Journal of Neurochemistry, 2007, **103**, 88–97 doi:10.1111/j.1471-4159.2007.04740.x

Platelet activating factor-induced neuronal apoptosis is initiated independently of its G-protein coupled PAF receptor and is inhibited by the benzoate orsellinic acid

Scott D. Ryan,*,[1] Cory S. Harris,*,†,[1] Fan Mo,* Haemi Lee,*,‡ Sheng T. Hou,‡ Nicolas G. Bazan,§ Pierre S. Haddad,¶ John T. Arnason† and Steffany A. L. Bennett*

**Department of Biochemistry, Microbiology, and Immunology, Neural Regeneration Laboratory and Ottawa Institute of Systems Biology, University of Ottawa, Ottawa, Ontario, Canada*

†*Department of Biology, University of Ottawa, Ottawa, Ontario, Canada*

‡*Experimental Therapeutics Laboratory, Institute for Biological Sciences, National Research Council of Canada, Ottawa, Ontario, Canada*

§*Neuroscience Center of Excellence, Louisiana State University, New Orleans, Louisiana, USA*

¶*Department of Pharmacology, Université de Montréal, Montréal, Quebec, Canada*

Abstract

The bioactive lipid mediator platelet activating factor (PAF) is recognized as a key effecter of neuronal apoptosis, yet it is not clear whether its G-protein coupled receptor (PAFR) initiates or prevents PAF neurotoxicity. Using PAFR–/– and congenic wild-type mice, we show that PAF triggers caspase-3/7 activity and neuronal death in PAFR–/– but not PAFR+/+ cerebellar granule neurons. Restoring receptor expression by recombinant adenoviral infection protected cells from PAF challenge. Neuronal death was not mediated by nitric oxide or *N*-methyl-D-aspartate receptor signaling given that *N*-nitro-L-arginine methyl ester and MK-801 did not inhibit PAF-induced neuronal loss in PAFR–/– neurons. To intervene in PAFR-independent neurotoxicity, the anti-apoptotic actions of three structurally distinct PAF antagonists were compared to a panel of plant and fungal benzoic acid derivatives. We found that the PAF antagonist BN 52021 but not FR 49175 or CV 3988 inhibited PAFR-independent neurotoxicity. Orsellinic acid, a fungal-derived benzoic acid, blocked PAF-mediated neuronal apoptosis without affecting PAFR-mediated neuroprotection. These findings demonstrate that PAF can transduce apoptotic death in primary neurons independently of its G-protein coupled receptor, that PAFR activation is neuroprotective, and that orsellinic acid effectively attenuates PAFR-independent neuronal apoptosis.

Keywords: apoptosis, benzoic acids, neurotoxicity, orsellinic acid, platelet activating factor, platelet activating factor receptor.

J. Neurochem. (2007) **103**, 88–97.

The platelet activating factor (PAF: 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) family of glycerophospholipids exerts potent pro-inflammatory and neuromodulatory effects in the CNS (Prescott *et al.* 2000). Under normal physiological conditions, PAF participates in long-term potentiation associated with learning and memory through stimulation of its G-protein coupled receptor (PAFR) (Izquierdo *et al.* 1995; Teather *et al.* 1998; Kato 1999; Chen *et al.* 2001). When concentrations are elevated under pathological conditions, PAF becomes neurotoxic and has been identified as a key mediator of neuronal death following ischemia, encephalitis, epileptic seizure, meningitis, and HIV-1 demen-

Received March 21, 2007; revised manuscript received May 3, 2007; accepted May 4, 2007.

Address correspondence and reprint requests to Steffany A. L. Bennett, Department of Biochemistry, Microbiology and Immunology, Neural Regeneration Laboratory, University of Ottawa, 451 Smyth Road, Ottawa, ON K1H 8M5, Canada. E-mail: sbennet@uottawa.ca

[1]Both authors contributed equally to this work.

Abbreviations used: BSA, bovine serum albumin; CGNs, cerebellar granule cells; DMSO, dimethylsulfoxide; EGFP, enhanced green fluorescent protein; ET, ethidium homodimer; L-NAME, *N*-nitro-L-arginine methyl ester; NMDA, *N*-methyl-D-aspartate; NO, nitric oxide; PAF, platelet activating factor; PAF-AH, PAF-acetylhydrolase; PAFR, platelet activating factor receptor; PARP, poly(ADP ribose) polymerase.

tia (Birkle *et al.* 1998; Perry *et al.* 1998; Bazan *et al.* 2002; Farooqui *et al.* 2006).

Targeting PAF neurotoxicity is complicated by the fact that bioactivity can be signaled through both PAFR-dependent and PAFR-independent pathways. In non-neuronal cells, PAFR expression exacerbates cell death initiated by etoposide and mitomycin C yet protects cells from tumor necrosis factor α, tumour necrosis factor-related apoptosis inducing ligand (TRAIL), and extracellular PAF exposure (Southall *et al.* 2001; Brewer *et al.* 2002; Li *et al.* 2003). PAF also interacts with intracellular binding sites (Marcheselli *et al.* 1990; Bratton *et al.* 1992; Sapir *et al.* 1997) initiating caspase-3-dependent DNA fragmentation in the absence of PAFR (Bonin *et al.* 2004). Although cyotoxic signaling can be reversed by ectopic PAFR expression (Brewer *et al.* 2002), in neurons, it is not known whether PAF triggers apoptosis through its G-protein coupled receptor or whether PAFR activation is neuroprotective.

To address this issue, cerebellar granule cells (CGNs) cultured from PAFR+/+ and PAFR−/− mice were treated with apoptotic concentrations of PAF in the absence of serum PAF-acetylhydrolases (PAF-AHs). Here, we show that PAF elicits caspase-dependent neuronal apoptosis in the absence of PAFR, that endogenous PAFR expression protects cells from PAF neurotoxicity, and that ectopic PAFR expression confers neuroprotection. To intervene in this death pathway without affecting PAFR-mediated neuroprotection, we used a 96-well screening approach to identify anti-apoptotic PAF inhibitors. We found that the non-competitive PAFR antagonist BN 52021 but not the PAF inhibitor FR 49175 or the competitive PAFR antagonist CV 3988 prevented PAF-induced neuronal apoptosis. Within a panel of naturally occurring benzoic acids, we identified a novel PAF inhibitor. Orsellinic acid, a fungal-derived benzoic acid derivative, selectively blocked PAF-induced neuronal apoptosis without inhibiting PAFR-mediated neuronal survival. Together, these data highlight a new anti-apoptotic role for PAFR signaling and a novel pharmacological means of inhibiting PAF-induced neuronal apoptosis.

Materials and methods

Reagents

All cell culture reagents were obtained from Invitrogen (Burlington, ON, Canada) and all chemicals were purchased through Sigma-Aldrich (St Louis, MO, USA) unless otherwise stated. Pure benzoic acid derivatives (>95% purity as determined by HPLC analysis) were obtained from the phytochemical collections of C. Nozzilillo (University of Ottawa, Canada) and J. T. Arnason (University of Ottawa, Canada). Cultures were treated with a final concentration of 1 μg/mL based on previous dose-response studies (C. Harris, J. T. Arnason and S. A. L. Bennett, unpublished data). Cells were also treated with the *N*-methyl-D-aspartate (NMDA) receptor antagonist MK-801 [(+)-5-methyl-10,11- dihydro-5*H*-dibenzo [a,d] cyclohepten-5,10-imine maleate, 10 μmol/L], *N*-nitro-L-arginine methyl ester (L-NAME, 5 mmol/L; Biomol Research Laboratories, Plymouth Meeting, PA, USA), or the PAF antagonists/inhibitors BN 52021 (21 μg/mL), FR 49175 (4 μg/mL), and CV 3988 (1 μg/mL) (Biomol Research Laboratories). Concentrations employed were based on previous reports (Malipiero *et al.* 1999; Brewer *et al.* 2002; Bonin *et al.* 2004; Hou *et al.* 2006). Benzoic acids and PAF inhibitors were dissolved in dimethylsulfoxide (DMSO). L-NAME and MK-801 were dissolved in EtOH. Cells were exposed to a final concentration of 0.1% vehicle. All treatments were performed in serum-free media as described below.

Congenic PAFR−/− and PAFR+/+ Mice

Breeding pairs of PAFR−/− mice in a hybrid C57BL/6J × 129/Ol background (Ishii *et al.* 1998) were the kind gift of Dr Takao Shimizu (University of Tokyo, Japan). To insure uniformity in genetic background, animals were backcrossed to C57BL/6 wild-type mice (Charles River Laboratories, Senneville, QC, Canada) for 10 generations. Congenic 10th generation PAFR+/+ and PAFR−/− colonies were established and maintained from PAFR+/− matings. Animals were genotyped as described by (Nagase *et al.* 1999).

Primary murine cell culture

Cerebellar granule cells were cultured from post-natal day 7–10 mice as previously described (Cregan *et al.* 2000). Briefly, cerebella were dissected, meninges removed, and tissue minced in ice-cold dissection solution [124 mmol/L NaCl, 5.37 mmol/L KCl, 1 mmol/L NaH_2PO_4, 1.2 mmol/L $MgSO_4$, 14.5 mmol/L D-glucose, 25 mmol/L HEPES, 3 mg/mL bovine serum albumin (BSA), pH.7.4]. Tissue was incubated in dissection solution containing 0.5 mg/mL trypsin (Sigma) at 37°C for 18 min. Trypsin was deactivated by addition of 0.52 mg/mL chicken egg white trypsin inhibitor and cells were pelleted. Cells were resuspended in dissection solution containing 0.75 mg/mL DNase I (Roche, Mississauga, ON, Canada) and triturated. $CaCl_2$ was added to a final concentration of 15 μmol/L. Following centrifugation, cells were plated in Eagle's minimum essential medium containing 25 mmol/L glucose, 10% fetal bovine serum, 1% gentamycin, 2 mmol/L L-glutamine, and 20 mmol/L KCl at 37°C in a 5% CO_2/95% air atmosphere. CGNs were seeded at a density of 2×10^5 cells/cm^2 in 96-well plates coated with laminin (20 μg/mL) and poly-D-lysine (100 mg/mL; Sigma-Aldrich) and maintained in this serum-containing media for 72 h before being exposed to PAF in serum-free media. Cultures were composed >90% neurons as established by immunocytochemistry (data not shown).

Cell survival assay

PAF (1-*O*-hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine; Biomol Research Laboratories) and *lyso*-PAF (1-*O*-hexadecyl-*sn*-glycero-3-phosphocholine; Biomol Research Laboratories) were dissolved in treatment media (Eagle's minimum essential medium containing 25 mmol/L glucose, 0.025% BSA, 1% gentamycin, 2 mmol/L L-glutamine, and 20 mmol/L KCl). CGNs were treated for 24 h with 1 μmol/L PAF, 1 μmol/L *lyso*-PAF or vehicle (treatment media alone). Note that all treatments were performed in serum-free media containing 0.025% BSA to ensure that cultures were not exposed to plasma PAF-AH. Where treatment with L-NAME, MK-801, test compounds, or respective vehicles (0.1%) are indicated, cultures

were pre-treated for 15 min prior to addition of PAF. CGN survival was assessed by Live/Dead viability/cytotoxicity assay (Invitrogen). Viable cells were identified by the enzymatic conversion by intracellular esterases of non-fluorescent calcein-AM to fluorescent calcein. Dead cells were identified by uptake of ethidium homodimer (ET) as a result of loss of membrane integrity. Cells were imaged using a DMIR epifluorescent inverted microscope (Leica, Richmond Hill, ON, Canada) equipped with a QICAM digital camera (Quorum Technologies, Guelph, ON, Canada) and captured using OpenLab software v5.05 (Improvision, Lexington, MA, USA). Percent survival was calculated as viable cell number$^{(\text{calcein}^+ - \text{calcein}^+/\text{ET}^+)}$/mean number of viable cells in vehicle control$^{(\text{calcein}^+ - \text{calcein}^+/\text{ET}^+)} \times 100$.

Recombinant adenovirus infection

Recombinant pADTrack-CMV adenoviral vectors carrying a 1 kb Flag-tagged human PAFR cDNA excised from pCDM8PAFR (Kunz *et al.* 1992) kindly providing by Dr Norma Gerard (Harvard Medical School, USA) and enhanced green fluorescent protein (EGFP) under separate cytomegalovirus promoters were prepared using the AdEasy adenoviral vector system (QBiogene Inc., Irvine, CA, USA) and titered as we have described previously (Huang *et al.* 2005). Control adenovirus contained EGFP only. Recombinant adenoviruses were added to cell suspensions at the time of plating. All experiments were performed at a multiplicity of infection of 100 pfu/cells. Efficiency of infection of both vectors was comparable as established by counting $EGFP^+$ cells immediately before treatment. Cell survival in serum-free media following PAF (1 μmol/L) treatment was calculated as $EGFP^+$ cell number following treatment/mean $EGFP^+$ cell number in the vehicle control × 100.

Screening assay for PAF inhibitors

PC12-AC cells, a clonal derivative of the PC12 pheochromocytoma cell line derived in our laboratory, were cultured in Roswell Park Memorial Institute 1640 medium supplemented with 10% horse serum and 5% newborn calf serum and maintained at 37°C and 5% atmospheric CO_2 and seeded in 96-well plates (1.25×10^4 cells/well) for treatment. After plating, cells were treated for 24 h with PAF (1 μmol/L, 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) or PAF vehicle (0.1% EtOH) in the presence of each benzoic acid (1 μg/mL), PAF antagonist, or treatment compound vehicle (0.1% DMSO) in Roswell Park Memorial Institute 1640 medium supplemented with 0.025% BSA. All treatments were carried out in serum-free media. Negative controls included cultures incubated in treatment media only. WST (Roche Diagnostics) was added to each well at the end of the treatment period and incubated for 60 min before spectrophotometric analysis at 420 nm (formazan) and at 620 nm (reference). Cultures were blanked against cell-free treatment media incubated for the same period of time. Cell number per well was calculated from standard curves derived from wells containing known cell density. Percent viability was calculated as cell number$^{(\text{treatment well})}$/mean cell number$^{(\text{vehicle control})} \times 100$.

Caspase activation

Executioner caspase activation in CGNs was determined using CaspaTag (caspase-3/7) assay (Chemicon, Temecula, CA, USA). CGNs capable of cleaving FAM-DEVD-FMK to its fluorescent product were scored as positive and reported as a percentage of total cell number after a 24 h treatment with PAF (1 μmol/L) or vehicle (treatment media). Cells were imaged using a DMIR epifluorescent inverted microscope (Leica) equipped with a QICAM digital camera (Quorum Technologies) and captured using OpenLab software v5.05 (Improvision). Activation was confirmed by western analysis. Proteins were isolated in radioimmunoprecipitation buffer (10 mmol/L phosphate-buffered saline, 1% nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 30 μl/mL aprotinin, 10 μl/mL Na orthovanadate, 10 μl/mL phenylmethylsulfonyl fluoride, and 1 μl/mL NaF). Protein samples (30 μg) were separated by SDS–PAGE under reducing conditions. Western analyses were performed using monoclonal anti-poly(ADP ribose) polymerase (PARP) (1 : 10 000, Clonetec, Mountain View, CA, USA) and monoclonal anti-actin (1 : 1000, Sigma). Secondary antibodies were horseradish peroxidase-conjugated anti-mouse IgG (1 : 2000, Jackson Immunolabs, Westgrove, PA, USA). Immunoreactive bands were visualized using SuperSignal West Pico (MJS BioLynx Inc., Brockville, ON, Canada).

Statistical analysis

Data were analyzed using one-way factorial ANOVA tests followed by *post hoc* Dunnett's *t*-tests or unpaired Student's *t*-tests, as applicable. *p*-values under 0.05 were considered statistically significant (shown as *); *p*-values under 0.01 were considered highly significant (shown as **).

Table 1 Names and molecular weights of benzoic acids screened for PAF inhibitor activity

Common name	IUPAC name	Molecular Weight
Salicylic acid	2-Hydroxybenzoic acid	138.1
3-Hydroxybenzoic acid	3-Hydroxybenzoic acid	138.1
Resorcyclic acid	2,4-Dihydroxybenzoic acid	154.1
Gentisic acid	2,5-Dihydroxybenzoic acid	154.1
Protocatechuic acid	3,4-Dihydroxybenzoic acid	154.1
Vanillic acid	4-Hydroxy-3-methoxybenzoic acid	168.2
Orsellinic acid	4,6-Dihydroxy-2-methylbenzoic acid	168.2
Gallic acid	3,4,5-Trihydroxybenzoic acid	170.1
Veratric acid	3,4-Dimethoxybenzoic acid	182.2
Syringic acid	4-Hydroxy-3,5-dimethoxybenzoic acid	198.2
m-Digallic acid	3,4-Dihydroxy-5-[(3,4,5-trihydroxy-benzoyl)oxy]benzoic acid	322.2

Results

PAF triggers neuronal apoptosis in the absence of PAFR

To evaluate the role PAFR in PAF-mediated neuronal cell death, primary CGN cultures derived from PAFR+/+ and PAFR−/− mice were treated with 1 μmol/L PAF in treatment media devoid of serum. Cell survival was assessed by Live/Dead assay. PAFR+/+ neuronal viability was not affected by 24 h of PAF treatment (Fig. 1a and c). In PAFR−/− neurons, PAF reduced cell survival by 40% compared to vehicle controls (Fig. 1c and d). To establish PAF specificity, CGNs were treated with the immediate PAF metabolite, *lyso*-PAF. *Lyso*-PAF (1 μmol/L) did not alter PAFR+/+ or PAFR−/− neuronal viability demonstrating that PAF and not its metabolite was responsible for the observed effects (Fig. 1). In complementary gain of function studies, PAFR−/− CGNs were infected with a recombinant adenoviral vector expressing EGFP or EGFP and PAFR. PAF significantly reduced survival of $EGFP^{+}$/PAFR−/− cells (Fig. 2a and c) whereas ectopic expression of PAFR inhibited PAF-induced cell death (Fig. 2b and d). To establish whether PAF triggers apoptosis in null-mutant neurons, caspase 3/7 activity was evaluated. In PAFR+/+ neurons, the percentage of cells exhibiting activated caspase-3/7 was minimal and the basal rate of DEVD substrate cleavage was comparable between vehicle and PAF treatment (Fig. 3a and c). By contrast, in PAFR−/− neurons, a significant increase in caspase activation was observed after 24 h exposure to PAF (Fig. 3b and d).

Platelet activating factor-induced cortical neuron death has been reported to involve nitric oxide (NO) pathways and glutamate excitotoxicity (Xu and Tao 2004). To determine whether these mechanisms underlie PAFR-independent apoptosis in CGNs, PAFR−/− neurons were exposed to PAF (1 μmol/L) in the presence of the NO synthase inhibitor L-NAME (5 mmol/L) or the NMDA receptor antagonist MK-801 (10 μmol/L). These concentrations have been shown previously to inhibit NO and glutamate toxicity in primary CGNs (Malipiero *et al.* 1999; Hou *et al.* 2006). We found no effect of either inhibitor on PAF-induced toxicity, suggesting that these pathways are not activated by PAF in neurons lacking PAFR (Fig. 4).

Screening plant and fungal metabolites for inhibitors of PAF-mediated apoptosis

To identify novel compounds capable of inhibiting PAFR-independent neuronal apoptosis, test compounds were selected from a larger library of ∼100 natural products, pure phenolics, and fungal extracts (Martineau *et al.* 2006; Spoor *et al.* 2006). Compounds were prioritized based on previously

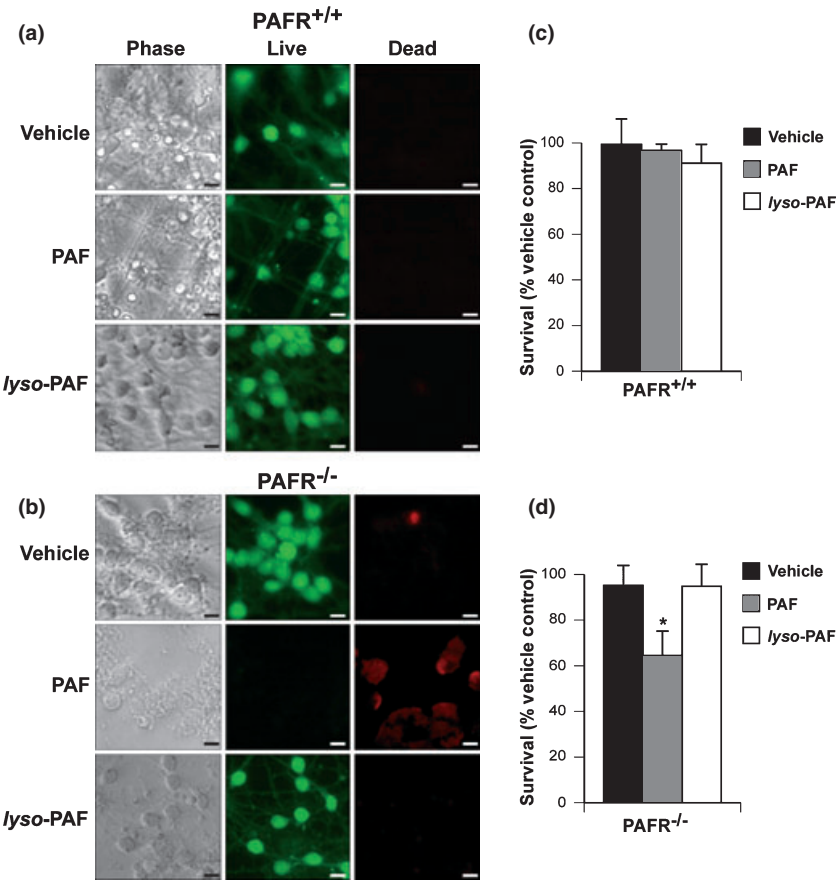

Fig. 1 PAF but not its immediate metabolite *lyso*-PAF triggers cell death in neurons that do not express PAFR. CGNs derived from PAFR+/+ and PAFR−/− mice were treated for 24 h with vehicle (serum-free treatment media), PAF (1 μmol/L) or *lyso*-PAF (1 μmol/L). Cell survival was assessed by Live/Dead staining. (a) PAFR+/+ CGNs were capable of cleaving calcein-AM to its fluorescent product (green cells) without loss of membrane integrity (red cells) following PAF, *lyso*-PAF, or vehicle treatment. (b) Cell viability was compromised in PAFR−/− cultures treated with PAF but not vehicle or *lyso*-PAF. Scale bars, 10 μm. (c, d) Quantification of neuronal survival by Live/Dead assay. PAF but not *lyso*-PAF elicited significant cell loss in PAFR−/− cultures without altering survival of PAFR+/+ cultures (*$p < 0.05$, ANOVA, *post hoc* Dunnett's *t*-test, $n = 10$–15 fields per condition performed over triplicate experiments). Data are reported as mean ± SE. Scale bars, 20 μm.

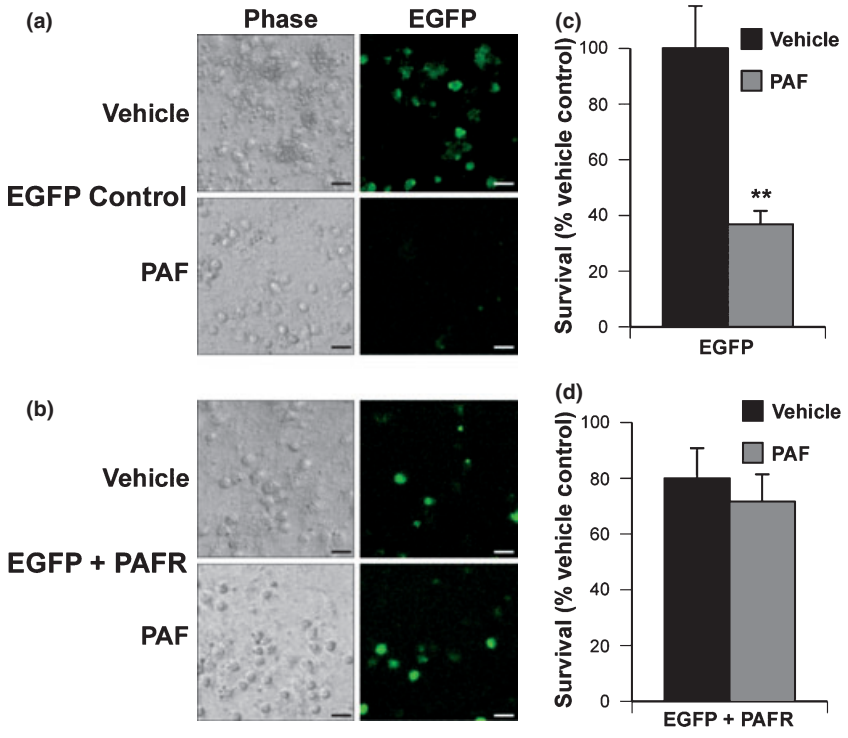

Fig. 2 PAFR expression protects PAFR–/– neurons from PAF-induced death. PAFR–/– CGNs were infected with recombinant adenovirus containing EGFP (a, c) or EGFP and PAFR (b, d). The number of EGFP$^+$ cells in both cultures was comparable prior to treatment indicating equivalent infection efficiency. (a, b) Cultures were treated with vehicle or PAF (1 μmol/L) for 24 h in serum-free treatment media. The number of EGFP$^+$ neurons were reduced in cultures infected with the EGFP control adenovirus (a) but not EGFP + PAFR (b). (c,d) In quantitative analyses, neuronal survival was expressed as percentage of the total number of EGFP$^+$ cells present in vehicle controls. Note that significant loss of EGFP$^+$ cells was detected in control cultures whereas PAFR expression protected PAFR–/– CGNs from PAF-induced death (**$p < 0.01$, Student *t*-test, $n = 5$–10 fields per condition performed over duplicate experiments). Data are reported as mean ± SE. Scale bars, 20 μm.

published studies demonstrating that (i) benzoic acid derivatives can alter PAF catabolism and thereby PAF-mediated biological activity and (ii) PAF-like lipids play a role in etoposide-induced apoptosis (Hattori *et al.* 1995; Huh *et al.* 1998; Southall *et al.* 2001; Li *et al.* 2003; Bonin *et al.* 2004). Based on this evidence, we prioritized 10 benzoic acid derivatives (Table 1) present in our library that enhanced PC12-AC cell viability following etoposide challenge (C. T. Harris, J. T. Arnason and S. A. L. Bennett, unpublished data). These compounds were screened for anti-PAF activity using the PAF-sensitive PC12-AC cell line (Brewer *et al.* 2002; Bonin *et al.* 2004). As positive controls, the non-competitive PAF antagonist ginkgolide B (BN 52021) and the fungal derivative, bis(methylthio)gliotoxin (FR 49175) (Brewer *et al.* 2002; Bonin *et al.* 2004) were included. As a negative control, we used the competitive PAF antagonist CV 3988 previously shown to block PAFR-dependent but not PAFR-independent PAF signaling in non-neuronal cells (Brewer *et al.* 2002).

The effects of test compounds on cell viability were established by mitochondrial dehydrogenase cleavage of the formazan dye WST. As expected, both BN 52021 and FR 49175 inhibited PAF-induced death without altering the viability of vehicle-treated cultures (Fig. 5a and b). CV 3988 had no significant effect on cell viability in the presence or absence of PAF (Fig. 5a and b). Five benzoic acids (gentisic acid, veratric acid, gallic acid, syringic acid, and salicylic acid) increased viable cell number in both vehicle- and PAF-treated cultures (Fig. 5a and b) likely indicative of mitogenic effects in growth factor-deprived treatment media. Two benzoates (resorcyclic acid and *m*-digallic acid) decreased viable cell number in vehicle-treated cultures (Fig. 5a). Interestingly, both compounds significantly increased viable cell number in PAF-treated cultures (Fig. 5b). Of the benzoic acids tested, only orsellinic acid significantly inhibited PAF-mediated death without affecting viability of vehicle-treated cells (Fig. 5a and b).

We next assessed the ability of orsellinic acid, BN 52021, FR 49175, and CV 3988 to inhibit PAF-induced caspase 3/7 activity. PAF-induced caspase 3/7 activity, denoted by appearance of the 85 kDa PARP cleavage fragment by western analysis, was reduced by BN 52021, FR 49175, and orsellinic acid but not CV 3988 (Fig. 5c).

Orsellinic acid inhibits PAF-mediated neuronal apoptosis

PC12-AC cells are tumor derived pheochromocytoma cells that can be differentiated to a peripheral nervous system neuronal phenotype. As such, their responses may not accurately reflect post-mitotic terminally differentiated neurons of the CNS. To establish whether the PAF inhibitors identified in our screen block PAF-induced apoptosis in CNS neurons without impacting on PAFR-mediated neuroprotection, PAFR+/+ and PAFR−/− CGNs were treated with vehicle (treatment media) or PAF (1 μmol/L) in the presence or absence of CV 3988 (1 μg/mL), BN 52021 (21 μg/mL), FR 49175 (4 μg/mL), or orsellinic acid (1 μg/mL) (Fig. 6). Cell survival was assessed by Live/Dead assay. BN 52021, FR 49175, CV 3988, and orsellinic acid had no effect on PAFR+/+ neurons treated with vehicle or PAF indicating that these

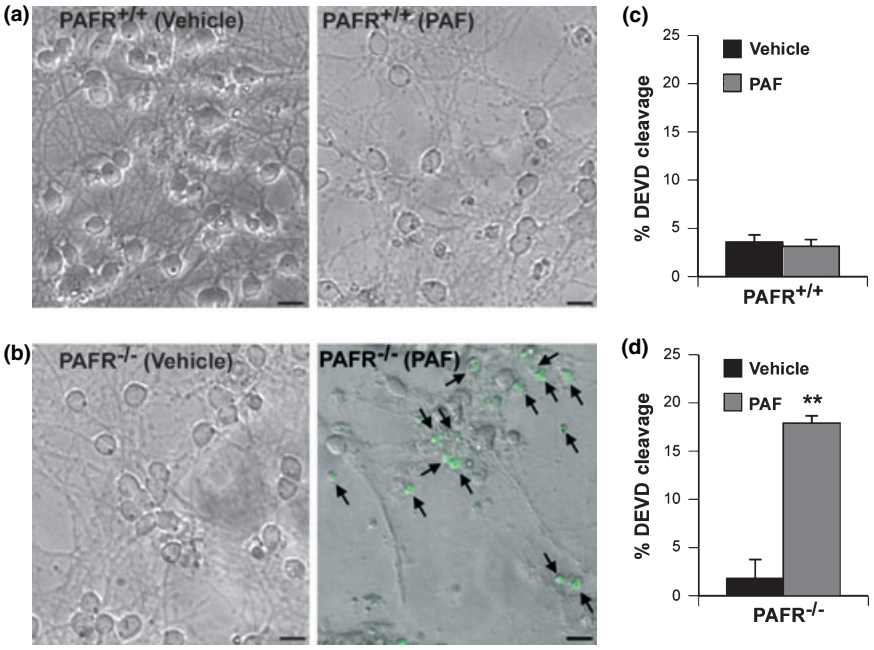

Fig. 3 PAF induces a caspase 3/7-dependent apoptotic pathway in PAFR−/− neurons. PAFR+/+ and PAFR−/− CGNs were treated with vehicle or PAF (1 μmol/L) for 24 h in serum-free media. (a, b) Activated caspase 3/7 was identified by the cleavage of FAM-DEVD-FMK to its fluorescent product. Photomicrographs depict overlays of cleaved FAM-DEVD-FMK and phase images. Note the condensed pyknotic neurons in PAFR−/− treated cultures exhibiting caspase 3/7 activity (arrows). Scale bars, 20 μm. Data were expressed as percentage activated caspase 3/7 positive cells relative to the total cell number per field. (a, c) PAFR+/+ CGNs show no change in caspase 3/7 activation as a result of PAF treatment whereas (b, d) PAFR−/− CGNs show a significant increase in caspase 3/7 activity relative to vehicle-treated controls (**$p < 0.01$, Student's *t*-test, $n = 6$ performed over duplicate experiments). Data are reported as mean ± SE.

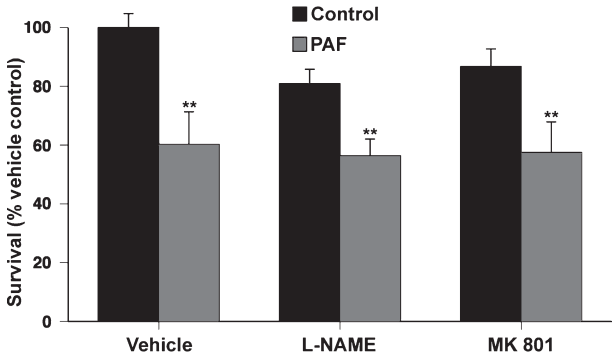

Fig. 4 PAFR−/− neurons are not protected by NO synthase inhibition or NMDA receptor blockade. Cell viability was assessed by Live/Dead viability/cytotoxicity kit. The effect of L-NAME (5 mmol/L) and MK-801 (10 μmol/L) on PAFR−/− survival was determined in the presence and absence of vehicle or PAF in serum-free media (**$p < 0.01$ relative to EtOH control, *post hoc* Dunnett's *t*-test, $n = 6$). Data represent mean + SE.

compounds will likely not impede PAFR-mediated neuroprotection (Fig. 6a). Likewise, test compounds had no effect on the viability of vehicle-treated PAFR−/− CGNs (Fig. 6b). Only BN 52021 and orsellinic acid increased neuronal survival (Fig. 6b) and inhibited caspase 3/7 activation following treatment of PAFR−/− cultures with PAF (Fig. 6c).

Discussion

In this study, we show for the first time that PAF triggers PAFR-independent caspase-dependent apoptosis in primary neurons. This toxic response was specific to PAF and not its immediate or subsequent metabolites as *lyso*-PAF did not initiate death. Interestingly, we found that PAFR-independent death does not involve NO or NMDA receptor signaling pathways. Neither L-NAME nor MK-801 protected PAFR−/− CGNs from PAF. These results are consistent with previous reports that NO production depends upon PAFR activation (Han *et al.* 2006; Santiago *et al.* 2006). Furthermore, we demonstrate that expression of the PAF G-protein coupled receptor can elicit neuroprotection and inhibit PAF-mediated cell death. To intervene in PAFR-independent apoptosis, we identified a benzoic acid derivative, orsellinic acid, and a non-competitive PAF antagonist, BN 52021, capable of reducing PAF neurotoxicity in PAFR−/− neurons without impacting upon the neuroprotection provided by PAFR expression.

The significance of these findings lies in converging evidence that PAF plays a key role in transducing neuronal death in multiple neurodegenerative conditions (Birkle *et al.* 1998; Perry *et al.* 1998; Bazan *et al.* 2002; Bate *et al.* 2004a,b; Farooqui *et al.* 2006), yet therapeutic strategies

targeting PAF signaling have met with limited success (Le Bars *et al.* 1997, 2000; van Dongen *et al.* 2000; Wettstein 2000). In rodent brain, PAFR is primarily expressed by activated microglia with neuronal expression restricted to subpopulations of cells in the hippocampus, notably the CA3 pyramidal cell field, cortex, and cerebellum (Mori *et al.* 1996, 1997; Bennett *et al.* 1998). Earlier studies have hinted at the possibility that PAFR-independent signaling pathways may participate in neuronal loss in regions where PAFR is not detected. For example, striatal neurons synthesize and bind PAF following ischemic insult (Domingo *et al.* 1994) yet express little to no PAFR mRNA (Mori *et al.* 1996). Here, we show that PAF triggers executioner caspase activation in PAFR−/− neurons providing conclusive evidence that PAF does not require its G-protein coupled receptor to initiate neuronal apoptosis. Moreover, we show that PAFR expression protects neurons from pathological PAF challenge. These data provide possible insight into why subpopulations of neurons, such as PAFR-expressing CA3 pyramidal neurons, are spared following ischemic insult (Cronberg *et al.* 2005). These findings also highlight the need to identify reagents that inhibit PAFR-independent apoptosis without impacting upon PAFR neuroprotection in injured brain.

To this end, our comparison of known PAF inhibitors demonstrates significant differences in their efficacies between cell types that could impact on potential *in vivo* use. First, we found that the PAF antagonist CV 3988 does not protect cells from PAF challenge in the absence of PAFR. Thus, it is likely that not all PAF antagonists will prove useful in inhibiting PAF-induced neuronal loss. Second, FR 49175, BN 52021, and orsellinic acid were found to protect PC12-AC cells from PAF challenge but, in this study, only BN 52021 and orsellinic acid inhibited PAFR-independent apoptosis in primary neurons. We have previously demonstrated that the ginkgolide BN 52021 and the fungal metabolite derivative FR 49175 accelerate PAF catabolism in PC12-AC cells (Bonin *et al.* 2004). Both compounds inhibit expression of the α_1 catalytic subunit of PAF-AH I complex thereby promoting dimerization of the α_2 subunit

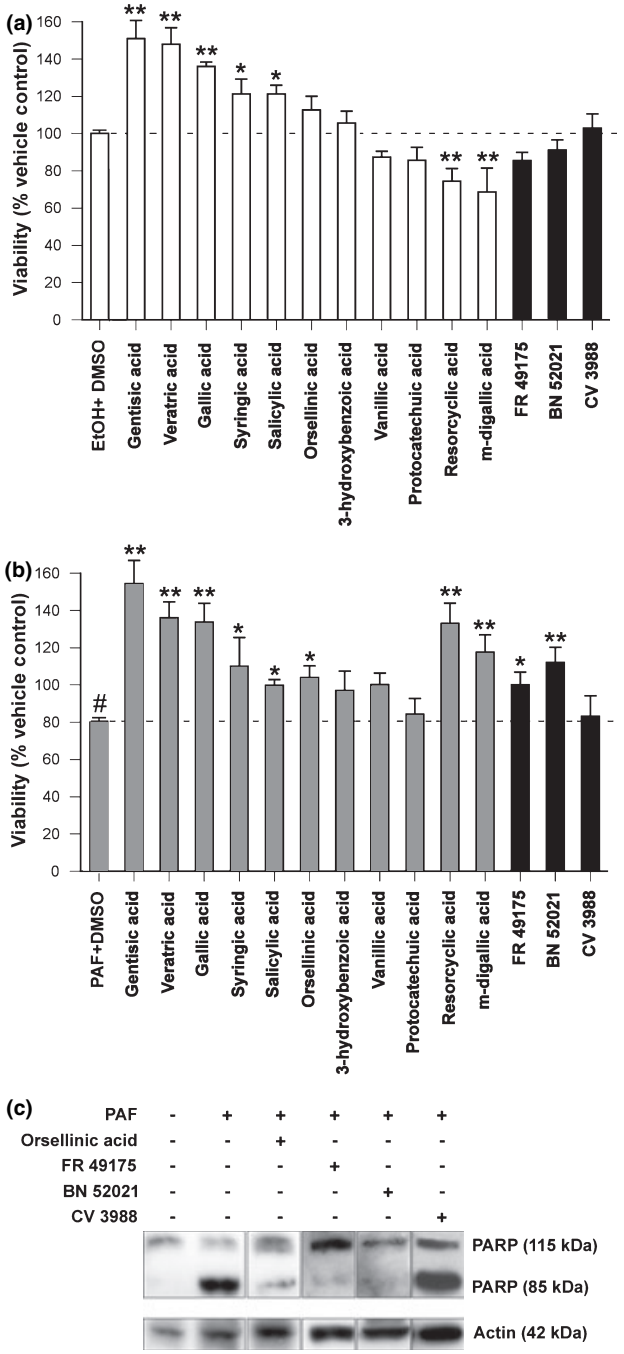

Fig. 5 Orsellinic acid, BN 52021, and FR 49175 protect PC12-AC cells from PAF-induced apoptosis without altering viability of vehicle-treated cells. Viable cell number was quantified by WST assay and standardized to wells of known cell densities after a 24 h treatment with vehicle (a) or PAF (b) and test compounds. Benzoic acids (a, white bars; b, grey bars), BN 52021, FR 49175, and CV 3988 (black bars) were tested in serum-free media at 1, 21, 4, and 1 μg/mL, respectively, previously shown to elicit maximal biological activity. (a) Toxic (<100% viability) and proliferative (>100% viability) activities were established by comparing the effects of test compounds on vehicle-treated controls. (*$p < 0.05$, **$p < 0.01$ relative to EtOH + DMSO (dashed line), *post hoc* Dunnett's *t*-test, $n = 10–20$.) (b) Effect of test compounds on PAF-induced PC12-AC cell loss. PAF + DMSO elicited significant loss in viable cell number compared to EtOH + DMSO treatment [#, $p < 0.05$ in (b) relative to vehicle-treated controls in (a), Student's *t*-test $n = 10–20$]. Only the positive controls (BN 52021 and FR 49175) and orsellinic acid significantly inhibited PAF-induced toxicity without altering viability of vehicle-treated cultures [*$p < 0.05$, **$p < 0.01$ relative to PAF + DMSO (dashed line), *post hoc* Dunnett's *t*-test, $n = 10–20$]. (c) Orsellinic acid, BN 52021, and FR 49175, but not CV 3988 prevented caspase 3/7 cleavage of PARP induced by PAF.

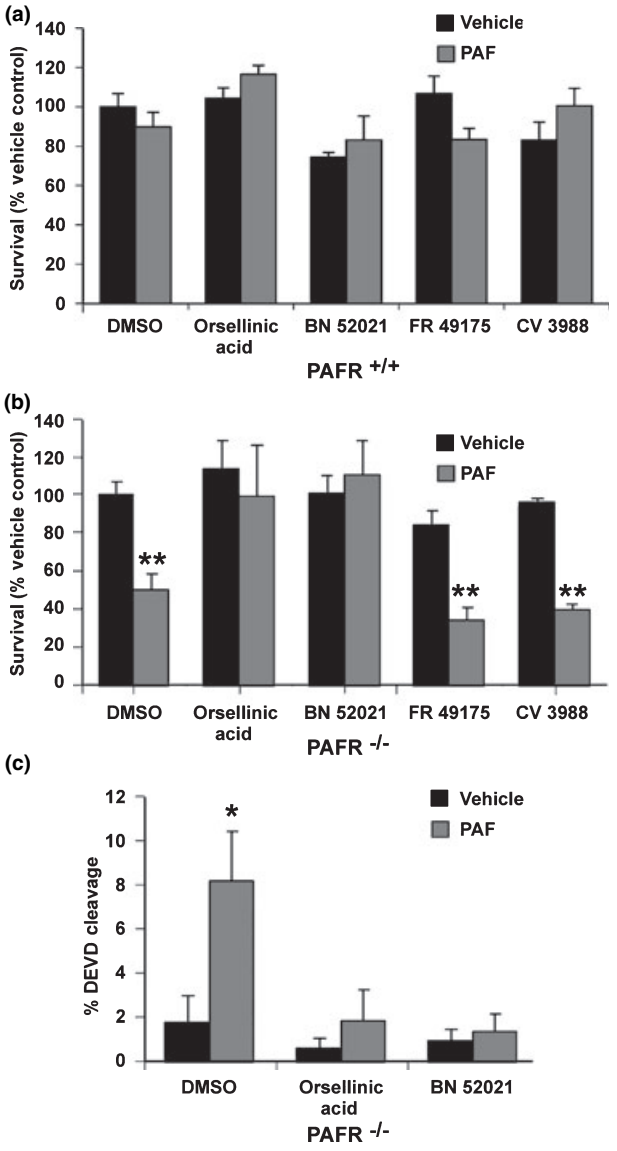

Fig. 6 Orsellinic acid and BN 52021 protect PAFR–/– neurons from PAF toxicity without perturbing PAFR-mediated neuroprotection. Cell viability was assessed by Live/Dead viability/cytotoxicity kit. The effect of orsellinic acid (1 μg/mL), BN 52021 (21 μg/mL), FR 49175 (4 μg/mL), and CV 3988 (1 μg/mL) on (a) PAFR+/+ and (b) PAFR–/– survival was determined in the presence and absence of vehicle or PAF in serum-free media. (c) PAF-induced executioner caspase activation was determined in PAFR–/– CGNs. Note that orsellinic acid and BN 52021 inhibited PAF-induced neuronal loss as indicated by an increase in the number of calcein$^+$ viable cells (b) and a reduction in the number of caspase 3/7$^+$ cells (c) in PAFR–/– cultures without perturbing PAFR-mediated neuroprotection in PAFR+/+ cultures (a) (*$p < 0.05$, **$p < 0.01$ relative to DMSO control, *post hoc* Dunnett's *t*-test, $n = 5$–8). Data represent mean ± SE.

(Bonin *et al.* 2004). Here, we show that this mechanism likely does not impact upon neuronal PAF catabolism. In embryonic brain, as in undifferentiated PC12-AC cells, PAF-AH I α_1 is expressed at higher levels than PAF-AH I α_2 (Manya *et al.* 1998; Bonin *et al.* 2004). Transiently reducing α_1 protein levels is sufficient to promote α_2 homodimerization. In mature brain, PAF-AH α_2 predominates over PAF-AH α_1 (Manya *et al.* 1998). Consequently, any pharmacological reduction in PAF-AH α_1 expression is unlikely to enhance formation of the anti-apoptotic α_2/α_2 homodimer. Thus, we predict that FR 49175 will not prove effective in strategies targeting adult neurons *in vivo* but may protect embryonic brain from pathological exposure to PAF. Third, BN 52021 has been shown to bind two distinct PAF intracellular sites as well as acting as a non-competitive inhibitor of PAFR and promoting PAF-AH I α_2/α_2 homodimer formation (Marcheselli *et al.* 1990; Bonin *et al.* 2004). The finding that BN 52021 but not FR 49175 blocks neuronal apoptosis suggests that PAFR-independent apoptosis may be initiated by one of these intracellular binding sites. The presence of these sites in CGNs is consistent with evidence that subtoxic concentrations of a non-metabolizable PAF analog alters neuronal migration in PAFR−/− CGNs (Tokuoka *et al.* 2003).

We also characterized a novel PAF inhibitor identified from a panel of benzoic acid derivatives produced by plants and fungi. We found that orsellinic acid selectively inhibits PAFR-independent death in neurons without altering PAFR-neuroprotection following PAF challenge. Orsellinic acid (2-methyl-4,6-dihydroxybenzoic acid) is a fungal metabolite structurally similar to the known anti-inflammatory compound acetylsalicylic acid (aspirin). While we have yet to identify the mechanism through which this benzoate elicits its biological effects, previous studies have shown that other benzoate family members modulate PAF catabolism (Hattori *et al.* 1995; Huh *et al.* 1998; Bonin *et al.* 2004; Zhu *et al.* 2004).

In summary, these data highlight a novel PAFR-independent pathway leading to caspase-dependent apoptosis in primary neurons following PAF challenge and identify two natural health product inhibitors, orsellinic acid, and BN 52021, capable of intervening in this pathway. These findings provide a pharmacological means of differentially targeting distinct PAFR-dependent and independent pathways that can be used to further elucidate underlying mechanisms of neurotoxicity and neuroprotection in PAF-associated disease.

Acknowledgements

This work was supported by an operating grant from the Ontario Mental Health Foundation (OMHF) to SALB and a Canadian Institute Heath of Research (CIHR) Team Grant to PSH, JTA, and SALB. PSH is a National Research Scientist of the Quebec Health Research Fund. SALB is a CIHR New Investigator and an OMHF Intermediate Investigator. SDR is funded by an OMHF graduate studentship. CSH is funded by a Canadian Graduate Studentship. FM was supported by a NSERC Undergraduate Research Studentship Award. We thank J. Bennett for editorial assistance and

Dr Shawn Whitehead for critical reading of this manuscript. We are very grateful to Dr T. Shimizu for the kind gift of PAFR−/− breeding pairs.

References

Bate C., Salmona M. and Williams A. (2004a) The role of platelet activating factor in prion and amyloid-beta neurotoxicity. *Neuroreport* **15**, 509–513.

Bate C., Reid S. and Williams A. (2004b) Phospholipase A2 inhibitors or platelet activating factor antagonists prevent prion replication. *J. Biol. Chem.* **279**, 36405–36411.

Bazan N. G., Tu B. and Rodriguez de Turco E. B. (2002) What synaptic lipid signaling tells us about seizure-induced damage and epileptogenesis. *Prog. Brain Res.* **135**, 175–185.

Bennett S. A. L., Chen J., Pappas B. A., Roberts D. C. S. and Tenniswood M. (1998) Alterations in platelet activating factor receptor expression associated with neuronal apoptosis in an in vivo model of excitotoxicity. *Cell Death Differ.* **5**, 867–875.

Birkle D. L., Kurian P., Braquet P. and Bazan N. G. (1998) Platelet activating factor antagonist BN52021 decreases accumulation of free polyunsaturated fatty acid in mouse brain during ischemia and electroconvulsive shock. *J. Neurochem.* **51**, 1900–1905.

Bonin F., Ryan S. D., Migahed L., Mo F., Lallier J., Franks D. J., Arai H. and Bennett S. A. (2004) Anti-apoptotic actions of the platelet activating factor acetylhydrolase I alpha 2 catalytic subunit. *J. Biol. Chem.* **279**, 52425–52436.

Bratton D. L., Dreyer E., Kailey J. M., Fadok V. A., Clay K. L. and Henson P. M. (1992) The mechanism of internalization of platelet-activating factor in activated human neutrophils. Enhanced transbilayer movement across the plasma membrane. *J. Immunol.* **148**, 514–523.

Brewer C., Bonin F., Bullock B., Nault M.-C., Morin J., Imbeault S., Shen T. Y., Franks D. J. and Bennett S. A. L. (2002) Platelet activating factor-induced apoptosis is inhibited by ectopic expression of the platelet activating factor G-protein coupled receptor. *J. Neurochem.* **18**, 1502–1511.

Chen C., Magee J. C., Marcheselli V., Hardy M. and Bazan N. G. (2001) Attenuated LTP in hippocampal dentate gyrus neurons of mice deficient in the PAF receptor. *J. Neurophysiol.* **85**, 384–390.

Cregan S. P., MacLaurin J., Gendron T. F., Callaghan S. M., Park D. S., Parks R. J., Graham F. L., Morley P. and Slack R. S. (2000) Helper-dependent adenovirus vectors: their use as a gene delivery system to neurons. *Gene Ther.* **7**, 1200–1209.

Cronberg T., Jensen K., Rytter A. and Wieloch T. (2005) Selective sparing of hippocampal CA3 cells following in vitro ischemia is due to selective inhibition by acidosis. *Eur. J. Neurosci.* **22**, 310–316.

Domingo M. T., Spinnewyn B., Chabrier P. E. and Braquet P. (1994) Changes in [3H]PAF binding and PAF concentrations in gerbil brain after bilateral common carotid artery occlusion: a quantitative autoradiographic study. *Brain Res.* **640**, 268–276.

van Dongen M. C., van Rossum E., Kessels A. G., Sielhorst H. J. and Knipschild P. G. (2000) The efficacy of ginkgo for elderly people with dementia and age-associated memory impairment: new results of a randomized clinical trial. *J. Am. Geriatr. Soc.* **48**, 1183–1194.

Farooqui A. A., Ong W. Y. and Horrocks L. A. (2006) Inhibitors of brain phospholipase A2 activity: their neuropharmacological effects and therapeutic importance for the treatment of neurologic disorders. *Pharmacol. Rev.* **58**, 591–620.

Han S. H., Kim J. H., Seo H. S., Martin M. H., Chung G. H., Michalek S. M. and Nahm M. H. (2006) Lipoteichoic acid-induced nitric oxide production depends on the activation of platelet-activating factor receptor and Jak2. *J. Immunol.* **176**, 573–579.

Hattori K., Hattori M., Adachi H., Tsujimoto M., Arai H. and Inoue K. (1995) Purification and characterization of platelet-activating factor acetylhydrolase II from bovine liver cytosol. *J. Biol. Chem.* **270**, 22308–22313.

Hou S. T., Jiang S. X., Desbois A., Huang D., Kelly J., Tessier L., Karchewski L. and Kappler J. (2006) Calpain-cleaved collapsin response mediator protein-3 induces neuronal death after glutamate toxicity and cerebral ischemia. *J. Neurosci.* **26**, 2241–2249.

Huang D., Desbois A. and Hou S. T. (2005) A novel adenoviral vector which mediates hypoxia-inducible gene expression selectively in neurons. *Gene Ther.* **12**, 1369–1376.

Huh H., Kim H. K. and Lee H. K. (1998) PAF antagonistic activity of 2-hydroxy-3-methoxybenzoic acid glucose ester from Gentiana scabra. *Arch. Pharm. Res.* **21**, 436–439.

Ishii S., Kuwaki T., Nagase T. *et al.* (1998) Impaired anaphylactic responses with intact sensitivity to endotoxin in mice lacking a platelet activating factor receptor. *J. Exp. Med.* **187**, 1779–1788.

Izquierdo I., Fin C., Schmitz P. K., Da Silva R. C., Jerusalinsky D., Quillfeldt J. A., Ferreira M. B. G., Medina J. H. and Bazan N. G. (1995) Memory enhancement by intrahippocampal, intraamygdala, or intraentorhinal infusion of platelet-activating factor measured in an inhibitory avoidance task. *Proc. Natl Acad. Sci. USA* **92**, 5047–5051.

Kato K. (1999) Modulation of long-term potentiation in the CA1 area of rat hippocampus by platelet-activating factor. *Adv. Exp. Med. Biol.* **469**, 221–227.

Kunz D., Gerard N. P. and Gerard C. (1992) The human leukocyte platelet-activating factor receptor. cDNA cloning, cell surface expression, and construction of a novel epitope-bearing analog. *J. Biol. Chem.* **267**, 9101–9106.

Le Bars P. L., Katz M. M., Berman N., Itil T. M., Freedman A. M. and Schatzberg A. F. (1997) A placebo-controlled, double-blind, randomized trial of an extract of Ginkgo biloba for dementia. North American EGb Study Group. *JAMA* **278**, 1327–1332.

Le Bars P. L., Kieser M. and Itil K. Z. (2000) A 26-week analysis of a double-blind, placebo-controlled trial of the ginkgo biloba extract EGb 761 in dementia. *Dement. Geriatr. Cogn. Disord.* **11**, 230–237.

Li T., Southall M. D., Yi Q., Pei Y., Lewis D., Al-Hassani M., Spandau D. and Travers J. B. (2003) The epidermal platelet-activating factor receptor augments chemotherapy-induced apoptosis in human carcinoma cell lines. *J. Biol. Chem.* **278**, 16614–16621.

Malipiero U., Heuss C., Schlapbach R., Tschopp J., Gerber U. and Fontana A. (1999) Involvement of the N-methyl-D-aspartate receptor in neuronal cell death induced by cytotoxic T cell-derived secretory granules. *Eur. J. Immunol.* **29**, 3053–3062.

Manya H., Aoki J., Watanabe M., Adachi T., Asou H., Inoue Y., Arai H. and Inoue K. (1998) Switching of platelet-activating factor acetylhydrolase catalytic subunits in developing rat brain. *J. Biol. Chem.* **273**, 18567–18572.

Marcheselli V. L., Rossowska M., Domingo M. T., Braquet P. and Bazan N. G. (1990) Distinct platelet-activating factor binding sites in synaptic endings and in intracellular membranes of rat cerebral cortex. *J. Biol. Chem.* **265**, 9140–9145.

Martineau L. C., Couture A., Spoor D. *et al.* (2006) Anti-diabetic properties of the Canadian lowbush blueberry Vaccinium angustifolium Ait. *Phytomedicine* **13**, 612–623.

Mori M., Aihara M., Kume K., Hamanoue M., Kohsaka S. and Shimizu T. (1996) Predominant expression of platelet-activating factor receptor in rat brain microglia. *J. Neurosci.* **16**, 3590–3600.

Mori M., Aihara M. and Shimizu T. (1997) Localization of platelet activating factor receptor messenger RNA in the rat eye. *Invest. Ophthalmol. Vis. Sci.* **38**, 2672–2678.

Nagase T., Ishii S., Kume K., Uozumi N., Izumi T., Ouchi Y. and Shimizu T. (1999) Platelet-activating factor mediates acid-induced lung injury in genetically engineered mice. *J. Clin. Invest.* **104**, 1071–1076.

Perry S. W., Hamilton J. A., Tjoelker L. W., Dbaibo G., Dzenko K. A., Epstein L. G., Hannun Y., Whittaker J. S., Dewhurst S. and Gelbard H. A. (1998) Platelet activating factor receptor activation. An initiator step in HIV-1 neuropathogenesis. *J. Biol. Chem.* **273**, 17660–17664.

Prescott S. M., Zimmerman G. A., Stafforini D. M. and McIntyre T. M. (2000) Platelet activating factor and related lipid mediators. *Annu. Rev. Biochem.* **69**, 419–445.

Santiago H. C., Braga Pires M. F., Souza D. G., Roffe E., Cortes D. F., Tafuri W. L., Teixeira M. M. and Vieira L. Q. (2006) Platelet activating factor receptor-deficient mice present delayed interferon-gamma upregulation and high susceptibility to Leishmania amazonensis infection. *Microbes Infect.* **8**, 2569–2577.

Sapir T., Elbaum M. and Reiner O. (1997) Reduction of microtubule catastrophe events by LIS1, platelet-activating factor acetylhydrolase subunit. *EMBO J.* **16**, 6977–6984.

Southall M. D., Isenberg J. S., Nakshatri H., Yi Q., Pei Y., Spandau D. F. and Travers J. B. (2001) The platelet-activating factor receptor protects epidermal cells from tumor necrosis factor (TNF) alpha and TNF-related apoptosis-inducing ligand-induced apoptosis through an NF-kappa B-dependent process. *J. Biol. Chem.* **276**, 45548–45554.

Spoor D. C., Martineau L. C., Leduc C. *et al.* (2006) Selected plant species from the Cree pharmacopoeia of northern Quebec possess anti-diabetic potential. *Can. J. Physiol. Pharmacol.* **84**, 847–858.

Teather L. A., Packard M. G. and Bazan N. G. (1998) Effects of post-training intrahippocampal injections of platelet-activating and PAF antagonists on memory. *Neurobiol. Learn. Mem.* **70**, 349–363.

Tokuoka S. M., Ishii S., Kawamura N., Satoh M., Shimada A., Sasaki S., Hirotsune S., Wynshaw-Boris A. and Shimizu T. (2003) Involvement of platelet-activating factor and LIS1 in neuronal migration. *Eur. J. Neurosci.* **18**, 563–570.

Wettstein A. (2000) Cholinesterase inhibitors and Gingko extracts–are they comparable in the treatment of dementia? Comparison of published placebo-controlled efficacy studies of at least six months' duration *Phytomedicine* **6**, 393–401.

Xu Y. and Tao Y. X. (2004) Involvement of the NMDA receptor/nitric oxide signal pathway in platelet-activating factor-induced neurotoxicity. *Neuroreport* **15**, 263–266.

Zhu P., DeCoster M. A. and Bazan N. G. (2004) Interplay among platelet-activating factor, oxidative stress, and group I metabotropic glutamate receptors modulates neuronal survival. *J. Neurosci. Res.* **77**, 525–531.

