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Heterogeneity in the *sn*-1 carbon chain of platelet-activating factor glycerophospholipids determines pro- or anti-apoptotic signaling in primary neurons[S]

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Abstract The platelet-activating factor (PAF) family of glycerophospholipids accumulates in damaged brain tissue following injury. Little is known about the role of individual isoforms in regulating neuronal survival. Here, we compared the neurotoxic and neuroprotective activities of 1-*O*-hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine (C_{16}-PAF) and 1-*O*-octadecyl-2-acetyl-*sn*-glycero-3-phosphocholine (C_{18}-PAF) in cerebellar granule neurons. We find that both C_{16}-PAF and C_{18}-PAF cause PAF receptor-independent death but signal through different pathways. C_{16}-PAF activates caspase-7, whereas C_{18}-PAF triggers caspase-independent death in PAF receptor-deficient neurons. We further show that PAF receptor signaling is either pro- or anti-apoptotic, depending upon the identity of the *sn*-1 fatty acid of the PAF ligand. Activation of the PAF G-protein-coupled receptor (PAFR) by C_{16}-PAF stimulation is anti-apoptotic and inhibits caspase-dependent death. Activation of PAFR by C_{18}-PAF is pro-apoptotic. These results demonstrate the importance of the long-chain *sn*-1 fatty acid in regulating PAF-induced caspase-dependent apoptosis, caspase-independent neurodegeneration, and neuroprotection in the presence or absence of the PAF receptor.—Ryan, S. D., C. S. Harris, C. L. Carswell, J. E. Baenziger, and S. A. L. Bennett. **Heterogeneity in the *sn*-1 carbon chain of platelet-activating factor glycerophospholipids determines pro- or anti-apoptotic signaling in primary neurons. *J. Lipid Res.* 2008.** 49: **2250–2258.**

Supplementary key words receptor • neurodegeneration • isoform • inhibitor • analog • antagonist • central nervous system • neurotoxicity • affinity • ligand

Platelet-activating factor (PAF) lipids are members of the 1-alkyl,2-acylglycerophosphocholine subclass (GP0102) of glycerophosphocholines (GP01) defined by an alkyl-ether linkage at the *sn*-1 position, an acetyl group at the *sn*-2 position, and a phosphocholine at the *sn*-3 position (1, 2) (**Fig. 1**). PAF species are synthesized through two enzymatic pathways (remodeling and de novo, Fig. 1). PAF-like lipids with longer *sn*-2 carbon chains or polar head group substitutions can also be produced by oxidation of structural membrane glycerophosphocholines or enzymatic modification of glycerophosphoethanolamines (Fig. 1). All three routes generate molecular species that differ in carbon chain length and degree of unsaturation at the *sn*-1 position (3). The impact of these *sn*-1 substitutions on neuronal function is not known.

The majority of PAF effects are attributed to interaction with a single G-protein-coupled receptor (PAFR). PAFR is expressed by multiple peripheral cell types but is regionally restricted to neuronal subpopulations in the central nervous system (4–6). Both PAFR-expressing and PAFR-deficient neurons are sensitive to the neurotoxic effects of PAF ligands (7, 8), yet it is not clear whether these pathways are mediated by different PAF isoforms. PAFR recognizes glycerophospholipids with an *sn*-2 acetyl moiety and an *sn*-3 phosphocholine group (1, 9). The length of the *sn*-2 carbon chain dictates PAFR affinity. Species with an *sn*-2 acetyl moiety exhibit the highest affinity (10–12). Substituting phosphoethanolamine for phosphocholine substantively reduces binding (9). The *sn*-1 ether linkage is not required for PAFR interaction but does increase potency several hundred-fold (1). The *sn*-1 carbon chain length and degree of unsaturation is implicated in activation of different PAFR signaling pathways (10, 13–15).

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Abbreviations: C_{16}-PAF, 1-*O*-hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine; C_{18}-PAF, 1-*O*-octadecyl-2-acetyl-*sn*-glycero-3-phosphocholine; CGN, cerebellar granule neuron; CMC, critical micellar concentration; EGFP, enhanced green fluorescent protein; EMEM, Eagle's minimum essential medium; PAF, platelet-activating factor; PAFR, platelet-activating factor G-protein-coupled receptor.

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[S] The online version of this article (available at http://www.jlr.org) contains supplementary data in the form of one figure.

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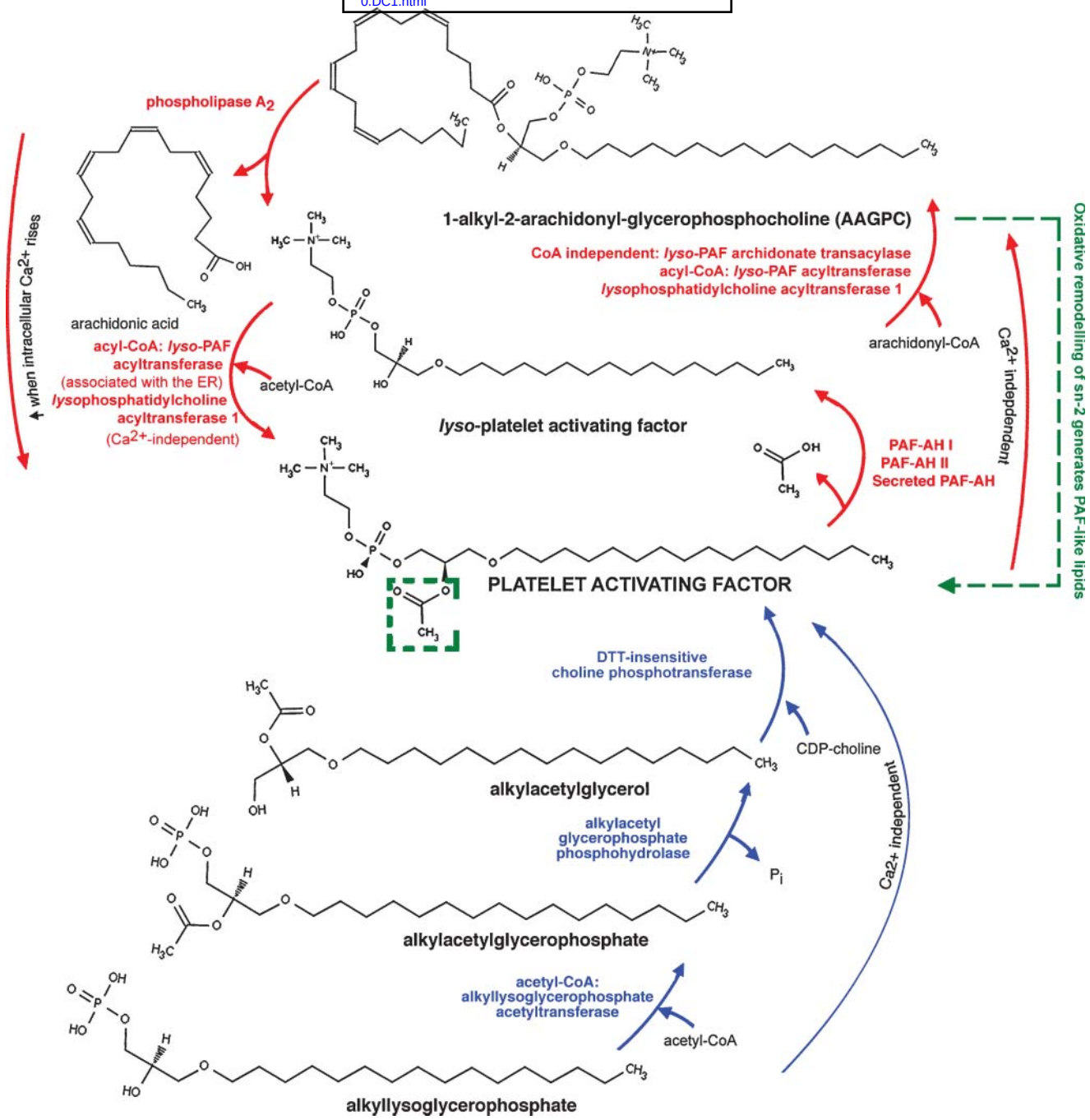

Fig. 1. Platelet-activating factor (PAF) metabolic pathways. PAF family members are produced through three main pathways. The primary enzymatic pathway is the remodeling pathway (red), whose kinetics in terminally differentiated neurons are faster than the de novo synthesis pathway (blue). Nonenzymatic oxidation of membrane lipids (green) generates PAF-like family members with longer-chain fatty acids (up to eight carbons) at the *sn*-2 position (hatched green box). The predominant molecular species in brain are C_{16}-PAF and C_{18}-PAF. Synthesis of C_{16}-PAF from the 1-alkyl-2-arachidonyl-glycerophosphocholine parental lipid is depicted in this schematic.

These isoform-specific differences may underlie observations that PAFR expression augments chemotherapeutic cytotoxicity yet protects cells from tumor necrosis factor α, TRAIL, and 1-*O*-hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine (C_{16}-PAF) (16–19). It is not known whether different PAF isoforms initiate different pro- or anti-apoptotic signaling pathways in neurons.

Under physiological conditions, PAFs act as retrograde neurotransmitters, contributing to hippocampal CA1 long-term potentiation by enhancing excitatory synaptic transmission (20, 21). When concentrations remain elevated, PAF lipids can initiate caspase-dependent neurotoxicity through PAFR-dependent and PAFR-independent pathways (7, 8, 22). In this regard, PAFs are recognized as primary mediators of neuronal apoptosis in acquired immune deficiency syndrome dementia, encephalitis, epileptic seizure, and ischemia (8, 22, 23). In progressive neurodegenerative disorders, PAF concentrations increase as a result of en-

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hanced phospholipase A_2 activity (24–27). Inhibition of the PAF remodeling pathway (Fig. 1) through dietary consumption of the omega-3 polyunsaturated fatty acid docosahexaenoic acid (28) blocks PAF-mediated apoptosis in nonneural cells (29) and reduces dendritic pathology in transgenic models of Alzheimer disease (30). These changes likely involve alterations in PAF *sn*-1 isoforms. Loss of apolipoprotein E4, a risk factor associated with early-onset Alzheimer disease, alters the traffic of polyunsaturated lipids from astrocytes to neurons, skewing the composition of long-chain fatty acids in synaptosomal phosphatidylcholines from octadecyl (18:0) to hexadecyl (16:0) species (31). In relapsing-remitting multiple sclerosis, increases in plasma concentrations of 1-*O*-hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine (C_{16}-PAF) and cerebrospinal fluid concentrations of 1-*O*-octadecyl-2-acetyl-*sn*-glycero-3-phosphocholine (C_{18}-PAF) are associated with enhanced blood-brain barrier injury (32). The role of these different isoforms in signaling neurodegeneration is not known.

Here, we show that the carbon chain length at the *sn*-1 position dictates whether neurons undergo caspase-dependent apoptosis, caspase-independent neurodegeneration, or are protected from PAF toxicity. In PAFR-deficient neurons, we find that submicellar concentrations of C_{16}-PAF initiate a caspase-dependent apoptotic death cascade, whereas C_{18}-PAF signals caspase-independent degeneration. Conversely, PAFR activation by C_{16}-PAF protects cells from PAF challenge, whereas PAFR activation by C_{18}-PAF signals pro-apoptotic caspase activation. Collectively, these data implicate heterogeneity in the *sn*-1 carbon chain of PAF species in control of neuronal fate, with signaling dependent upon PAFR expression profile.

MATERIALS AND METHODS

Reagents

All cell culture reagents were obtained from Invitrogen (Burlington, ON) and all chemicals were purchased through Sigma-Aldrich (St. Louis, MO) unless otherwise stated. 1-*O*-hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine (C_{16}-PAF), 1-*O*-octadecyl-2-acetyl-*sn*-glycero-3-phosphocholine (C_{18}-PAF), and PAF antagonists were obtained from Biomol Research Laboratories (Plymouth Meeting, PA). Stock solutions of 10 mM PAF were prepared by adding vehicle (treatment media or EtOH) directly to lyophilized material in source glassware. For concentration-response studies, PAF stock and working solutions were serially diluted prior to treatment. Concentrations were verified by phosphorus assay before treatment as described in (33). In some experiments, cultures were pretreated with the PAF antagonist CV 3988 (2 μM) or antagonist vehicle (0.1% DMSO).

Determination of critical micellar concentrations

Critical micellar concentrations (CMCs) were estimated from dynamic light-scattering data recorded on a DynaPro instrument (Wyatt Technologies, Santa Barbara, CA). For C_{16}-PAF and C_{18}-PAF species, 12 solutions were prepared over the concentration range of 10^{-7} to 10^{-3} M in 0.9 mM $MgSO_4$, 5.4 mM KCl, 26 mM $NaHCO_3$, 116 mM NaCl, and 1 mM NaH_2PO_4 at pH 7.2. The mean light scattering from each solution was calculated from 10 independent measurements, each recorded at 4°C with a 10 s acquisition time and a constant laser power. The data were analyzed using Dynamics V6 software (Wyatt Technologies). The concentration range at which micelle formation induced a dramatic increase in light scattering defined the CMC for both PAF lipid species.

Primary murine cell culture and treatment

Breeding pairs of $PAFR^{-/-}$ mice (34) were kindly provided by Dr. Takao Shimizu (University of Tokyo) and Dr. Nicolas Bazan (Louisiana State University). Mice were backbred into a C57BL/6 background for 10 generations (N10), and cerebellar granule neurons (CGNs) were extracted from congenic $PAFR^{-/-}$ and $PAFR^{+/+}$ mice as previously described (7). Briefly, cerebella from postnatal day 7–10 pups were removed. The meninges were dissected, and tissue was minced in ice-cold dissection solution (124 mM NaCl, 5.37 mM KCl, 1 mM NaH_2PO_4, 1.2 mM $MgSO_4$, 14.5 mM D-glucose, 25 mM Hepes, 3 mg/ml BSA, pH.7.4). Cells were dissociated in dissection media containing 0.5 mg/ml trypsin (Sigma-Aldrich) at 37°C for 18 min. Trypsin was deactivated by addition of 0.52 mg/ml chicken egg white trypsin inhibitor. Pelleted cells were resuspended in dissection solution containing 0.75 mg/ml DNase I (Roche, Mississauga, ON) and triturated to obtain a single cell suspension. $CaCl_2$ was added to a final concentration of 15 μM. CGNs were plated in Eagle's minimum essential medium (EMEM) containing 25 mM glucose, 10% FBS, 1% gentamycin, 2 mM L-glutamine, and 20 mM KCl at 37°C in a 5% CO_2 atmosphere 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). Cells were cultured in the serum-containing media for 72 h and washed extensively with PBS before being exposed to PAF in serum-free treatment media (EMEM, 25 mM glucose, 1% gentamycin, 2 mM L-glutamine, 20 mM KCl, and delipidated 0.025% BSA) as previously described (7, 35). C_{16}-PAF and C_{18}-PAF (0.5–1.5 μM) were dissolved in treatment media. CGNs were treated for 24 h. Where treatment with PAF antagonist CV 3988 or antagonist vehicle (0.1% DMSO) was indicated, cultures were pretreated for 15 min with test compound prior to addition of PAF isoforms. All treatments were performed in serum-free media to ensure that exogenous PAF was not hydrolyzed by serum-derived PAF acetylhydrolases. CGN survival was assessed by Live/Dead viability/cytotoxicity assay (Invitrogen). Viable cells were identified by the enzymatic conversion by intracellular esterases of nonfluorescent calcein-acetoxymethylester to fluorescent calcein. Dead cells were identified by uptake of ethidium homodimer as a result of loss of membrane integrity. Cells were imaged using a DMIR epifluorescent inverted microscope (Leica, Richmond Hill, ON) equipped with a QICAM digital camera (Quorum Technologies, Guelph, ON) and captured using OpenLab software v5.05 (Improvision, Lexington, MA). Percent survival was calculated as [viable cell number$^{(\text{calcein}+\ -\ \text{calcein}+/\text{ET}+)}$/mean number of viable cells in vehicle control$^{(\text{calcein}+\ -\ \text{calcein}+/\text{ET}+)} \times 100$]. All procedures were carried out in agreement with the guidelines of the Canadian Council for Animal Care and as approved by the University of Ottawa Animal Care Committee.

Recombinant adenovirus infection

Recombinant adenovirus preparation, characterization, and infection were performed as previously described (7). Briefly, recombinant adenovirus carrying enhanced green fluorescent protein (EGFP) and PAFR under separate cytomegalovirus promoters or EGFP alone was 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 follow-

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ing C_{16}-PAF or C_{18}-PAF (1 μM) treatment was calculated as $EGFP^+$ cell number following treatment/mean $EGFP^+$ cell number in the vehicle control × 100.

Assessment of caspase activation

Cleavage and activation of pro-caspase 3 and pro-caspase 7 were determined by Western analysis. Proteins were isolated in RIPA buffer (10 mM PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 30 μl/ml aprotinin, 10 mM sodium orthovanadate, 100 μl/ml PMSF). Protein samples (30 μg) were separated by SDS-PAGE under reducing conditions. Antibodies were diluted in 1% heat-denaturated casein in 10 mM PBS (10 mM sodium phosphate, 2.7 mM KCl, 4.3 mM NaCl, pH 7.5). Western analyses were performed using polyclonal anti-cleaved caspase 3 and anti-cleaved caspase 7 (1:1,000; Cell Signaling Technology, Inc., Danvers, MA) and monoclonal βIII-tubulin (1:10,000; Sigma-Aldrich, Oakville, ON). Secondary antibodies were HRP-conjugated anti-rabbit IgG (1:5,000; Jackson ImmunoResearch, Burlington, ON). Immunoreactive bands were visualized using SuperSignal West Pico (MJS BioLynx, Inc.). Caspase activation was confirmed by CaspaTag (caspase 3/7) activity assay (Chemicon, Temecula, CA) according to the manufacturer's protocol. CGNs capable of cleaving FAM-DEVD-FMK to its fluorescent product were counted and reported as a percentage of total cell number after a 24 h treatment with C_{16}-PAF or C_{18}-PAF (1 μM) or vehicle (treatment media). Cells were imaged as described above. Activation was confirmed by pretreatment and exposure to PAF in the presence of the caspase 3/7 inhibitor Z-DEVD-FMK (50 μM).

Statistical analysis

Data were analyzed using one-way factorial ANOVA tests followed by post hoc Dunnett's *t*-tests. *P* values under 0.05 were considered statistically significant (shown as *); *P* values under 0.01 or 0.001 were considered highly significant (shown as ** and ***, respectively).

RESULTS

PAFR expression protects primary CGNs from C_{16}-PAF but not C_{18}-PAF

PAF has been reported to elicit cell death through PAFR-dependent and PAFR-independent pathways (7, 16–18). To assess potential isoform specificity in these neurotoxic pathways, we compared the sensitivity of primary neuronal cultures endogenously expressing PAFR with cultures derived from null-mutant mice. CGNs from $PAFR^{-/-}$ and $PAFR^{+/+}$ mice were treated for 24 h with 0.5–1.5 μM C_{16}-PAF or C_{18}-PAF in serum-free media containing 0.025% BSA as a lipid carrier. Cell survival was assessed by Live/Dead assay. C_{16}-PAF elicited significant concentration-dependent neuronal loss in $PAFR^{-/-}$ but not $PAFR^{+/+}$ cultures (**Fig. 2A**). C_{18}-PAF initiated neuronal loss in both $PAFR^{+/+}$ and $PAFR^{-/-}$ cultures (Fig. 2B).

To confirm ligand specificity, we performed complementary gain-of-function experiments. $PAFR^{-/-}$ neuronal cultures were adenovirally infected with either EGFP or EGFP and human PAFR and treated with 1 μM C_{16}-PAF or C_{18}-PAF. C_{16}-PAF and C_{18}-PAF elicited neuronal death in $PAFR^{-/-}$ cells infected with EGFP alone (Fig. 2C, D). It should be noted that both C_{16}- and C_{18}-PAF (1 μM) toxicity was enhanced in cultures infected with the control virion (Fig. 2C, D), compared with uninfected cultures (Fig. 2A, B), likely due to the added stress placed on cul-

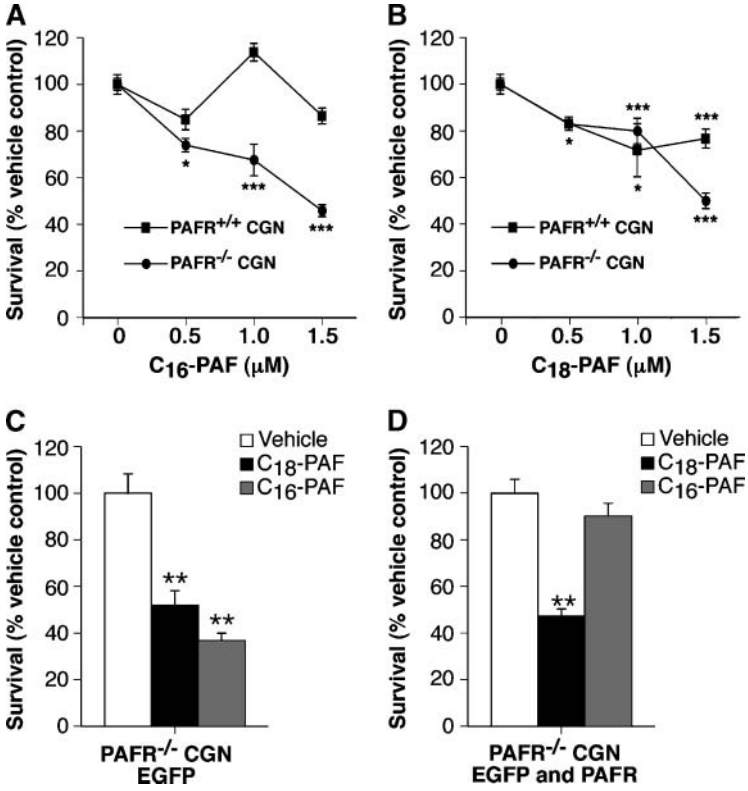

Fig. 2. PAF G-protein-coupled receptor (PAFR)-expressing neurons are resistant to C_{16}-PAF but not C_{18}-PAF toxicity. Cell survival was assessed by Live/Dead viability/cytotoxicity assay. Data are expressed as mean ± SEM. A: Both C_{16}-PAF (0.5–1.5 μM) and C_{18}-PAF (1–1.5 μM) were toxic to $PAFR^{-/-}$ cerebellar granule neurons (CGNs). B: Wild type $PAFR^{+/+}$ neurons were protected from C_{16}-PAF at all concentrations tested but not C_{18}-PAF (0.5–1.5 μM). C: Both C_{16}-PAF (1 μM) and C_{18}-PAF (1 μM) were toxic to $PAFR^{-/-}$ CGNs infected with pAdTrack-CMV adenoviral vector containing enhanced green fluorescent protein (EGFP) alone. Note that PAF-induced neuronal loss was greater in (C) compared with (A) as a result of adenoviral infection. D: Ectopic expression of PAFR using pAdTrack-CMV adenoviral vector containing EGFP and human platelet activating factor receptor-protected $PAFR^{-/-}$ CGNs from C_{16}-PAF but not C_{18}-PAF. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; ANOVA, post hoc Dunnett's *t*-test of PAF treatment versus vehicle treatment (n = 30–75 fields conducted in duplicate experiments).

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TABLE 1. Impact of *sn*-1 chain length on critical micelle concentration of PAF species

PAF Species Member	Critical Micelle Concentration
	μM
C_{16}-PAF	2.5–5.0
C_{18}-PAF	2.5–5.0

PAF, platelet-activating factor. Critical micelle concentration (CMC) was determined on the basis of dynamic light scattering. The mean light scattering from 12 solutions of each PAF species ranging from 10^{-7} to 10^{-3} M was calculated from 10 independent measurements. CMC was defined as the concentration range at which a dramatic increase in light scattering was observed.

tures by the adenoviral infection itself (see supplementary Fig. I). These data show that C_{16}-PAF and C_{18}-PAF elicit neuronal death in the absence of PAFR and that PAFR expression protects neurons from C_{16}-PAF but not C_{18}-PAF toxicity.

To address issues of nonspecific toxicity, we assessed CMC. Altering the chain length of the fatty acid present at the *sn*-1 position of a glycerophospholipid may impact on the physicochemical properties of that molecule and thus the potential for nonspecific lytic effects. By measuring the dynamic light-scattering ability of both C_{16}-PAF and C_{18}-PAF at concentrations from 10^{-3} to 10^{-7} M, we determined that both isoforms exhibit comparable CMCs (**Table 1**). Because the concentrations used in assessing neurotoxicity were well below the CMCs of both lipid species, we concluded that the opposing effects of C_{16}-PAF and C_{18}-PAF on $PAFR^{+/+}$ neurons were the result of differential lipid signaling and not the result of nonspecific lytic effects.

The identity of the PAF *sn*-1 chain dictates whether neurons undergo caspase-dependent apoptosis or caspase-independent neurodegeneration

Canonical apoptotic death is defined by caspase activation. To test whether PAF isoforms elicit neuronal apoptosis, $PAFR^{-/-}$ and $PAFR^{+/+}$ CGNs were exposed to 1 μM C_{16}-PAF, C_{18}-PAF, or vehicle (treatment media). Execu-

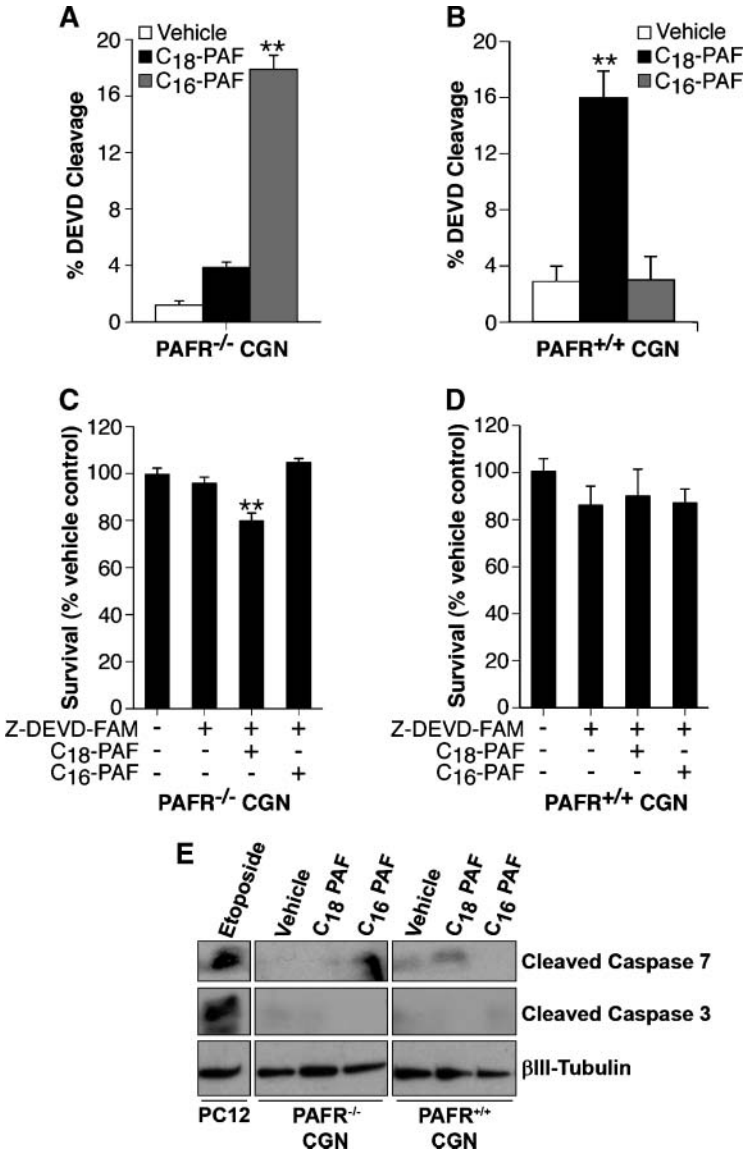

Fig. 3. PAF isoforms differentially induce caspase-dependent death in the presence or absence of PAFR. Executioner caspase 3 and 7 activation was determined using CaspaTag assay. The number of cells capable of cleaving FAM-DEVD-FMK to its fluorescent product after addition of PAF isoforms (1 μM) or vehicle (treatment media) was quantified after 24 h treatment. Data are expressed as the percentage of cells exhibiting DEVD cleavage (mean ± SEM). A: $PAFR^{-/-}$ CGNs exhibited a significant increase in caspase 3/7 activity following C_{16}-PAF but not C_{18}-PAF treatment. B: C_{18}-PAF but not C_{16}-PAF elicited a significant increase in caspase 3/7 activity in $PAFR^{+/+}$ CGNs. C: Pretreatment with the irreversible caspase inhibitor Z-DEVD-FMK (50 μM) protected $PAFR^{-/-}$ CGNs from C_{16}-PAF but not C_{18}-PAF toxicity. D: Z-DEVD-FMK (50 μM) protected $PAFR^{+/+}$ neurons from C_{18}-PAF toxicity. E: To confirm caspase activation, Western analysis for the active caspase 3 and caspase 7 isoforms was performed following treatment with vehicle or PAF isoform (1 μM). C_{16}-PAF activated caspase 7, as indicated by cleavage of the pro-protein to its active form but not caspase 3 in $PAFR^{-/-}$ neurons. C_{18}-PAF activated caspase 7 but not caspase 3 in $PAFR^{+/+}$ neurons. Loading control was neuron-specific βIII-tubulin. The positive control for both caspase 3 and caspase 7 activation was etoposide-treated PC12 cells. ** Statistically significant ANOVA and post hoc Dunnett's *t*-test compared with vehicle with $P < 0.01$ (n = 19–30 fields conducted in triplicate experiments).

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tioner caspase 3/7 activity was determined by quantifying cleavage of the caspase 3/7 substrate FAM-DEVD-FMK (**Fig. 3A, B**). Despite comparable toxicity (Fig. 2A, B), C_{16}-PAF but not C_{18}-PAF elicited a significant increase in the number of caspase-positive $PAFR^{-/-}$ neurons following a 24 h treatment (Fig. 3A). By contrast, in $PAFR^{+/+}$ cultures, a statistically significant increase in caspase 3/7 activity was detected following C_{18}-PAF treatment of $PAFR^{+/+}$ CGNs (Fig. 3B). Consistent with the observed protection conferred by PAFR expression (Fig. 2B, D), C_{16}-PAF did not significantly activate caspase 3/7 in $PAFR^{+/+}$ CGNs (Fig. 3B). These data demonstrate that C_{16}-PAF initiates caspase-dependent apoptosis in the absence of PAFR and that neurotoxicity shifts from a caspase-independent pathway to a caspase-dependent apoptotic pathway when C_{18}-PAF activates PAFR.

To confirm that PAF isoforms differentially signal caspase-mediated apoptosis in the presence or absence of PAFR, $PAFR^{-/-}$ CGNs were pretreated for 15 min with the irreversible caspase 3/7 inhibitor Z-DEVD-FAM prior to PAF addition. The inhibitor was maintained in media for the duration of the PAF challenge. Preventing caspase 3/7 activation protected $PAFR^{-/-}$ CGNs from C_{16}-PAF-induced apoptosis but not C_{18}-PAF-mediated, caspase-independent death (Fig. 3C). In $PAFR^{+/+}$ neurons, caspase inhibition effectively blocked the C_{18}-PAF-induced caspase-dependent apoptosis (Fig. 3D) without impacting on neuroprotection initiated by C_{16}-PAF activation of PAFR (Fig. 3D).

Caspases 3 and 7 cleave the same consensus sequence and the same substrates (36). As such, activity assays are unable to distinguish between these caspases. However, both caspases 3 and 7 must themselves be cleaved to be activated. Using an antibody specific to cleaved caspase 7, we found that C_{16}-PAF activated caspase 7 but not caspase 3 in $PAFR^{-/-}$ neurons, whereas C_{18}-PAF induced caspase 7 but not caspase 3 cleavage in $PAFR^{+/+}$ neurons (Fig. 3E). Taken together, these data demonstrate that C_{16}-PAF signals the activation of executioner caspase 7 independently of PAFR, whereas C_{18}-PAF activation of caspase 7 is PAFR dependent.

CV 3988 inhibits PAFR-mediated cell death activated by C_{18}-PAF

To evaluate the effect of a competitive PAFR antagonist on these cell death pathways, $PAFR^{-/-}$ and $PAFR^{+/+}$ CGNs were exposed to PAF isoforms (1 μM) in the presence of the structural PAF analog CV 3988 (**Fig. 4A, B**). Control cultures were treated with antagonist vehicle (0.1% DMSO) and PAF vehicle (treatment media) for 24 h (Fig. 4A, B). Consistent with our previous results, both C_{16}-PAF and C_{18}-PAF were toxic to $PAFR^{-/-}$ CGNs (Fig. 4A, DMSO) but only C_{18}-PAF elicited neuronal loss in $PAFR^{+/+}$ CGNs (Fig. 4B, DMSO). CV 3988 protected CGNs from C_{18}-PAF-induced PAFR-dependent apoptosis (Fig. 4A, CV3988).

DISCUSSION

In this study, we show for the first time that the length of the *sn*-1 carbon chain of PAF glycerophospholipids dictates neuronal fate. We find that both C_{16}-PAF and C_{18}-PAF are toxic to neurons that lack PAFR but initiate different cell death pathways. When PAFR-deficient neurons are exposed to lipid ligand, C_{16}-PAF triggers a signaling cascade culminating in the activation of executioner caspase 7, whereas C_{18}-PAF-induces caspase-independent neurodegeneration. Neurotoxicity is further modulated by PAFR expression. Ectopic or endogenous PAFR expression pro-

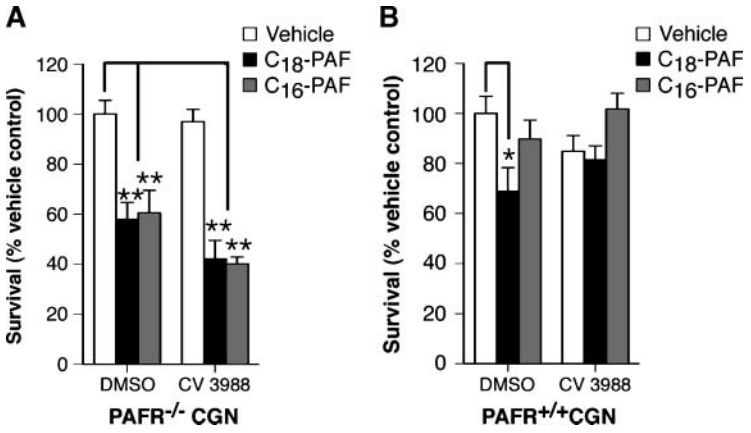

Fig. 4. Cell death pathways initiated by discrete PAF isoforms are inhibited by specific PAF antagonists. $PAFR^{-/-}$ CGNs (A) and $PAFR^{+/+}$ CGNs (B) were treated with C_{16}-PAF (1 μM), C_{18}-PAF (1 μM), or PAF vehicle (treatment media) in the presence of the competitive PAFR antagonist CV 3988 (2 μM) or antagonist vehicle (DMSO). Cell survival was assessed by Live/Dead viability/cytotoxicity assay 24 h after treatment. C_{16}-PAF and C_{18}-PAF were toxic to $PAFR^{-/-}$ neurons (A) when cultures were treated with antagonist vehicle (DMSO). Only C_{18}-PAF was toxic to $PAFR^{+/+}$ neurons treated with antagonist vehicle (B, DMSO). CV 3988 protected CGNs from C_{18}-PAF toxicity in $PAFR^{+/+}$ (B, CV 3988) but not $PAFR^{-/-}$ neurons (A, CX 3988). Data are expressed as mean ± SEM. $^*P < 0.05$; $^{**}P < 0.01$; ANOVA, post hoc Dunnett's *t*-test of all treatments versus PAF vehicle treated in the presence of DMSO (antagonist vehicle). (n = 25 fields conducted in duplicate experiments.)

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tects neurons from C_{16}-PAF, whereas PAFR expression shifts C_{18}-PAF-mediated neurotoxicity from a caspase-independent to a caspase-dependent signaling pathway. These data provide insight into the underlying cell death pathways initiated following PAF challenge that, in part, reconciles reports of both pro- and anti-apoptotic PAF signaling (7, 16–18, 37, 38).

Converging evidence suggests that regional variations in the synthesis of discrete PAF isoforms are associated with neurodegenerative disease (24–28, 30–32). C_{16}-PAF and C_{18}-PAF are the predominant PAF species present in uninjured tissue (39, 40) but differentially accumulate over development of or following injury. Recent studies from our laboratory have shown that C_{16}-PAF concentrations increase during neuronal differentiation (41), whereas local C_{18}-PAF concentrations have only been shown to increase in the injured central nervous system (32). Here, we show that both C_{16}-PAF and C_{18}-PAF trigger neuronal death in PAFR-negative cells, but through isoform-specific apoptotic or neurodegenerative signaling pathways, respectively. Surprisingly, C_{16}-PAF was not toxic when neurons expressed PAFR, whereas C_{18}-PAF promoted neuronal apoptosis, shifting from caspase-independent to caspase-dependent signaling, in PAFR-competent cells. We have yet to test whether the anti-apoptotic actions of C_{16}-PAF-PAFR are sufficient to inhibit PAF death elicited by other isoforms; however, these findings suggest that the changes in predominant *sn*-1 PAF isoforms observed over the course of neurodegenerative disease progression likely impact on the efficacy of strategies to intervene in PAF synthesis (28).

In support of such a combinatorial approach, we find that the PAFR-specific PAF analog and competitive antagonist (42) CV 3988 cannot inhibit all downstream PAF signaling pathways. CV 3988 partially rescues neurons from PAFR-dependent apoptosis resulting from C_{18}-PAF signal transduction, consistent with its PAFR specificity; however, CV 3988 has no impact on PAF signaling in the absence of PAFR, regardless of the PAF isoform present. These data may help explain the mixed success of single PAF antagonists in therapeutic intervention (43–48). We were surprised to see that PAFR antagonism had no impact on the survival of $PAFR^{+/+}$ neurons treated with C_{16}-PAF, inasmuch as we have previously reported pro-apoptotic activity in nonneuronal PC12 cells (18). Reports that CV 3988 displays inverse agonist activity may reconcile this observation (49) if inhibition of constitutive PAFR activity impacts on mitotic cell viability but not postmitotic neurons. Alternatively, C_{16}-PAF may be more effective in competing for PAFR binding with CV 3988 than is C_{18}-PAF.

Previous studies have attributed PAF isoform-specific bioactivity in peripheral tissue to differences in PAFR affinity (10, 13–15, 50, 51). C_{16}-PAF has, at maximum, a 1.5- to 3-fold higher affinity for PAFR than C_{18}-PAF, albeit with some controversy (10, 50–54). PAF stimulation of PAFR activation has been shown to elicit receptor/ligand internalization and rapid degradation of the internalized PAF to *lyso*-PAF (55–57). Thus, ligands with higher PAFR affinity (i.e., C_{16}-PAF) are likely internalized and degraded faster than lower-affinity isoforms (i.e., C_{18}-PAF). The net effect would be to prevent initiation of apoptotic pathways induced by a rise in intracellular C_{16}-PAF concentrations. In nonneuronal cells, pro- and anti-apoptotic PAF actions have been attributed to the relative ratio of NFκB-dependent pro- and anti-apoptotic gene products expressed in response to PAFR stimulation (16, 58). It may be that the lower affinity of C_{18}-PAF for PAFR, and thus the potentially longer PAF half-life compared with C_{16}-PAF, is sufficient to impact upon NFκB induction and that this balance shifts C_{18}-PAF-mediated cell death from a caspase-independent to a caspase-dependent event, as has been shown in other cell systems with other pro-death ligands (59, 60).

In summary, these data illustrate that the *sn*-1 identity of PAF isoforms dictates pro- or anti- apoptotic signaling dependent upon PAFR expression status. Together, these data indicate that strategies to reduce PAF-mediated neuronal loss must take into consideration the regional expression of PAFR over the course of disease etiology as well changes in the predominant PAF isoform present in degenerating tissue.

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