAPDH.

Western blot analysis

Total protein lysates were prepared from subconfluent cultures in RIPA buffer (10 mM phosphate buffer, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 30 μL/mL aprotinin, 1 mM sodium orthovanadate, 0.1 mg/mL phenylmethysulfonyl fluoride). Protein (30 μg) was separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions on 10% gels and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore Corporation, Bedford, MA, USA). Western analysis was performed using monoclonal anti-V5 (1 : 5000; Invitrogen) detected by horseradish peroxidase-conjugated goat antimouse IgG (1 : 2000; Jackson Immuno-Research, West Grove, PA, USA). Antibodies were diluted and membranes were blocked in 10 mM PBS containing 1% heat-denatured casein. Immunoreactive bands were visualized using SuperSignal West Pico chemiluminescent substrate (Pierce, Rockford, IL, USA) and exposed to Biomax X-ray film (Kodak). Densitometric analysis of replicate immunoblots was performed using AlphaImager-1220 software.

Treatments

PC12 cells (8800 cells/cm^2) were plated overnight in 6 cm diameter tissue culture plates (VWR, Montréal, Quebéc, Canada). For microscopic analyses, 8800 cells/cm^2 were plated in 10 cm-diameter plates containing four 22 cm^2 glass coverslips coated with 0.1% gelatin. Cells were treated for 24 h with EtOH (0.1% or 10%), PAF (10 nM–10 μM, Biomol), or *lyso*-PAF (10 nM–10 μM, Biomol) in RPMI containing 0.5% heat-inactivated HS (low serum media). In some experiments, DMSO (0.1%) or the PAF antagonists CV3988 (200 nM–20 μM, Biomol) and BN52021 (1 μM–100 μM, Biomol) were added to cells 10 min before treatment with EtOH (0.1%) or PAF (1 μM).

Cell death assays

DNA fragmentation was determined by terminal deoxytransferase (TdT) dUTP nick-end labeling (TUNEL) of cultures fixed for 20 min in 4% paraformaldehyde in 10 mM PBS (10 mM sodium phosphate buffer, pH 7.5, 154 mM NaCl) as described in Bennett *et al*. (1998). Negative controls included sections incubated with FITC-labeled dUTP in the absence of TdT. Oligonucleosomal fragmentation was confirmed using the Cell Death ELISA kit according to the protocol provided by the manufacturer (Roche). Morphological evidence of apoptotic loss was provided by assessment of Hoechst 33258-stained nuclei. Cells, processed for TUNEL, were double-labeled with Hoechst 33258 (0.2 μg/mL) for 20 min at room temperature. To differentiate between apoptosis and necrosis, cultures were incubated with ethidium bromide homodimer (0.75 μM) prior to fixation and TUNEL processing. Ethidium bromide homodimer is a membrane impermeant DNA-binding dye that can enter cells only if the plasma membrane has been compromised or artificially permeabilized. Positive necrotic controls were induced by treatment with 10% EtOH. Cell survival was also assessed by hemocytometer cell counts of Trypan Blue-excluding cells.

Statistical analysis

Data were analyzed using one-way factorial analysis of variance (ANOVA) tests or unpaired Student's *t*-tests, as applicable. Following detection of a statistically significant *F*-value, post hoc Dunnett's *t*-tests were used to identify which treatment condition differed statistically from control. *p*-values of < 0.05 were considered statistically significant (shown as * or †); *p*-values of < 0.01 were considered highly statistically significant (shown as ** or ††).

Results

PC12 cells do not express PAFR

To establish whether PC12 cells express PAFR transcript, we performed northern analysis of total RNA using a DIG-labelled cDNA encompassing 581 bp of the human PAFR open reading frame. This probe is 80% homologous to rat PAFR. The 4-kb PAFR transcript was not detected in PC12 cells (Fig. 1a, left panel, PC12 lane), or the negative control (Fig. 1a, right panel, HL60 lane) but could be detected in the positive control (Fig. 1a, right panel, DMSO-differentiated HL60 lane). To increase sensitivity, northern analysis of polyA$^+$ mRNA was performed using a ^{32}P-labelled 1-kb human PAFR cDNA probe encompassing the entire PAFR open reading frame. Transcript was detected in rat brain (positive control) but not PC12 cells (Fig. 1b). To definitively establish lack of PAFR transcript in PC12 cells, RT-PCR analysis was performed using primers that amplify a 381-bp fragment of human and rat PAFR. Amplicon was not detected in PC12 cells (PC12 lane, Fig. 1c) but was detected in rat brain (Rat Brain lane, Fig. 1c) and in PC12 cells transfected with human PAFR (HPAFR-3 lane, Fig. 1c). The same random-primed RT products were amplified for GAPDH to demonstrate template integrity (Fig. 1c).

Characterization of stable HPAFR and empty vector transfectants

PC12 cells were transfected with pcDNA3.1/GS/PAFR or pcDNA 3.1/GS generating stable clonal lines expressing human PAFR tagged at the C-terminus with V5 epitope or empty vector. Expression of the 1-kb transgene was verified by northern analysis (Fig. 1a, HPAFR-7, HPAFR-4 lanes) and by RT-PCR (Fig. 1c, HPAFR-3 lane). Western analysis detected a single 68-kDa single band in transfectants but not PC12 cells (Fig. 2). This size is consistent with previous reports (Muller *et al*. 1993; Garcia Rodriguez *et al*. 1995; Ishii *et al*. 1998; Tiemann *et al*. 2001).

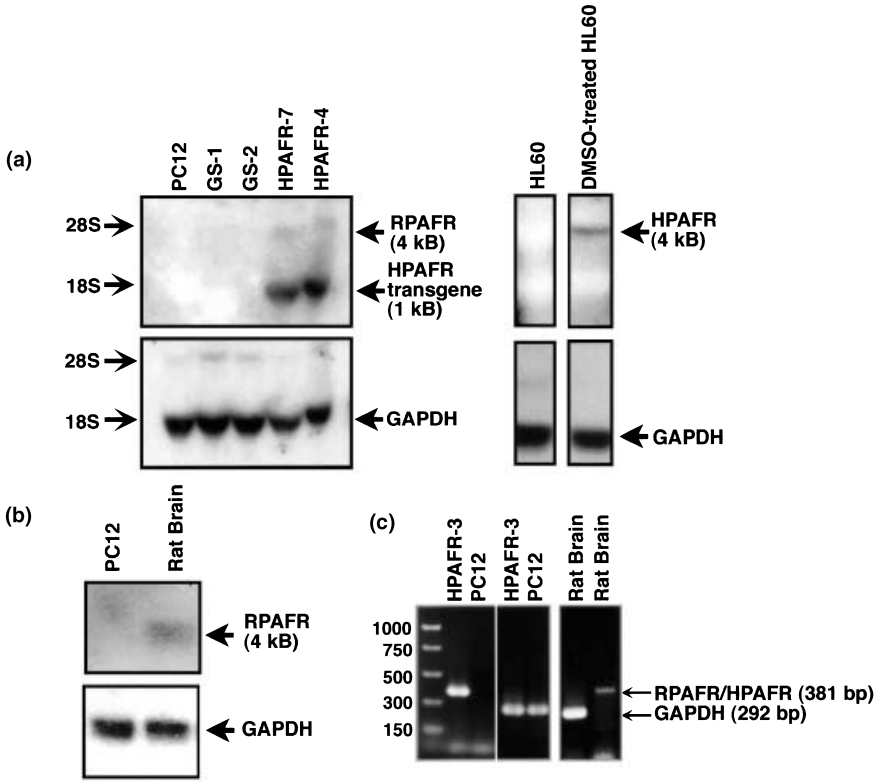

Fig. 1 PC12 cells do not express PAFR-GPCR. (a) Non-radioactive northern analysis of total RNA (30 μg) detected the expected 4 kb PAFR transcript in the positive control (right panel, DMSO-treated HL60 lane) and the 1 kb PAFR transgene in PC12 cells transfected with HPAFR (left panel, HPAFR-7, HPAFR-4 lanes) but not in PC12 cells (left panel, PC12 lane), empty vector transfectants (left panel, GS-1 and GS-2 lanes), or the negative control (right panel, HL60 lane). Hybridization to a GAPDH probe was used as a loading control. (b) To increase sensitivity, radioactive analysis of polyA$^+$ mRNA detected PAFR in rodent cerebrum (Rat brain lane) but not PC12 cells (PC12 lane). (c) RT-PCR analysis confirmed that PC12 cells do not express PAFR (PC12 lane). Amplicon is detected in HPAFR transfectants (HPAFR-3 lane) and in the same-species rat brain control (Rat brain lane) but not PC12 cells (PC12-lane). Random-primed cDNA was amplified with primers defining a 381-bp PAFR amplicon or a 292-bp GAPDH amplicon (internal control).

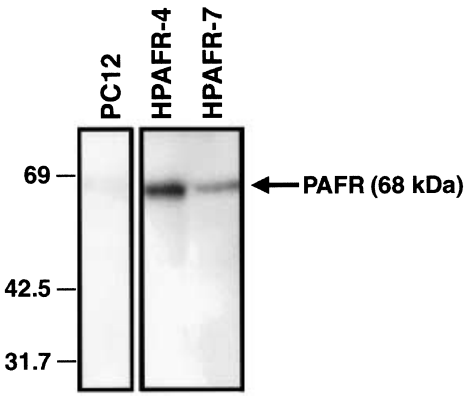

Fig. 2 HPAFR transfectants express the 68 kDa PAFR protein. Protein (30 μg) was separated by SDS-PAGE and immunoblotted using an anti-V5 antibody as described in Materials and methods.

PAF is cytotoxic to PC12 cells

To determine whether PAF triggers cell death, PC12 cells were exposed to ligand for 24 h (Fig. 3). A dose-dependent decrease in cell number was observed following administration of 100 nM, 1 μM and 10 μM PAF relative to vehicle (EtOH, 0.1%) (Fig. 3a). Cell loss increased when cells were plated at low cell densities (900 cells/cm^2) with a 53% reduction in cell number observed after 24 h of PAF (1 μM) treatment compared to a 24% cell loss when cultures were plated at higher cell densities (Fig. 3b). No difference in cytotoxicity was observed in cultures plated at densities of 1800–8800 cells/cm^2 (Fig. 3b).

To test whether PAF-induced cell loss could be blocked by PAF antagonists, cultures were treated with the neuroprotective PAF antagonist BN52021 (ginkgolide B) or with the structural PAF analog CV3988. Treatment with CV3988 (0.2–20 μM) and BN52021 (1–100 μM) in the presence of vehicle (0.1% EtOH) had no effect on cell survival (Fig. 3c, Vehicle). Cell death induced by 1 μM PAF could be inhibited by BN52021 (1–10 μM) but not by BN52521 (100 μM) or by CV3988 (0.2–20 μM) (Fig. 3c, PAF). BN52021 (1–100 μM) had no effect on PAF-mediated cytotoxicity at higher PAF concentrations (10 μM) (Fig. 3d).

PAF (1 μM) elicits apoptosis

Nuclear condensation, DNA strand breaks, and oligonucleosomal DNA fragmentation were assessed 24 h after PAF administration. Cells were double-labeled with TUNEL and Hoechst 33258. Nuclei of PAF (1 μM)-treated PC12 cells were smaller and more brightly stained (Fig. 4c) compared to vehicle (0.1% EtOH)-treated cells (Fig. 4a), characteristics consistent with apoptotic nuclear and chromatin condensation. Rare TUNEL-positive cells were detected in vehicle-treated cultures (arrow, Fig. 4b). Multiple TUNEL-positive cells were observed in PAF-treated cultures (Fig. 4d). Because TUNEL can detect both apoptotic oligonucleosomal fragmentation and random DNA damage associated with necrotic loss, the

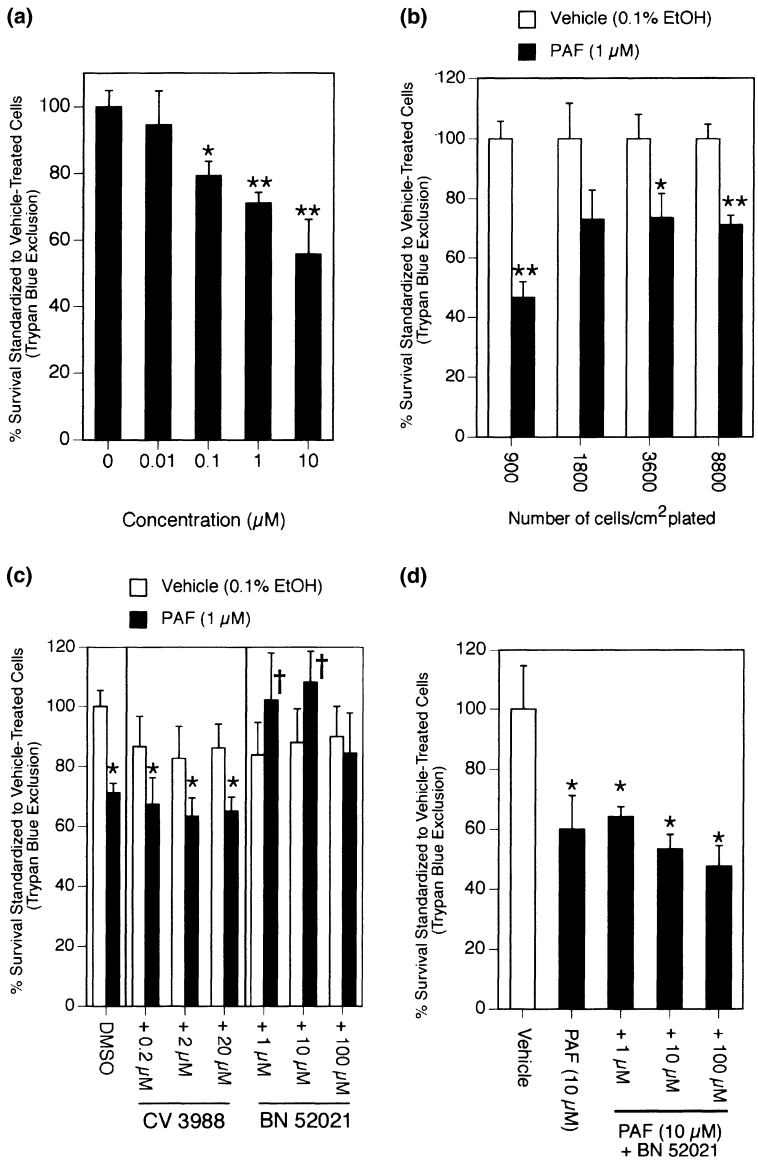

Fig. 3 PAF dose-dependently elicits PC12 death. (a) PC12 cells plated at 8800 cells/cm^2 were treated for 24 h with vehicle (0.1% ETOH, 0 lane) or PAF (0.01–10 μM) in low serum media. A dose-dependent decrease in cell number was observed after 24 h of treatment (*$p < 0.05$, **$p < 0.01$, ANOVA, *post-hoc* Dunnett's *t*-test). Data are expressed as per cent survival of vehicle-treated cultures assessed by hemocytometer counts of Trypan Blue excluding cells. (b) An increased susceptibility to PAF (1 μM)-induced cell death was observed when cells were plated at low cell densities (900 cells/cm^2). No difference in cytotoxicity was detected in cultures plated at 1800–8800 cells/cm^2. (*$p < 0.05$, **$p < 0.01$, Student's *t*-test). (c) PC12 cells were pre-treated with the PAF antagonist BN52021 or CV3988 before addition of vehicle (0.1% EtOH) or PAF (1 μM). PAF antagonists had no effect on vehicle-treated cell viability. A significant decrease in cell viability was detected after PAF or PAF + CV3988 treatment compared to vehicle (* indicates a significant decrease in viability relative to vehicle-treated cells, $p < 0.05$, ANOVA, *post-hoc* Dunnett's *t*-test). PAF-mediated cell death was inhibited by the PAF antagonist BN52021 (1–10 μM) († indicates a significant increase in survival relative to PAF-treated cells, $p < 0.05$, ANOVA, *post-hoc* Dunnett's *t*-test). (d) BN52021 did not inhibit cell death induced by higher concentrations of PAF (* indicates a significant decrease in viability relative to vehicle-treated cells $p < 0.05$ ANOVA, *post-hoc* Dunnett's *t*-test). Results are reported as mean ± standard error of measurement (SEM) of $n = 5$–42 cultures per data point.

morphology of nuclei double-labeled with Hoechst and TUNEL was examined in more detail. Condensation of damaged chromatin along the nuclear periphery (asterisks, Figs 4c and d), nuclear blebbing (arrows, Figs 4c and d), and nuclear fragmentation into apoptotic bodies (arrowhead, Figs 4c and d) were detected in PAF-treated cultures.

To quantify apoptotic DNA damage triggered by PAF, histone-associated DNA fragments were measured using a

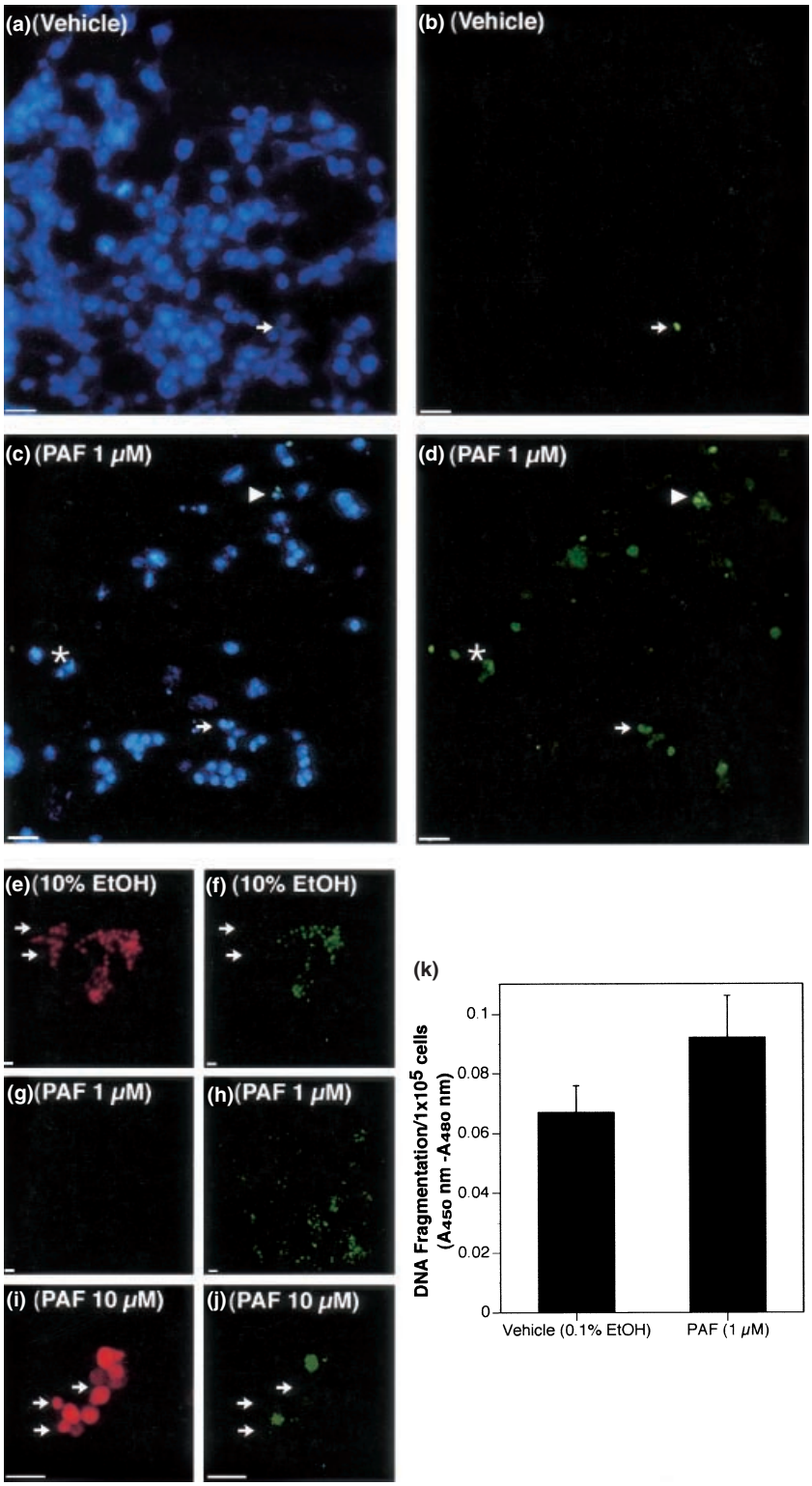

Fig. 4 PAF elicits nuclear condensation, DNA strand breaks, and oligonucleosomal DNA fragmentation in PC12 cells. PC12 cells plated at 8800 cells/cm^2 were treated for 24 h with (a and b) vehicle (0.1% ETOH) (c, d, g and h) PAF 1 μM (e and f) 10% EtOH, or (g and h) PAF 10 μM in low serum media. In (a)–(d), cells were double-labeled with Hoechst 33258 and TUNEL. TUNEL-positive cells in vehicle-treated cultures are indicated by arrows (a and b). An increase in the number of TUNEL-positive cells was detected in PAF-treated cultures (c and d). In addition to TUNEL, PAF-treated cells exhibited condensation of chromatin along the nuclear periphery (asterisks, c and d), nuclear blebbing (arrows, c and d), and apoptotic bodies (arrowhead, c and d). To determine whether DNA damage precedes loss of plasma and nuclear membrane integrity, cultures were stained with ethidium bromide homodimer prior to fixation and TUNEL processing. As a positive control for necrotic death, cultures were treated for 24 h with 10% EtOH (e and f). Arrows indicate cells in which plasma membrane breakdown preceded DNA damage. The vast majority of PAF (1 μM)-treated cells were TUNEL-positive (h) and ethidium bromide-negative (g). Necrosis was detected in cultures treated with higher concentrations of PAF (10 μM) (arrows, I and j). (k) Oligonucleosomal DNA fragmentation was quantified using a commercial ELISA protocol as described in Materials and methods. Data represent mean ± SEM of a single experiment conducted in triplicate.

commercial enzyme-linked immunoabsorbent assay (ELISA) (Fig. 4k). A 37% increase in oligonucleosomal fragmentation was detected following PAF (1 μM) treatment relative to vehicle (0.1% EtOH) treatment (Fig. 4k).

PAF (10 μM) elicits necrotic cell lysis

To further differentiate between apoptotic and necrotic cell loss, cultures were stained with ethidium bromide homodimer before processing for TUNEL. Ethidium bromide

homodimer is a membrane-impermeant dye that can only enter cells if the plasma membrane has been compromised. Apoptotic death is indicated when DNA fragmentation (TUNEL) precedes loss of plasma membrane integrity (ethidium bromide staining). Necrosis is indicated when ethidium bromide staining precedes TUNEL. As a positive control for necrosis, cultures were treated for 24 h with 10% EtOH (Figs 4e and f). In many cells, ethidium bromide-positive cells (Fig. 4f, arrows) were TUNEL-negative (Fig. 4e, arrows) – evidence that a breakdown in membrane integrity preceded DNA damage. By contrast, TUNEL-positive PAF (1 μM)-treated cells (Fig. 4h) did not stain with ethidium bromide (Fig. 4g) – a phenotype consistent with apoptotic death. When cells were exposed to higher concentrations of PAF (10 μM), damage to cell membranes indicated by ethidium bromide permeability (Fig. 4i, arrows) preceded DNA fragmentation in multiple cells (Fig. 4j, arrows). These data indicate that cytotoxicity observed following treatment with PAF (1 μm) is apoptotic in nature while higher concentrations of PAF (10 μM) exert lytic effects on the plasma membrane resulting in necrosis.

PAF-induced necrosis but not apoptosis can be elicited by downstream metabolites

In addition to PAFR-mediated signaling, biological activity can also be elicited by downstream PAF metabolites. To test the specificity of the apoptotic and necrotic responses, PC12 cells were treated with *lyso*-PAF, the immediate metabolite of PAF (Fig. 5). *Lyso*-PAF had no effect on the survival of PC12 cells when administered at concentrations below 10 μM (Fig. 5). At 10 μM, *lyso*-PAF was cytotoxic (Fig. 5) and caused necrosis as assessed by TUNEL and ethidium bromide homodimer double-labeling (data not shown).

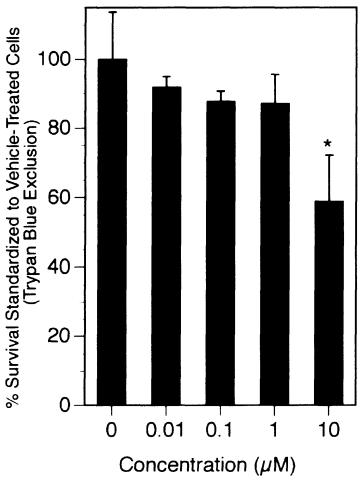

Fig. 5 PAF-induced cell death is not mediated by downstream metabolites. PC12 cells, plated at 8800 cells/cm^2, were treated for 24 h with *Lyso*-PAF (0.01–10 μM). *Lyso*-PAF had no effect on PC12 cell survival at concentrations below 10 μM. Data are expressed as per cent survival of vehicle-treated cultures assessed by hemocytometer counts of Trypan Blue excluding cells. Results are reported as mean ± SEM of $n = 5$ cultures per data point. Details are as described in Materials and methods. (*$p < 0.05$, ANOVA, *post-hoc* Dunnett's-test).

Ectopic expression of PAFR protects cells from PAF-induced apoptosis

To determine whether PAFR expression regulates cell loss, the empty vector transfectant GS-2 and the PAFR transfectant HPAFR-7 were treated for 24 h in low serum media with PAF (1 μM). As with wild-type PC12 cells (Fig. 3), PAF elicited apoptotic loss of GS-2 cells (Fig. 6). Ectopic expression of HPAFR was shown to protect cells from PAF (1 μM)-induced cell death (Fig. 6). HPAFR-mediated protection could be blocked by pre-treatment with the PAF antagonist CV 3988 (10 μM) (Fig. 6).

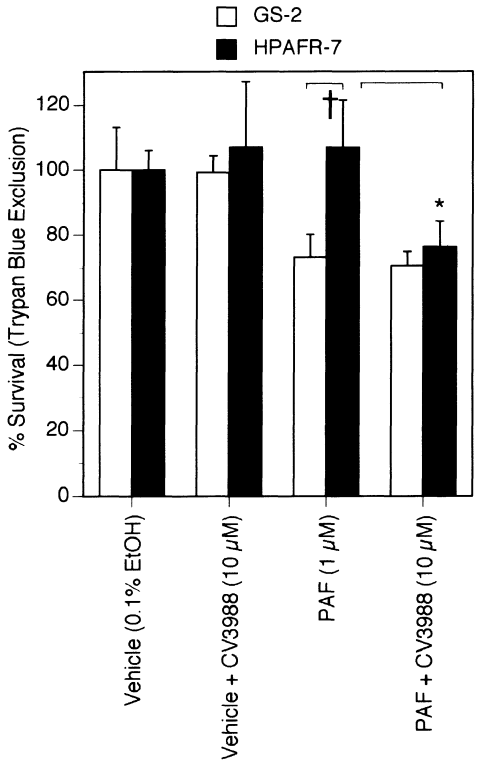

Fig. 6 Expression of PAFR-GPCR protects cells from PAF-induced apoptosis. Stable empty vector (GS-2) or PAFR-GPCR (HPAFR-7) transfectants were treated for 24 h with vehicle (0.1% EtOH) or PAF (1 μM) in low serum media. Data are expressed as per cent survival of vehicle-treated cultures as assessed by hemocytometer counts of Trypan Blue excluding cells. Results are reported as mean ± SEM of $n = 7$ cultures per data point. († indicates a significant increase in survival relative to PAF-treated empty vector transfectants, * indicates a significant decrease in survival of HPAFR-7 transfectants treated with CV3988, $p < 0.05$, Student's *t*-test). CV3988 had no effect on the viability of vehicle-treated HPAFR-7 or GS-2 cultures.

Discussion

This study provides evidence that PAF can elicit apoptotic and necrotic death in cells that do not express the PAF G-protein coupled receptor. Necrosis is observed when concentrations of PAF or its immediate metabolite *lyso*-PAF exceed their critical micelle concentrations and exert lytic detergent effects on cell membranes. Apoptosis is observed at physiologically relevant concentrations of PAF, is not initiated by *lyso*-PAF, and, in absence of PAFR, can be inhibited by BN52021 (ginkgolide B), a PAF antagonist that is not structurally related to PAF. Ectopic expression of PAFR blocks PAF-induced apoptosis and receptor-mediated cell survival can be pharmacologically inhibited by the structurally related PAF antagonist CV3988. Together, these results suggest that PAF can trigger apoptotic cell loss independently of its G-protein-coupled receptor and that PAFR activation protects PC12 cells from PAF-mediated apoptosis.

These data are important in light of one of the proposed mechanisms for HIV-dementia. It is hypothesized that neuronal loss is exacerbated by protracted exposure to pro-inflammatory agents, specifically PAF, synthesized and secreted by productively-infected brain-resident macrophages/microglia (Gelbard *et al*. 1994; Perry *et al*. 1998; Tong *et al*. 2001). There are two PAFR transcripts, driven by different promoters, generated by alternative splicing of the 5′-non-coding exons 1 and 2 to a common acceptor site on exon 3 (Mutoh *et al*. 1993). Both transcripts code for the same PAFR protein (Ishii and Shimizu 2000). Transcript 1 (or the leukocyte-type transcript) is expressed by leukocytes, eosinophils, monocytes, and microglia (Mutoh *et al*. 1993; Pang *et al*. 1995; Kotelevets *et al*. 1998). Consensus sequences for nuclear factor (NF)-κB, SP-1, and initiator sequence mediate up-regulation by inflammatory stimuli including PAF (Mutoh *et al*. 1993; Ishii and Shimizu 2000). Our data indicate that PAFR activation can protect cells from apoptotic loss and it is plausible that activated microglia are able to resist the toxic actions of PAF through PAFR up-regulation. This hypothesis is consistent with the recent findings that PAFR can inhibit TRAIL and TNFα-induced apoptosis in a NF-κB-dependent process and that PAFR-expressing macrophages can inactivate PAF more rapidly than macrophages cultured from PAFR-deficient mice (Southall *et al*. 2001; Ohsima *et al*. 2002). Conversely, neurons express PAFR transcript 2 (or the tissue-type transcript). Transcript 2 lacks NF-κB consensus sequences and is not up-regulated by inflammatory stimuli (Mutoh *et al*. 1993; Pang *et al*. 1995; Kotelevets *et al*. 1998). Thus, neurons may be more susceptible to PAF-mediated apoptosis following HIV-infection, ischemic injury, or epileptic seizure because they lack the ability to regulate toxicity by increasing anti-apoptotic PAFR expression.

To understand how PAF elicits cytotoxic effects on PC12 cells, we examined morphological, biochemical, and molecular indices of apoptosis and necrosis. At concentrations of 100 nM to 1 μM, PAF induces nuclear and chromatin condensation, DNA strand breaks, oligonucleosomal DNA fragmentation, and nuclear disintegration into apoptotic bodies. At equimolar concentrations, *lyso*-PAF, the immediate metabolite of PAF, does not affect PC12 viability. These findings suggest that PAF-mediated apoptosis is not elicited by downstream lipid metabolites. Although we have yet to determine how cell death is initiated by PAF in the absence of PAFR, the differential effects of two PAF antagonists, BN52021 and CV3988, on PC12 survival are intriguing. BN52021, (ginkgolide B) is a terpenoid derived from Ginkgo biloba leaves that inhibits PAF-induced glutamate release and protects neurons from excitotoxic damage following ischemia-reperfusion injury (Clark *et al*. 1992; Langley *et al*. 1999; Yoshikawa *et al*. 1999). In addition to interacting with synaptosomal PAF binding sites (Marcheselli *et al*. 1990), BN52021 is also a potent antioxidant (Westman *et al*. 2000). Given that PAF triggers downstream oxyradical production (Peplow 1999) and that reactive oxygen species have repeatedly been implicated in apoptotic signaling (Carmody and Cotter 2001), it is plausible that the antioxidant properties of BN52021 protect against PAF-mediated cell death. Another possibility is that PAF-mediated apoptosis is initiated by a second PAFR. This hypothesis is strengthened by the finding that BN52021 blocks PAF-mediated apoptosis at concentrations below but not including 100 μM, a concentration with proven antioxidant effects (Ahlemeyer *et al*. 1999; Westman *et al*. 2000). Three PAF binding sites have been identified pharmacologically (Marcheselli *et al*. 1990). One synaptosomal site localizes to plasma membrane (Marcheselli *et al*. 1990). Two high affinity binding sites localize to microsomal membranes (Marcheselli *et al*. 1990). The molecular identity of these binding sites has yet to be determined although it is likely that one of the synaptosomal sites is PAFR. It is interesting that, in contrast to BN52021, CV3988, a PAF analog that shares the glycerol backbone with PAF, failed to inhibit PAF-mediated apoptosis in PC12 cells despite administration at concentrations up to 20 times that of its reported IC_{50} antagonist activities (Valone 1985; Nunez *et al*. 1986; Terashita *et al*. 1987). However, CV3988 effectively blocked the anti-apoptotic actions of PAFR activation in keeping with its affinity for synaptosomal PAF binding sites. These data suggest that it may be possible to reduce phagocytic activation and production of neurotoxic inflammatory mediators in pathological conditions using antagonists capable of blocking PAF/PAFR interaction while inhibiting PAF-mediated neuronal death using antagonists that exhibit anti-apoptotic activity. A reassessment of the relative apoptotic protection activity relative to PAFR binding affinity of known PAF antagonists would therefore be important to test this hypothesis. We believe that the PC12 model characterized in this study should prove useful in this respect.

Finally, to establish whether higher concentrations of PAF (> 1 μM) exert physicochemical effects on the plasma

membrane, we evaluated whether a breakdown in plasma membrane precedes DNA damage in PAF-treated PC12 cells. Membrane permeability characteristic of necrosis was apparent only when PC12 cells were treated with 10 μM PAF or equimolar concentrations of its immediate metabolite *lyso*-PAF. Necrotic loss was not inhibited by PAF antagonists. These findings are consistent with non-specific lytic detergent actions on the plasma membrane observed when phospholipid levels exceed their critical micelle concentration. The critical micelle concentration of PAF is estimated to be 2.5–3 μM (Blank *et al*. 1981).

In summary, the present results demonstrate that PAF can elicit apoptotic death in PC12 cells independently of PAFR at physiological concentrations, and necrotic death when lipid levels exceed their critical micelle concentration. Furthermore, we found that ectopic expression of PAFR protected PC12 cells from PAF-mediated apoptosis suggesting that receptor activation is cytoprotective. Further studies are required to elucidate the downstream signaling pathways triggered by PAF in PC12 cells and to characterize the relative anti-apoptotic activity of existing PAF antagonists. However, these data support the growing consensus that targeting PAF signaling may reduce the rate of cell death during chronic cerebral inflammation (Bazan *et al*. 1995; Bazan 1998; Birkle *et al*. 1998; Perry *et al*. 1998; Langley *et al*. 1999; Schifitto *et al*. 1999) and demonstrate, for the first time, that PAF can elicit apoptotic death in cells lacking PAFR.

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