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Molecular Mechanisms Underlying Platelet Activating-Factor-mediated Neuronal Death

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MOLECULAR MECHANISMS UNDERLYING PLATELET ACTIVATING FACTOR-MEDIATED NEURONAL DEATH

Scott D Ryan

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Abstract

Aberrant glycerophosphocholine metabolism is associated with neuronal loss in multiple neurodegenerative diseases. In addition to being central to activation of apoptosis, aberrations in steady state levels of the platelet activating factor (PAF) family of glycerophospholipids influence production and degradation of multiple other bioactive lipids making PAF a promising target for disease intervention. In this thesis, I show that concentrations of C_{16}-PAF, and its immediate metabolite C_{16}-*lyso*-PAF, are elevated in post-mortem Alzheimer disease and transgenic (TgCRND8) mouse temporal cortex. It has been assumed that activation of the PAF G-protein coupled receptor (PAFR) was required for PAF-induced apoptosis. Here, I show that C_{16}-PAF triggers neuronal apoptosis in neurons devoid of PAFR and that PAFR expression protects neurons from C_{16}-PAF but not C_{18}-PAF demonstrating that carbon chain length at the *sn*-1 position has significant impact on biological activity. As PAFR is only expressed in discrete neuronal populations yet PAF-induced neuronal loss is widespread, both PAFR-dependent and PAFR-independent mechanisms must be involved. This thesis sought to elucidate downstream PAFR-independent signalling pathways in neurodegenerative disease. I delineated a novel signal transduction pathway whereby rising concentrations of C_{16}-PAF induce endoplasmic reticulum (ER) stress and activate Cdk5 and Gsk3β, resulting in the phosphorylation of microtubule associated proteins that elicits apoptosis in human neurons devoid of PAFR. Finally, to intervene in PAF toxicity, I identified a novel PAF inhibitor (orsellinic acid) that protects neurons from C_{16}-PAF without impacting on PAFR-mediated neuroprotection. Together, these data provide new mechanistic insight into how aberrant lipid metabolism compromises neuronal function in Alzheimer disease.

Acknowledgements

I would like to thank my supervisor Dr. Steffany Bennett for her support over the last five years. Her constant availability and support were instrumental in the completion of this thesis. I would also like to thank the members of my thesis advisory committee, Dr. David Park and Dr. Michel Chrétien whose comments and suggestions have improved the experimental design of investigations conducted herein. Several people were kind enough to provide reagents and other tools used in experiments described in this thesis, to those people I extend my sincere thanks. To all members of the Bennett laboratory both past and present that added to my graduate experience both personally and professionally; it has been my pleasure to know you all. I would like to give special thanks to Cory Harris whose assistance and advice over the course of my graduate studies was very helpful. Finally, I would like to extend my deepest thanks to my friends and family who supported me throughout this process.

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List of Abbreviations

Aβ	amyloid beta
AD	Alzheimer disease
APP	amyloid precursor protein
ATF-6	activating transcription factor
BH3 only	Bcl-2 homology 3-only,
BIM	Bcl-2 interacting mediator of cell death
BMF	Bcl-2 modifying factor
B-PAF	Bodipy-platelet activating factor
B-*lyso*-PAF	Bodipy *lyso*-PAF
BSA	bovine serum albumin
C_{16}-PAF	1-O-hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine
C_{18}-PAF	1-O-octadecyl-2-acetyl-*sn*-glycero-3-phosphocholine
Cdk5	cyclin-dependent kinase 5
CGN	cerebellar granule neuron
CHO	Chinese hamster ovary
CHOP	C/EBP Homologous Protein
CNS	central nervous system
$cPLA_2$	cytosolic phospholipase A_2
CREB	cAMP response element-binding protein
d_4-C_{16}-PAF	Deuterated C_{16}-PAF
Dab-1	disabled protein 1
DAG	diacylglycerol
DFP	diisopropyl fluorophosphates
DHA	docosahexaenoic acid
DMSO	dimethylsulfoxide
DTNB	5,5'-dithiobis(2)-nitrobenzoic acid
DTT	dithiothreitol
EGFP	enhanced green fluorescent protein
EMEM	Eagle's minimum essential medium
ER	endoplasmic reticulum
ERK	extracellular signal related kinase
ET	ethidium homodimer
FBS	fetal bovine serum
GAPDH	glyceraldehydes phosphate dehydrogenase
GSK3β	glycogen synthase kinase 3β
HDL	high-density lipoprotein
HIV	human immunodeficiency virus
IP_3	inositol 1,4,5-trisphosphate
LC-ESI-MS	high performance liquid chromatography electrospray ionization tandem mass spectrometry
LDL	low-density lipoprotein
L-NAME	N-nitro-L-arginine methyl ester
lyso-PAF	alkyl*lyso*glycerophosphocholine
*lyso*PLC	*lyso*phospholipase C

*lyso*PLD	*lyso*phospholipase D
MAP	mitogen-activated protein
MAPK	mitogen-activated protein kinase
MCAO	middle cerebral artery occlusion
mc-PAF	methyl-carbamyl platelet activating factor
NMDA	N-methyl-D-aspartate
NOS	nitric oxide synthase
PAF	platelet activating factor
PAF-AH	PAF acetylhydrolase
PAFR	platelet activating factor receptor
PARP	poly ADP-ribose polymerase
PBS	phosphate buffered saline
PI3K	phosphatidylinositol 3-kinase
PI3P	phosphatidylinositol-3,4,5-trisphosphate
PKC	protein kinase C
PLA_1	phospholipase A_1
PLA_2	phospholipase A_2
PLC	phospholipase C
PLD	phospholipase D
PP2A	protein phosphatase 2A
PTK	protein tyrosine kinases
S1P	site-1 protease
S2P	site-2 protease
SE	standard error
SFK	Src-family-of-tyrosine-kinase
siRNA	small interfering RNA
ROS	reactive oxygen species
RPMI 1640	Roswell Park Memorial Institute medium
RT-PCR	reverse transcriptase-polymerase chain reaction
$t_{1/2}$	time to reach half maximal concentrations
TLC	thin-layer chromatography
TNFα	tumour necrosis factor α
TUNEL	terminal deoxytransferease dUTP nick-end labeling
UPR	unfolded protein response
VLDLR	very low density lipoprotein receptor
WT	wild-type

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Chapter 1: Bioactive glycerophospholipid mediators in neurodegenerative disease

1.1 Bioactive neuromodulators produced by phospholipase A_2

Phospholipids are unique from other macromolecules. They establish a barrier between the cell and the extracellular space via a lipid bi-layer known as the plasma membrane as well as define the boundary of intracellular organelles wherein specific cellular functions can be carried out in separate yet interrelated microenvironments. Each membrane contains multiple potential signalling molecules critical to cell function and survival that can be rapidly mobilized by the activation of phospholipase A_1 (PLA_1,) A_2, C, and D and non-enzymatic oxidation (Farooqui et al., 2006). Cellular events from protein phosphorylation to gene transcription are regulated through lipid signalling and thus control of lipid signalling, in many ways, dictates cell fate. The first step in the mobilization of bioactive lipid mediators from structural glycerophospholipids requires engagement of the PLA_2 superfamily of enzymes (for a review see (Gelb et al., 1994)). The PLA_2 family of enzymes removes the fatty acyl chain from glycerophospholids generating a free fatty acid and a *lyso*-phospholipid. While this process is a simple one step enzymatic conversion, the potential outcome with respect to downstream signalling events is vast and will be discussed throughout this thesis. The fact that PLA_2 activity is enhanced in multiple neurodegenerative disease states underlines the importance of studying the consequences of aberrant bioactive lipid metabolism on cellular processes (Farooqui and Horrocks, 2006). Here, we will focus on one family of bioactive lipid mediators, the PAF subclass of 1-alkyl, 2-acylglycerophosphocholines generated under disease conditions and their impact on neurodegeneration.

As demonstrated in Fig 1.1, the unifying nature of lipid metabolism makes aberrant lipid signalling difficult to target. Altering steady state levels of any one phospholipid impacts on the synthesis and degradation of multiple fatty acids, phospholipids, and glycerides that, in turn, alter synthesis of downstream inflammatory eicosanoid metabolites such as prostaglandins and leukotrienes (Nicolaou, 2004; Snyder, 1987). Conversely, the therapeutic promise of identifying lipid second messengers that can be targeted to manipulate neurodegenerative disease pathology through their global impact on lipid metabolism represents a new strategy for the treatment of neurodegenerative disease. Clearly, this promise requires rigorous validation. In this thesis, I focus on manipulating synthesis and degradation of the PAF family of glycerophospholipids to highlight how these pathways might be targeted to combat neurodegeneration.

1.2 The PAF family of glycerophospholipids:

Enhanced synthesis of PAF glycerophospholipids has been associated with numerous neurodegenerative conditions including epileptic seizure, encephalitis, ischemia reperfusion injury, HIV-1 associated dementia and Alzheimer-like dementia (Bate et al., 2006; Bate et al., 2004b; Bazan et al., 2002a; Bazan et al., 2002b; Birkle et al., 1998; Farooqui and Horrocks, 2006; Hershkowitz and Adunsky, 1996; Ishii and Shimizu, 2000; McLarnon et al., 2005; Perry et al., 1998a; Stafforini et al., 2003). Common to all neurodegenerative conditions are several key pathways of cell loss that involve generation of pro-inflammatory mediators and oxidative stress (Bazan, 2006).

Figure 1.1: The unifying nature of lipid metabolism. This schematic diagram illustrates the central nature of PAF in phospholipid synthesis and degradation from membrane components to signalling molecules. Cellular impacts of PAF, PAF synthetic precursors, and PAF synthesis bi-products and metabolites are highlighted in red.

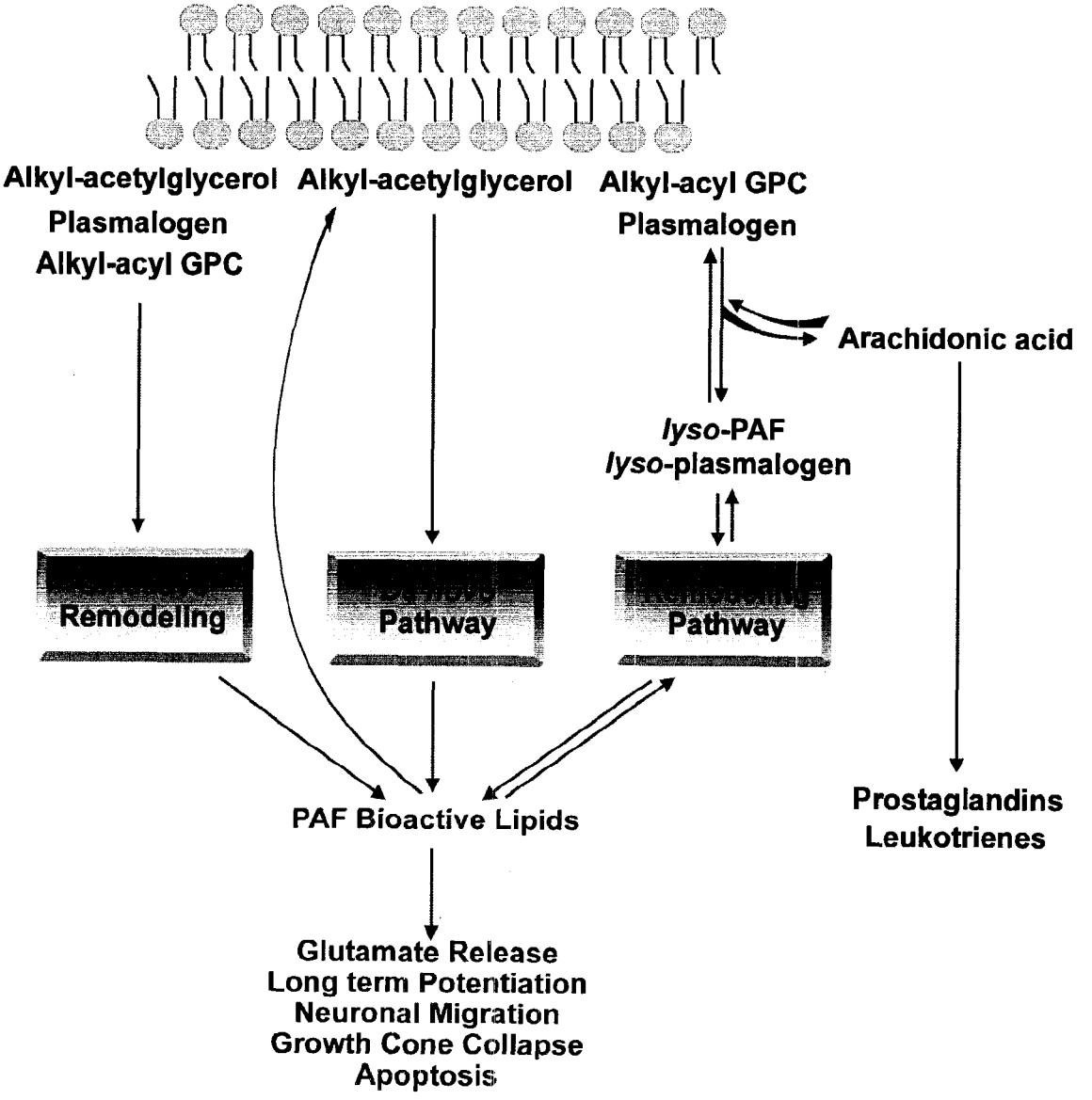
Alkyl-acetylglycerol
Plasmalogen
Alkyl-acyl GPC
Alkyl-acetylglycerol
Alkyl-acyl GPC
Plasmalogen
Arachidonic acid
lyso-PAF
lyso-plasmalogen
Remodeling
Pathway
Pathway
PAF Bioactive Lipids
Prostaglandins
Leukotrienes
Glutamate Release
Long term Potentiation
Neuronal Migration
Growth Cone Collapse
Apoptosis

Figure 1.1

One family of glycerophospholipids that play a critical role in neurodegenerative cell loss is the PAF family. PAF lipids are potent pro-inflammatory autacoids produced via three metabolic pathways (*de novo*, remodelling and oxidative remodelling) (Fig 1.1) (Prescott et al., 2000). As part of the inflammatory response, PAF signals to other cells via PAFR (Sugimoto et al., 1992). Receptor-mediated signalling is transduced by several second messengers that include calcium as well as lipid messengers such as inositol 1,4,5-trisphosphate (IP_3), and diacylglycerol (DAG). Collectively these messengers alter multiple cellular functions, in part, through activation of mitogen-activated protein (MAP) kinase, protein kinase C (PKC), phosphatidylinositol 3-kinase (PI3K), and protein tyrosine kinases (PTK) (for reviews see (Ishii and Shimizu, 2000; Prescott et al., 2000)). PAF can also signal intrinsically with intracellular PAF metabolism mobilizing DAG and mitogen activated protein kinase signalling, independently of PAFR (Baker and Chang, 2001; Brose and Rosenmund, 2002; Miguel et al., 2001).

1.2.1 PAFR-dependent signalling

PAFR is a G-protein coupled receptor with seven trans-membrane spanning domains (Ishii and Shimizu, 2000). These trans-membrane domains are important for PAF binding (Prescott et al., 2000) and their localization within the lipid bi-layer places them in close proximity to exogenously produced PAF as well PAF-like lipids present in the plasma membrane. In the brain, PAFR is highly expressed by glia (microglia and astrocytes) while low levels of expression have been reported in neurons (Mori et al., 1996). Furthermore, those neurons that do express PAFR are diffusely located throughout the hippocampus, cerebellum and neocortex (Bennett et al., 1998).

PAFR-dependent signalling has been the subject of extensive investigation. Activation of PAFR through binding of PAF family members results in rapid turnover of inositol phospholipids by PI3K and PLC to generate phosphatidylinositol-3,4,5-trisphosphate (PI3P) and IP_3 that leads to increased intracellular calcium (Ali et al., 1994; Chao and Olson, 1993; Shukla, 1991; Shukla, 1992). PLC activity removes the phosphocholine group from glycerophospholipids and thus also results in enhanced DAG production. Concurrently, PAFR activation enhances PLD activity (Liu et al., 1994). This enzyme cleaves the choline head group from phospholipids generating phosphatidic acid that can be further metabolised into DAG. As a second messenger DAG that, in and of itself, represents a larger family of multiple isoforms, is known to induce PKC activation (Kishimoto et al., 1980; Takai et al., 1979) that results in downstream MAP kinase activation (Honda et al., 1994; van Biesen et al., 1996). Investigations into the role of MAP kinases in PAFR signalling revealed that the MAP kinase extracellular signal related kinase (ERK) is activated by tyrosine phosphorylation upon PAF exposure (Ali et al., 1994). This was confirmed by ectopic expression of PAFR in Chinese hamster ovary (CHO) cells that responded to PAF by increasing ERK phosphorylation and activity (Honda et al., 1994). MAP kinase activation has ultimately been implicated in the regulation of transcription factor activation underlying synaptic plasticity (Thomas and Huganir, 2004). PAFR-mediated activation of ERK also triggers increased activation of PLA_2 (Lin et al., 1993) that, coupled with rises in intracellular calcium levels, further enhances membrane phospholipid metabolism, and increases PAF synthesis via the remodelling pathway (Fig 1.2). As a means of regulating this PAF synthesis, PKC was found to

Figure 1.2: Remodelling PAF synthesis. This is a schematic illustration of the enzymatic process through which PAF is synthesized from an alkylacylglycerophosphocholine parental lipid. This reversible process centers on *lyso*-PAF as both the immediate precursor of PAF synthesis (blue) and the immediate metabolite of PAF degradation (red).

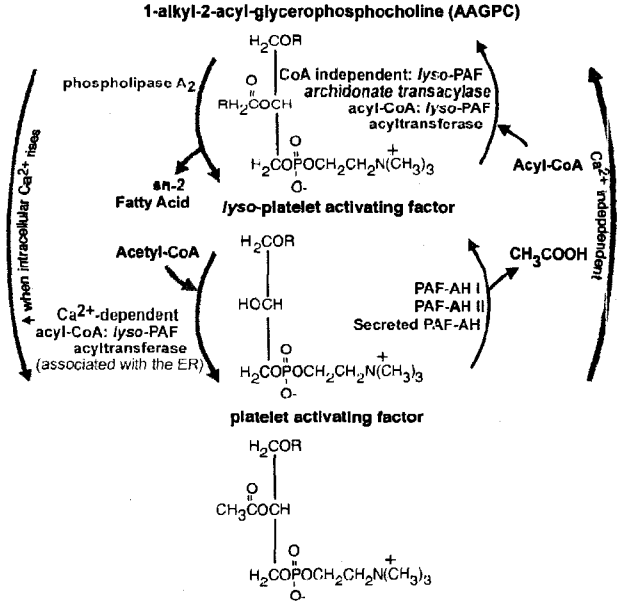
1-alkyl-2-acyl-glycerophosphocholine (AAGPC)
phospholipase A2
sn-2
Fatty Acid
CoA independent: lyso-PAF
archidonate transacylase
acyl-CoA: lyso-PAF
acyltransferase
Acyl-CoA
lyso-platelet activating factor
Acetyl-CoA
Ca2+-dependent
acyl-CoA: lyso-PAF
acyltransferase
(associated with the ER)
PAF-AH I
PAF-AH II
Secreted PAF-AH
CH3COOH
platelet activating factor
when intracellular Ca2+ rises
Ca2+ independent

Figure 1.2

phosphorylate and desensitize PAFR creating a negative feedback loop following PAFR activation (Ali et al., 1994).

PAFR activation has been repeatedly associated with neuronal loss. PAF production following excitotoxin challenge enhances glutamate release (Bazan and Allan, 1996). Excitotoxic injury has been shown to impact specifically on PAFR-expressing neurons (Bennett et al., 1998). Furthermore, neuronal loss due to glutamate and nitric oxide production has been critically linked to PAFR activation in cortical neurons (Xu and Tao, 2004). Given the diffuse expression of PAFR in the brain, however, these mechanisms likely underlie selective neuronal loss. Thus, the widespread loss of neurons, including populations devoid of PAFR, in neurodegenerative pathologies, implicates other PAFR-independent mechanisms. These signalling pathways have yet to be elucidated.

1.2.1 PAFR-independent signalling

Much less is known about PAF signalling in the absence of PAFR given the prevailing assumption that all PAF signalling is PAFR mediated. Three putative PAF binding sites have been identified in the rat cerebral cortex (Marcheselli et al., 1990); a plasma membrane binding site and two microsomal PAF binding sites. The plasma membrane binding site is likely PAFR and it has been argued that at least one of the microsomal PAF binding sites results from ligand-displacement from PAFR following ligand-activated internalization (Hurst et al., 1999). The other site has yet to be identified. Nuclear PAF binding sites have also been characterized in non-neuronal cell types. These sites may reflect a novel translocation of PAFR to the nuclear membrane underlying the effects of PAF on gene transcription (Baker and Chang, 2000b; Lukiw et al.,

1998, Gobeil et al., 2006; Zhu et al., 2006, Marrache et al., 2002). Nuclear PAFR localization has yet to be observed in neurons. Nonetheless, PLC mediated DAG generation following exogenous phospholipid administration is maintained in isolated neuronal nuclei (Baker and Chang, 2001). While this is likely to neutralise rising intra-nuclear levels of PAF and other bioactive lipids by shuttling excess PAF down other lipid pathways, the DAG isoforms produced may still lead to PKC activation in intact neurons devoid of PAFR. To this end, Tsuda et al., 2007 has shown that in $PAFR^{-/-}$ dorsal root ganglion neurons, PAF-mediated ERK phosphorylation activation is impeded relative to wild type neurons but still occurs. Furthermore, Chen et al., 2003b have shown that PAF mediated ERK activation occurs in cell systems that express mutant PAFR either incapable of internalizing or incapable of G-protein signalling, supporting the notion that PAF also signals independently of its receptor. Thus DAG mediated ERK activation may underlie PAF signalling in the absence of PAFR.

The neurotoxic effects of PAF are likely signalled through both PAFR-dependent and –independent pathways. As elevated PAF levels are neurotoxic in a manner similar to that observed under conditions of neurotransmitter excitotoxicity (Bennett et al., 1998; Clark et al., 1992, 1994; Zhu et al., 2004), it was assumed that PAFR activation was required for PAF-induced apoptosis (Ishii and Shimizu, 2000). This is, however, not absolute as PAFR expression can prevent PAF-induced cell death *in vitro* (Brewer et al., 2002). In epidermal and PC12 adrenal-derived cells, ectopic PAFR expression exacerbates cell death induced by chemotherapeutic agents but protects cells from tumour necrosis factor α, TRAIL, and PAF itself (Brewer et al., 2002; Li et al., 2003; Southall et al., 2001). These opposing effects likely depend upon PAF mediated changes in gene

transcription and the relative ratio of NFκB-dependent pro- and anti-apoptotic gene products elicited in response to an external apoptotic inducer and PAF (Li et al., 2003). Moreover, recent studies indicate that PAFR expression is downregulated following ischemic insult before apoptotic signal transduction is initiated and subsequently restored in penumbral neurons that survive the ischemia/reperfusion event (Zhang et al., 2007). As elevated PAF levels are attributed to the progression of multiple neurodegenerative diseases (see section 1.6) regulation of PAF synthesis and degradation, as well as receptor-dependent and receptor-independent signalling is of the utmost importance. The impact of elevated PAF levels in central nervous system (CNS) neurons that do not express PAFR, however, has not been systematically assessed and became a primary focus of investigation in this thesis.

1.3 PAF synthesis in the brain

PAF is synthesized primarily by neurons and microglia (Aihara et al., 2000; Mori et al., 1996). PAF synthesized by neurons functions as a retrograde neurotransmitter released by the post-synaptic cell in response to glutamate mediated activation of the NMDA receptor to act on PAFR expressed by the pre-synaptic neuron and signal further glutamate release during long-term potentiation (Aihara et al., 2000; Clark et al., 1992; Kato et al., 1994; Wieraszko et al., 1993). The synthesis and secretion of PAF by microglia occurs in response to inflammatory stimuli to activate astrocytes and further recruit additional microglia to the site of injury (Aihara et al., 2000; Teather and Wurtman, 2003). In hyper-inflammatory neurodegenerative conditions such as Alzheimer disease (AD) and HIV dementia, chronic microglia activation likely leads to PAF overproduction

and secretion (Gelbard et al., 1994; McLarnon et al., 2005; Walker and Lue, 2005). This combination of inflammatory PAF synthesis and secretion by microglia, coupled with PAF synthesis and secretion by neurons following excitotoxic challenge, can result in PAF excess and leads to apoptotic neuronal loss (Bennett et al., 1998; Lustig et al., 1992; Pulliam et al., 1998). Underlying signalling pathways mediating PAF-induced neurotoxicity are unclear.

1.3.1 Remodelling PAF synthesis

The remodelling pathway is the primary pathway of PAF synthesis in neurons (Fig 1.2) (Prescott et al., 2000; Snyder, 1987). It is a cyclic pathway that centres on *lyso*-PAF (alkyllysoglycerophosphocholine) as both the immediate PAF metabolite and the immediate precursor of PAF synthesis. In this pathway, PAF originates from the cellular pool of alkylacylglycerophospholipids. These lipids are most abundant in microsomal membranes (Baker and Chang, 1996). Alkylacylglycerophospholipids are acted upon in this pathway by cytosolic PLA_2 enzymes that cleave the fatty acid from the *sn*-2 position of the glycerol backbone (Balboa et al., 2002). The *sn*-2 position of alkylacylglycerophospholipids is most commonly occupied by a polyunsaturated fatty acid (Gaposchkin and Zoeller, 1999; Thomas et al., 1990). To this end, the most common alkylacylglycerophospholipid to be shuttled into the remodelling pathway is alkylarachidonylglycerophosphocholine (Prescott et al., 2000). PLA_2 action on alkylarachidonylglycerophosphocholine produces *lyso*-PAF and arachidonic acid (Fig 1.2). This is also a key step in prostaglandin synthesis and further propagates the inflammatory response as arachidonic acid can be metabolized by Cox-2 into prostaglandin E_2 (Fig 1.1) (Bazan, 2006).

Lyso-PAF is a substrate for acetyl-CoA: *lyso*-PAF acetyltransferase which, facilitates the transfer of an acetyl group to *lyso*-PAF generating PAF (Fig 1.2) (Shindou et al., 2007). This enzyme localizes to the ER and is calcium regulated (Record and Snyder, 1990). Under basal conditions acetyl-CoA: *lyso*-PAF acetyltransferase preferentially transfers arachidonic acid to *lyso*-PAF from arachidonyl-CoA (Shindou et al., 2007). In this respect this enzyme is responsible for the synthesis of the parental lipid alkylarachidonylglycerophosphocholine and membrane biogenesis. As demonstrated using lipopolysaccharide as a stressor, when intracellular calcium levels are elevated acetyl-CoA becomes the preferred enzymatic substrate for acetyl-CoA: *lyso*-PAF acetyltransferase making the synthesis of PAF favoured (Fig 1.2) (Record and Snyder, 1990; Shindou et al., 2007). Although elevation of intracellular calcium is essential for propagation of the cellular stress response, should PAF concentrations already be high due to secretion by activated microglia, further acetylation of *lyso*-PAF to PAF may be undesired. In this instance, excessive PAF synthesis would lead to apoptotic loss of uninjured neurons thus implicating PAF in the pathology of neurodegenerative disease.

As such, alternative routes exist for exit of PAF and *lyso*-PAF from the remodelling pathway. Regeneration of membrane phospholipids from *lyso*-PAF can be achieved by CoA independent *lyso*-PAF transacylase although this enzyme also has high affinity for 1-O-alk-1'-enyl-2-*lyso*-*sn*-glycero-3-phosphoethanolamine and thus is also directly involved in plasmalogen synthesis (Lee et al., 1992). PLC and lysophospholipase C (*lyso*PLC) are able to remove the phosphocholine group from PAF and *lyso*-PAF respectively generating di- and mono-glycerides (Fig 1.3). These enzymes inhibit PAF signalling and have been associated with cessation of the inflammatory response in several sys-

Figure 1.3: *De Novo* PAF synthesis. This illustration depicts the process whereby alkylysoglycerophosphate is enzymatically modified to PAF through generation of two stable membrane components; alkylacetylglycerophosphate and alkylacetylglycerol (blue). PAF can be converted back into alkylacetylglycerophosphate and alkylacetylglycerol by enzymatic actions of PLD and PLC respectively (red).

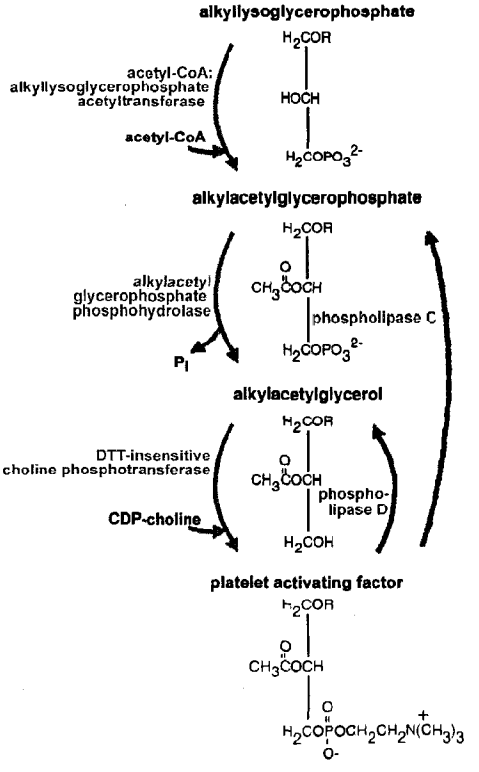
alkyllysoglycerophosphate
acetyl-CoA:
alkyllysoglycerophosphate
acetyltransferase
acetyl-CoA
alkylacetylglycerophosphate
alkylacetyl
glycerophosphate
phosphohydrolase
phospholipase C
alkylacetylglycerol
DTT-insensitive
choline phosphotransferase
CDP-choline
phospho-
lipase D
platelet activating factor

Figure 1.3

tems (Baker and Chang, 2000a; Baker and Chang, 2000b; Wang and Dennis, 1999). Enzymatic generation of DAG second messengers by PLC, however, leads to activation of the ERK/MAPK signalling cascade that may also be undesired. PLD and microsomal lysophospholipase D (*lyso*PLD) are able to remove the choline group from PAF and *lyso*-PAF respectively generating alkylacetylglycerophosphate and alkyllysoglycerophosphate (Fig 1.3). This metabolic route may be advantageous in that these products would maintain the PAF precursor pool as substrates of the *de novo* PAF synthesis pathway.

1.3.2 De novo PAF synthesis

De novo PAF synthesis is a pathway by which alkyllysoglycerophosphate is acetylated (Blank et al., 1990; Lee et al., 1986) and dephosphorylated (Snyder, 1987) prior to the addition of a phosphocholine head group by dithiothreitol (DTT) insensitive cholinephosphotransferase to generate PAF (Fig 1.3) (Snyder, 1997; Woodard et al., 1987). While PAF generation in this manner is believed to be essential in spermatozoa and embryo development (Muguruma and Johnston, 1997; Roudebush, 2001; Roudebush and Diehl, 2001), it may serve a different function in the mature neuron. This pathway may serve to store PAF intermediates at times of PAF abundance. Alkyllysoglycerophosphate is found in microsomal membranes and can be produced by enzymatic action of *lyso*PLD on alkyllysoglycerophosphocholine (*lyso*-PAF) by removal of the choline group (Wang and Dennis, 1999). As the kinetics of *de novo* PAF synthesis are significantly slower than that of the remodelling pathway this process may serve as a means of PAF precursor storage in the mature neuron. Rather than shuttling *lyso*-PAF into the remodelling pathway for immediate PAF synthesis, conversion to alkyllysoglycerphosphate would allow

for further acetylation by acetyl-CoA: alkyllysoglycerphosphate acetyl transferase (Fig 1.3) (Blank et al., 1990; Lee et al., 1986). The resulting alkylacetylglycerophosphate, that may be generated by PLD action on PAF directly, is a stable membrane component and may remain as such until dephosphorylation by alkylacetylglycerophosphate phosphohydrolase to alkylacetyl glycerol for further processing to PAF (Fig 1.3) (Snyder, 1987). This *lyso*PLD mediated shuttling of *lyso*-PAF away from PAF synthesis may prove to be essential should PAF levels become pathophysiological as seen in multiple neurodegenerative disorders. In ischemic brain, where PAF levels are known to increase (Nishida and Markey, 1996), *lyso*PLD is inhibited by increased intracellular calcium, fatty acid and *lyso*-phosphatidic acid imbalance (Baker and Chang, 2000a; Baker and Chang, 2000b), thus inhibiting a potential neuroprotective metabolic response.

1.3.3 Oxidative remodelling of PAF related lipid mediators

Oxidative remodelling synthesis of PAF is a process whereby membrane phospholipids are oxidized resulting in PAF and PAF-like lipids (Fig 1.4). This process involves the reaction of polyunsaturated fatty acid side chains with a reactive species such as oxygen. This means of synthesis likely underlies PAF production following reperfusion injury (Umemura et al., 1997) as the rapid return of oxygen to the brain following reperfusion has been shown to oxidize arachidonic acid containing membrane phospholipids (Murin et al., 2001; Wakatsuki et al., 1999). Other means of oxidative PAF generation in neurodegenerative disorders such as AD and prion-associated diseases may involve protein-lipid interaction (Barnham et al., 2006; Hayashi et al., 2007; Lau et al., 2006; Murray et al., 2007). Non-fibrillar amyloid beta (Aβ) has been shown to accelerate

Figure 1.4: Oxidative remodelling generation of PAF. This illustration depict the non-enzymatic generation of PAF by means of oxidative cleavage of the fatty acid present at the sn-2 side position of alkylacylglycerophosphocholine (Green). Cleavage of the sn-2 side chain may produce fatty acids up to three carbons in length that possess PAF bioactivity.

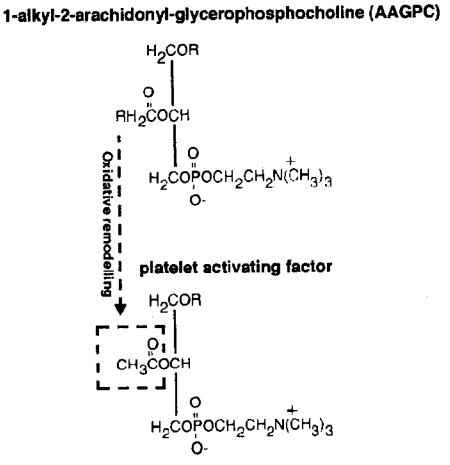
1-alkyl-2-arachidonyl-glycerophosphocholine (AAGPC)
Oxidative remodelling
platelet activating factor

Figure 1.4

membrane lipid oxidation by physiological concentrations of ascorbate and submicromolar concentrations of copper(II) ion (Murray et al., 2005). In turn, membrane lipid oxidation by Aβ has been shown to enhance amyloidosis (Mandal et al., 2004) setting up a pathogenic negative feedback loop.

Oxidative lipid remodelling results in the release of a small fatty acyl "tail" leaving a truncated phospholipid mediator (Fig 1.4) (Prescott et al., 2000). As polyunsaturated fatty acids are energetically favoured in this reaction (and most commonly present at the *sn*-2 position of the glycerol backbone), these truncated phospholipid mediators can possess PAF-like bioactivity. Key structural features that underlie PAF activity include an alkyl-linked 16 carbon fatty acid at the *sn*-1 position, an acetyl-group at the *sn*-2 position, and phosphocholine head group at the *sn*-3 position (Prescott et al., 2000). These characteristics define PAF affinity for PAFR (Ishii and Shimizu, 2000). Varying fatty acid chain length, degree of saturation, ether linkage or head group type decreases affinity for PAFR and potency resulting in altered PAF bioactivity (Carolan and Casale, 1990; Hwang et al., 1989; Ishii et al., 1997; Marathe et al., 1999; Snyder, 1987). PAF-like lipids with longer *sn*-2 carbon chains than canonical PAF have also been shown to exert direct cytotoxic effects through activation of mitochondrial-dependent death pathways (Chen et al., 2007). Thus, aberrant synthesis of both PAF species and PAF-like oxidative products may result in signalling that directly impacts on disease pathology.

While PAF isoforms are defined by an ether-linked *sn*-1 chain, a *sn*-2 acetyl group, and a *sn*-3 phosphocholine (Fig 1.2), other related PAF species also exert considerable bioactivity. The plasmalogen family is defined by an ether-linked alkene at the *sn*-1 position. The major constituents of the plasmalogen family are plasmenylcholine and

plasmenylethanolamine (Gaposchkin and Zoeller, 1999) and are substrates for oxidative remodelling into PAF-like lipids. Plasmalogens have PAF bioactivity when truncated at the *sn*-2 position (Nakayama et al., 1988; O'Flaherty et al., 1994). This can occur through enzymatic acetylation of *lyso*-plasmenylethanolamine or through oxidative remodelling of plasmenylcholine and plasmenylethanolamine (Baker and Chang, 2000b; Khaselev and Murphy, 1999, 2000a,b). Alternatively alkylacylethanolamine may also be remodeled into a PAF-like lipid although this lipid species is much less abundant in the cell as the majority of ethanolamine is contained within the plasmalogen pool (Gaposchkin and Zoeller, 1999). These mediators have affinity for PAFR that is 10-100 fold lower than putative PAF species (Prescott et al., 2000), however, given their abundance in brain tissue and their potential to elicit second messenger production independently of PAFR, the importance of their production cannot be ignored.

1.4 PAF Degradation

Rapid inactivation of PAF signalling has been shown to be neuroprotective following acute rises in intracellular PAF levels (Ogden et al., 1998; Umemura et al., 2007). While it has been discussed that PLC and PLD (Fig 1.3) in addition to *lyso*PLC and *lyso*-PLD can carry out this function, the primary and most rapid means of terminating PAF signalling is by deacetylation of the *sn*-2 side chain and production of *lyso*-PAF. Enzymes responsible for this action are group VIIA,B and group VIIIA,B PLA_2 enzymes, or PAF-acetylhydrolases (PAF-AHs) (Fig 1.2) (Schaloske and Dennis, 2006). Three PAF-AH enzymes have been identified consisting of two intracellular PAF-AH isoforms and one extracellular PAF-AH isoform.

1.4.1 Extracellular PAF-AH

Plasma PAF-AH, also known as lipoprotein PLA_2, is a 45 kDa monomer secreted into circulation by endothelial and hematopoietic cells (Asano et al., 1999; Tjoelker et al., 1995). While plasma PAF-AH protein has not been reported in neurons (Arai et al., 2002), the enzyme has nonetheless been associated with neurodegenerative disease progression. In so much as stroke develops as a cardiovascular ailment, plasma PAF-AH has been critically linked to its progression. Localized to lipoproteins, low-density lipoprotein (LDL) and high-density lipoprotein (HDL) (Okamura et al., 2007), plasma PAF-AH has been shown to hydrolyze oxidized phospholipids associated with circulating LDL and is thus believed to be anti-atherosclerotic, albeit with controversy (Carlquist et al., 2007). Other investigators have shown that plasma PAF-AH increases the *lyso*phosphotidic acid composition of LDL exacerbating inflammation and, as such, may actually augment the progression of atherosclerosis and stroke (Karabina and Ninio, 2006). The enzyme recognizes PAF and PAF analogs with short to medium *sn*-2 chains including oxidatively modified phospholipids. Oxidized LDL is an abundant source of these molecules in circulating plasma and can result in PAF induced hyper-inflammation associated with obesity and diabetes (Chen et al., 2003a; de Castro et al., 2007; Tzotzas et al., 2007).

1.4.2 Intracellular PAF-AH

Two cytosolic PAF-AH enzymes are present within the cell, PAF-AH I and PAF-AH II (Arai et al., 2002). PAF-AH II is a single 40 kDa polypeptide specific for phospholipids with both alkyl and acyl linked fatty acids at the *sn*-1 position and with moder-

ate length *sn*-2 chains as well as short chain diacylglycerols, triacylglycerols, and acetylated alkanols (Hattori et al., 1996; Min et al., 2001). Ectopic expression reduces cell death triggered by oxidative stress (Marques et al., 2002; Matsuzawa et al., 1997). *In vivo* studies using PAF-AH II transgenic mice have shown that overexpression significantly reduces tissue damage following middle cerebral artery occlusion (MCAO) and loss of PAF-AH II significantly increases susceptibility to membrane oxidative damage (Kono et al., 2007; Umemura et al., 2007). The multiple possible substrates of PAF-AH II make it an attractive candidate for PAF signal intervention following reperfusion injury as both PAF and oxidatively generated PAF-like family members would be inactivated through PAF-AH II engagement.

Cytosolic PAF-AH I cleaves the acetyl group at the *sn*-2 position of PAF and PAF-like lipids of either the glycerophosphocholine or glycerophosphoethanolamine classes (Manya et al., 1999). The enzymatic complex is a tetramer composed of 29 kDa and 31 kDa α_1 and α_2 catalytic subunits (Hattori et al., 1993). The α subunits form homodimers or heterodimers that complex with homodimers of the non-catalytic 45 kDa regulatory β □subunit identified as LIS1 (Ho et al., 1997; Tarricone et al., 2004). Genetic links between LIS1 and abnormal cerebral development raised the possibility that PAF and PAF acetylhydrolase activity regulate cortical lamination (Hattori et al., 1994). In the brain, mutations of the LIS1 gene result in aberrant cortical layer formation and lisencephaly know as Miller Dieker Syndrome (Reiner et al., 1995; Reiner et al., 1993). These two lines of evidence implicate PAF-AH I with a more fundamental role in development that has yet to be fully investigated. PAF-AH I function may be key linking in-

tracellular PAF accumulation to the progression of many neurodegenerative disease conditions. This possibility will be explored in this thesis.

1.4.3 Multifunctional roles of LIS1

LIS1 protein expression is crucial to cerebral development, neuronal migration, axonal transport and axonal growth (Reiner et al., 1993; Liu et al., 2000; Smith et al., 2000). In mammals, LIS1 is enriched in neurons relative to other cell types and is most abundant as part of the dynein motor complex (Smith et al., 2000). Within this complex, LIS1 co-localizes with doublecortin to enhance microtubule polymerization (Caspi et al., 2000). In mature neurons LIS1 overexpression induces retrograde movement of cytoplasmic dynein and leads to peripheral accumulation of microtubules (Smith et al., 2000). LIS1 deficiency on the other hand deregulates the cytoskeleton and results in microtubule catastrophe (Kholmanskikh et al., 2003; Sapir et al., 1997). LIS1 has also been found to colocalize with both dynein and dynactin at the mitotic kinetochore of cerebral progenitors (Faulkner et al., 2000). Loss of LIS1 function leads to uncoupling of the nucleus and centrosome and impaired mitotic division (Shu et al., 2004; Tsai et al., 2005). Dynein function in this manner was found to be regulated by NUDEL protein that facilitates the interaction of LIS1 with dynein (Shu et al., 2004).

With respect to PAF-AH function and activity, crystalography studies of mouse NUDEL have revealed that NUDEL competes with the PAF-AH I α_2 homodimer for LIS1 binding (Kitagawa et al., 2000; Tarricone et al., 2004). LIS1 has been shown to accelerate the PAF-AH I catalytic complex when the complex exists as a PAF-AH I α_2 homodimer (Manya et al., 1999). Recent investigation as to whether PAF-AH I α_2

homodimer might compete for LIS1 binding in transfected CHO cells have shown that α_2 overexpression can pull LIS1 from the dynein complex effectively inactivating dynein, uncoupling the centriole from the nucleus and halting cell division (Yamaguchi et al., 2007). This mechanism may exist for the purpose of enhancing PAF catalysis under hyper-inflammatory conditions. In the case of neurodegenerative disease, however, where the inflammatory stimulus is chronic, loss of LIS1 mediated dynein function may accentuate disease pathology. This hypothesis will also be tested in this thesis.

1.5 PAF and apoptotic signalling

Apoptosis is a process by which a cell undergoes regulated disassembly and death in a controlled manner (Vila and Przedborski, 2003). This process is a normal cellular function that is essential for the survival of an organism. Regardless of the initiating stressor, apoptosis leads to convergence of several key death pathways. Classic apoptosis is characterized by chromatin condensation, phosphatidylserine exposure, cytoplasmic shrinkage, zeiosis and the formation of apoptotic bodies (Leist and Jaattela, 2001). These pathways often involve organelle specific activation of caspases and calpains as well as mitochondrial membrane permeabilization (Ferri and Kroemer, 2001). The mitochondria is of specific interest to apoptosis initiated by oxidatively generated PAF-like lipids while the ER and Golgi are particularly important to apoptotic signalling potentially associated with enzymatic PAF synthesis. A general summary PAF-related mediators involvement in apoptosis is provided in Fig 1.5.

Figure 1.5: PAF related lipid mediators in apoptogenic signal transduction.
Apoptotic signalling can be initiated via extrinsic FAS-receptor activation or by intrinsic apoptotic signals. The later can result from aberrations in bioactive lipid generation induced by oxidative stress. Among these bioactive lipids are PAF related lipid mediators. This schematic depicts the apoptotic process highlighting (red) the involvement of PAF-related lipid mediators and their downstream effectors in murine neurons.

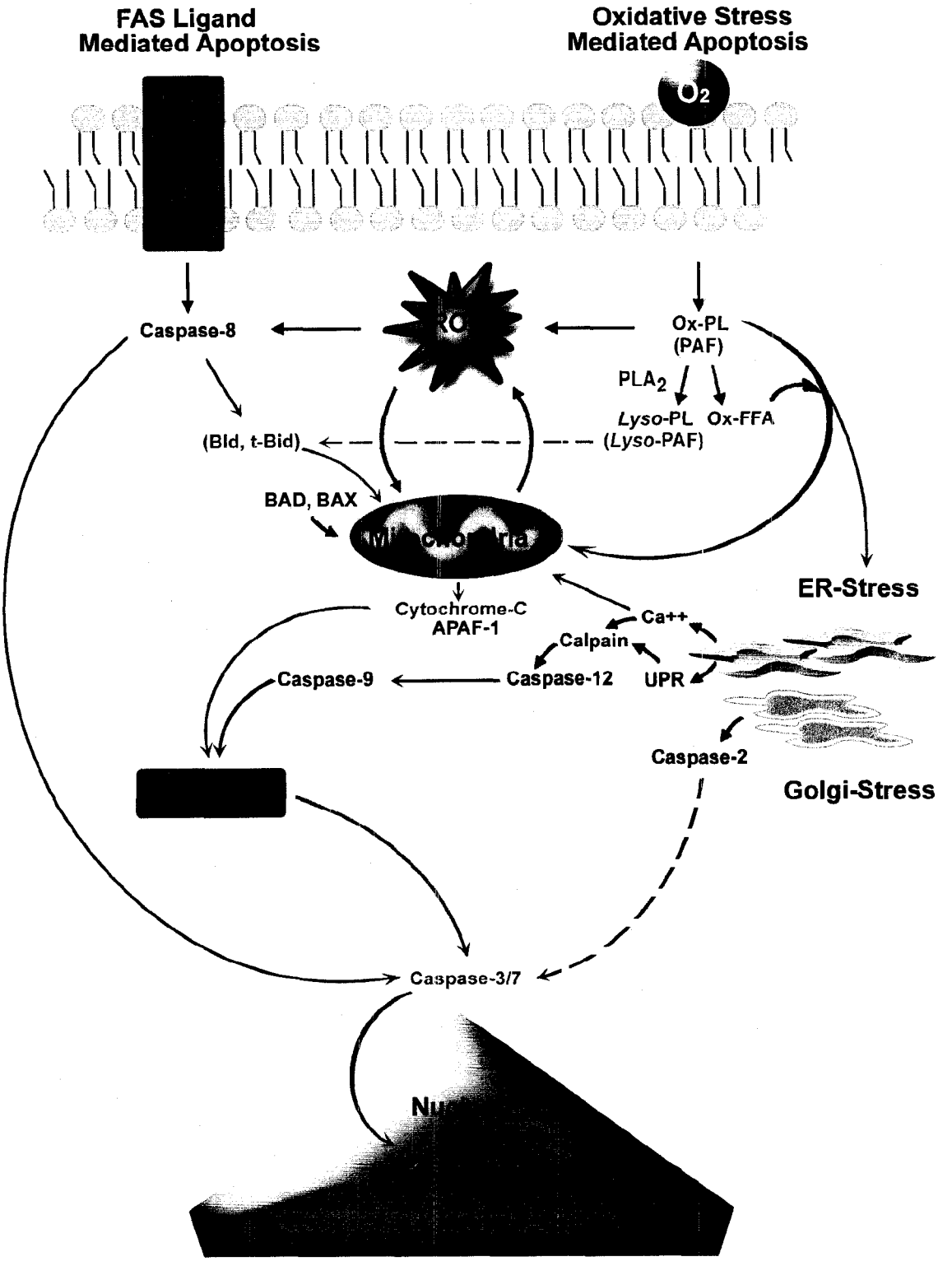
FAS Ligand
Mediated Apoptosis
Oxidative Stress
Mediated Apoptosis
O2
Caspase-8
Ox-PL
(PAF)
PLA2
Lyso-PL
Ox-FFA
(Bld, t-Bid)
(Lyso-PAF)
BAD, BAX
ER-Stress
Cytochrome-C
APAF-1
Ca++
Calpain
Caspase-9
Caspase-12
UPR
Caspase-2
Golgi-Stress
Caspase-3/7

Figure 1.5

1.5.1 Mitochondrial stress response

As the site of lipid beta-oxidation, mitochondria are a major source of reactive oxygen species (ROS) production and degradation (Ott et al., 2007). Mitochondrial impairment can exacerbate cellular damage. Exogenous ROS, as occurs following ischemia, interacts with membrane phospholipids resulting in oxidative remodelling to PAF-like lipid species. Recent investigations into the type of stressor able impede mitochondrial regulation of ROS production point to oxidized phospholipids as a major factor (Chen et al., 2007). Oxidized phospholipids alter mitochondrial functions, such as oxidative phosphorylation, membrane permeability and calcium regulation (Albano et al., 1991; Landar et al., 2006; Zini et al., 2007). Of these oxidized phospholipids, alkylglycerophosphocholines have been shown to be the primary subclass responsible for mitochondrial damage. Furthermore, ectopic overexpression of PAF-AH II suppressed mitochondrial apoptotic signalling following oxidized phospholipid exposure (Chen et al., 2007). (Matsuzawa et al., 1997) has shown that PAF-AH II translocates to the plasma membrane following oxidative stress thus preventing the oxidative modification of bioactive phospholipids and the ensuing death cascade (Marques et al., 2002). Collectively, these data link oxidative remodelling generation of PAF-like lipids with the mitochondrial-activated death cascade.

The primary role of mitochondria in the apoptotic signalling cascade induced by ROS and oxidized lipids is induction of mitochondrial permeability transition. The mitochondrion plays a crucial role in the regulation of intracellular calcium levels. When intracellular calcium levels exceed the threshold of mitochondrial retention, however, they are released via the voltage-dependent anion channel known as the mitochondrial perme-

ability transition pore (Crompton, 1999). Pore opening is followed by swelling of the mitochondrial matrix, rupture of the outer mitochondrial membrane, and the release of cytochrome *C* from the intermembrane space. Cytochrome *C* release initiates formation of the apoptosome complex formed from cytosolic APAF-1, cytochrome *C*, ATP, and procaspase 9 (Riedl and Salvesen, 2007). Apoptosome formation enhances caspase 9 activity leading to activation of executioner caspases 3 and 7 (Riedl and Salvesen, 2007). The calcium threshold for mitochondrial permeability transition decreases following oxidative stress (Kowaltowski et al., 1996a; Kowaltowski et al., 1996b). Cytochrome *C* is capable of binding acidic phospholipids and can be found bound to the inner mitochondrial membrane through association with the phospholipid cardiolipin (Tuominen et al., 2002). Oxidation of cardiolipin or calcium binding of cardiolipin facilitates the release of cytochrome *C* from the mitochondrion and activation of the caspase cascade (Ott et al., 2002; Petrosillo et al., 2001).

An alternative means of inducing mitochondrial dysfunction is through extrinsic activation of the FAS receptor that involves engagement of members of the Bcl-2 family of proteins, notably Bax, Bak, and Bid as well as through ER stress (Antonsson et al., 1997; Eskes et al., 2000; Korsmeyer et al., 2000; Wei et al., 2000). These alternate pathways may be exacerbated by oxidative remodelling of PAF family members. During apoptosis the Bcl-2 protein Bid is cleaved to t-Bid by either by FAS receptor activated caspase 8 or ER stress-activated calpain (Li et al., 1998; Mandic et al., 2002). t-Bid then translocates to the mitochondria where it facilitates oligomerization of Bax and Bak to induce mitochondrial permeability transition, thus triggering release of pro-apoptotic mitochondrial proteins such as cytochrome *C* (Narita et al., 1998). Investigation into the

regulators of Bid and t-Bid translocation found that both isoforms have lipid transfer activity. t-Bid is targeted to the mitochondria through *lyso*glycerophospholipid binding (although the lipid species involved have yet to be identified at the molecular level) (Esposti et al., 2001). Once at the mitochondria, lipids are deposited into the mitochondrial membrane completing the translocation process (Esposti et al., 2001). *Lyso*glycerophosphocholine accumulation in the outer mitochondrial membrane is associated with permeability transition. Similarly, in its non-truncated form, Bid has been suggested to be involved in the apoptotic process by transfering *lyso*glycerophosphocholines into the outer mitochondrial membrane where they accumulate during apoptosis (Goonesinghe et al., 2005). Furthermore, cells expressing mutant forms of Bid unable to facilitate this transfer are resistant to apoptosis (Goonesinghe et al., 2005). A dramatic increase in oxidatively generated PAF species would lead to increased *lyso*glycerophosphocholine production by PAF-AH. Thus, PAF may confer pro-apoptotic signalling through non-truncated Bid in addition to promoting t-Bid translocation. Recent studies have shown that a potential PAF-AH II enzymatic product, *lyso*phosphatidic acid, also enhances FAS receptor expression on the surface of the plasma membrane of leukocytes by inducing Cox-2 activity (Kang et al., 2006) thus making the cell more susceptible to extrinsic FAS-receptor-mediated death signalling and Bid truncation. Finally, PAF has been shown to act on isolated mitochondria directly to induce permeability transition and cytochrome *C* release although the exact mechanism of action is unclear (Parker et al., 2002). Taken together these data directly implicate production and degradation of PAF-related lipids in activation of the mitochondrial death cascade.

1.5.2 ER and Golgi stress responses

Excess lipid of any family or class can result in lipotoxicity and apoptosis (Unger, 2002), however, several lipid mediators have been associated specifically with induction of ER stress (Gregor and Hotamisligil, 2007). Stearic acid (C_{18}) and palmitic acid (C_{16}), the primary alkyl linked groups in PAF, are known inducers of ER stress (Borradaile et al., 2006; Wang et al., 2006; Wei et al., 2006). The ER is of particular interest to the study of PAF imbalance in neurodegenerative disorders, as evidence has shown that the major enzymes involved in PAF synthesis localize to the ER. These observations suggest that the ER may serve as the initial sensor of perturbations in PAF synthesis.

The ER is involved in transduction of apoptotic signalling through two pathways. The unfolded protein response (UPR) and regulation of calcium homeostasis. The UPR is initiated by accumulation of unfolded proteins in the ER to restore protein folding (Fig 1.6) (Hoyer-Hansen and Jaattela, 2007). Apoptosis follows when ER function cannot be restored (Hoyer-Hansen and Jaattela, 2007; Patil and Walter, 2001). The UPR has also been linked to induction of autophagy (Hoyer-Hansen and Jaattela, 2007). Accumulation of unfolded proteins in the ER can occur due to inhibition of N-linked protein glycosylation, impairment of disulphide bond formation, or loss of ER-Golgi transport (Ferri and Kroemer, 2001). BIP (GRP78) is a chaperone of protein folding that localizes to the ER and is an early marker of ER stress-mediated activation of the UPR (Gulow et al., 2002; Munro and Pelham, 1986). Binding of BIP to unfolded proteins induces its dissociation away from Ire1-α/β and activating transcription factor (ATF-6). Ire1-α then autophosphorylates and oligomerizes leading to activation of the JNK pathway through recruitment of TRAF-2 (Urano et al., 2000). Meanwhile Ire1-β, cleaves 28S rRNA, thereby in-

Figure 1.6: The UPR mediates activation of the caspase cascade. While the UPR is initiated to augment protein folding in the ER, prolonged activation can trigger an apoptotic caspase cascade. BIP is recruited to chaperone protein folding following accumulation of unfolded proteins within the ER lumen. This inhibits BIP association with ATF-6, IRE1-α and IRE1-β. Activated IRE1-β inhibits further protein translation while ATF-6 induces genes necessary for UPR. IRE1-α can recruit Traf-2 which results in calpain-mediated cleavage of ER pro-caspase, initiating the caspase cascade.

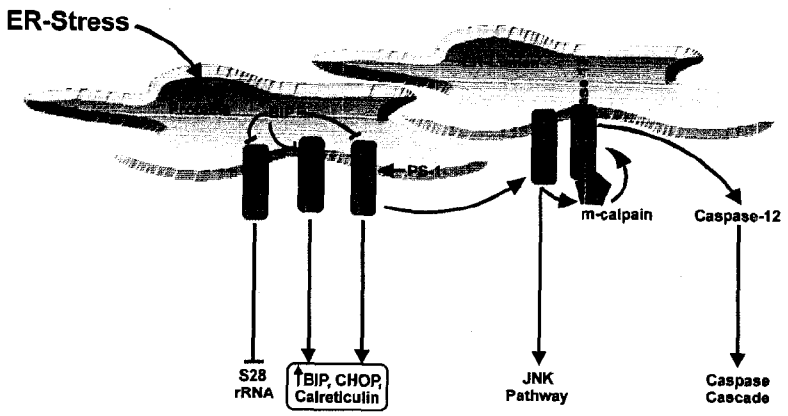
ER-Stress
PS-1
m-calpain
Caspase-12
S28
rRNA
BIP, CHOP,
Calreticulin
JNK
Pathway
Caspase
Cascade

Figure 1.6

hibiting protein translation (Iwawaki et al., 2001). UPR signalling is enhanced by presinillin-1 mediated cleavage of Ire1-α that translocates to the nucleus upon cleavage (Katayama et al., 1999). Nuclear localized Ire1-α functions as an endoribonuclease promoting translation of ER-stress induced genes (Lee et al., 2002; Niwa et al., 1999; Tirasophon et al., 2000; Tirasophon et al., 1998). Following loss of BIP association, ATF-6 translocates to the Golgi where it is processed to its active form by site-1 (S1P) and site-2 proteases (S2P), implicating ER-Golgi cross talk in UPR signalling (Chen et al., 2002; Nadanaka et al., 2007; Shen et al., 2002). Following activation, ATF-6 translocates to the nucleus where it associates with cAMP response element-binding protein (CREB) and leads to the activation of genes possessing an ER stress element (Li et al., 2000). These genes include those critical to protein folding in the ER lumen such as BIP as well those involved in cell death such as calreticulin and C/EBP Homologous Protein (CHOP). CHOP is a transcription factor that decreases the expression of anti-apoptotic Bcl2 family members promoting apoptosis (McCullough et al., 2001).

Alternative apoptotic signal transduction from ER may be facilitated through calcium signalling. Calcium mobilization results in activation of m-calpain, a cytosolic cysteine protease, which cleaves anti-apoptotic Bcl-2 family member Bcl-XL, converting it into a pro-apoptotic protein (Orrenius et al., 2003). m-calpain activation leads to cleavage and activation of ER pro-caspase 12 and initiation of the caspase cascade. These two pathways of ER-stress transduction are not mutually exclusive. Cross-talk occurs with respect to activation of the caspase cascade and commitment to programmed cell death. While calcium release can induce caspase activation directly and alter mitochondrial

permeability, TRAF-2 recruitment by Ire1-α also leads to calpain-mediated caspase-12 activation (Orrenius et al., 2003).

The putative ER stress caspase in rodents is caspase 12, however, the human homologue of caspase-12 has a loss-of-function mutation within the SHG box that prohibits it from acting catalytically (Fischer et al., 2002). While it has been suggested that the functional caspase 12 homologue in humans is either caspase 4 or caspase 2, the two enzymes have very distinct consensus sequences (LEVD and VDVAD respectively) (Dahmer, 2005; Hitomi et al., 2004). Caspase 4 has not been detected in human brain making caspase 2 the likely initiator of the ER-stress-dependent caspase cascade in neurodegenerative disease (Lin et al., 2000). Caspase 2 localizes to both ER and Golgi membranes (Cheung et al., 2006; Mancini et al., 2000). This further underscores that ER-Golgi cross talk following ER-stress is critical to apoptotic signalling in human neurons (Nakagomi et al., 2007).

The PAF-AH I regulatory subunit, LIS1, may represent another means of ER-Golgi apoptotic signalling. As part of the dynein complex LIS1 is involved shuttling of endosomes and lysosomes as well as shuttling of intermediate compartments from the ER to the Golgi (Harada et al., 1998; Hirokawa, 1998; Hirokawa et al., 1998). Prolonged loss of LIS1 from the dynein complex has been shown to cause microtubule catastrophe and loss of dynein mediated Golgi distribution (Sapir et al., 1999; Sapir et al., 1997; Smith et al., 2000) that can manifest in ER stress (Gonatas et al., 2006; Nakagomi et al., 2008). Several studies have shown that pro-apoptotic proteins such as BH3 only (Bcl-2 homology 3-only), BIM (Bcl-2 interacting mediator of cell death) and BMF (Bcl-2 modifying factor) are sequestered by the dynein complex in healthy cells (Day et al.,

2004; Puthalakath et al., 1999; Puthalakath et al., 2001; Strasser et al., 2000). ER stress has been shown to induce BIM translocation from the dynein complex to the ER where it subsequently activates an apoptotic caspase cascade (Morishima et al., 2004), thus implicating dynein malfunction in both ER-mediated and Golgi-mediated apoptosis.

1.6 Neurodegeneration and PAF metabolism

PAF has been repeatedly associated with learning and memory in the central nervous system. *In vivo*, under normal physiological conditions, PAF lipids act as retrograde neurotransmitters participating in long-term potentiation and enhancing behavioural criteria of learning and memory (Chen et al., 2001; Francescangeli et al., 2002; Izquierdo et al., 1995; Kato, 1999; Teather et al., 1998). However, PAF becomes neurotoxic when concentrations exceed 100 nM (Brewer et al., 2002; DeCoster et al., 1998; Tong et al., 2001). Pathophysiological production of PAF has been identified as a key mediator of neuronal death in a multitude of neurodegenerative disorders *in vivo* including ischemia, HIV-1 associated dementia and Alzheimer's like dementia (Bate et al., 2004b; Bazan, 1998; Birkle et al., 1998; McLarnon et al., 2005; Perry et al., 1998b). Both converging death pathways and converging pathways of PAF production and secretion are at play in these conditions. Thus, it will be essential to identify predominant PAF species present in diseased tissue and underlying mechanisms of production if therapeutic intervention is to prevent a bioactive lipid death signalling cascade.

1.6.1 PAF in AD

Progressive memory dysfunction in AD is associated with numerous pathological

changes. While genetic mutations are known to underlie familial or early onset AD, in 90% of patients there is no known genetic determinant (2005). Central to disease progression is the accumulation of Aβ peptide. The amyloid hypothesis of disease progression proposes that the sequence of pathogenic events that are thought to lead to AD stem from the accumulation or lack of clearance of Aβ (Fig 1.7) (Citron, 2004). Improper processing of amyloid precursor protein (APP) results in the generation of 40 and 42 amino acid Aβ peptides (LaFerla et al., 2007). While Aβ oligomers may impact on neuronal synapses and neurites directly (Volker Nimmrich, 2008), the extracellular aggregation of Aβ peptides into fibrillar plaques leads to activation of microglia and astrocytes (Hu et al., 1998; Jekabsone et al., 2006; Sebastiani et al., 2006). Aβ accumulation also initiates mitochondrial and ER stress pathways in neurons that alter multiple kinase cascades (LaFerla et al., 2007). Aberrant kinase activation causes Tau-protein hyperphosphorylation, causing intracellular neurofibrillary tangles and cytoskeletal abnormalities (Blurton-Jones and Laferla, 2006). These pathologies underscore memory dysfunction in AD and culminate in neuronal loss. Increased PAF levels in AD may result from multiple sources. Non-aggregated Aβ increases PLA_2 activity enhancing production of bioactive glycerophospholipids, that further enhance Aβ aggregation (Kriem et al., 2005; Mandal et al., 2004). In addition, plasmalogens are rapidly converted to ethanolamine PAF by a plasmalogen-specific PLA_2 that is reportedly up regulated in AD brain (Farooqui and Horrocks, 2001). Thus, the possibility that PAF and PAF-like lipids are increased in AD brain is consistent with both PLA_2-mediated increases in glycerophosphocholine and ethanolamine levels and the decrease in ethanolamine plasmalogens observed in AD brain. Pharmacological inhibition of PAF-mediated neurotoxicity as well as re-

Figure 1.7: The role of PAF in the amyloid cascade hypothesis. The amyloid cascade hypothesis (black) postulates that AD pathology and dementia are the end result of accumulation and lack of clearance of Aβ peptide. This schematic illustrates how this sequence of pathological events leads to enhanced PAF production (red) and accelerated AD pathology (blue). Modified from Citron 2004.

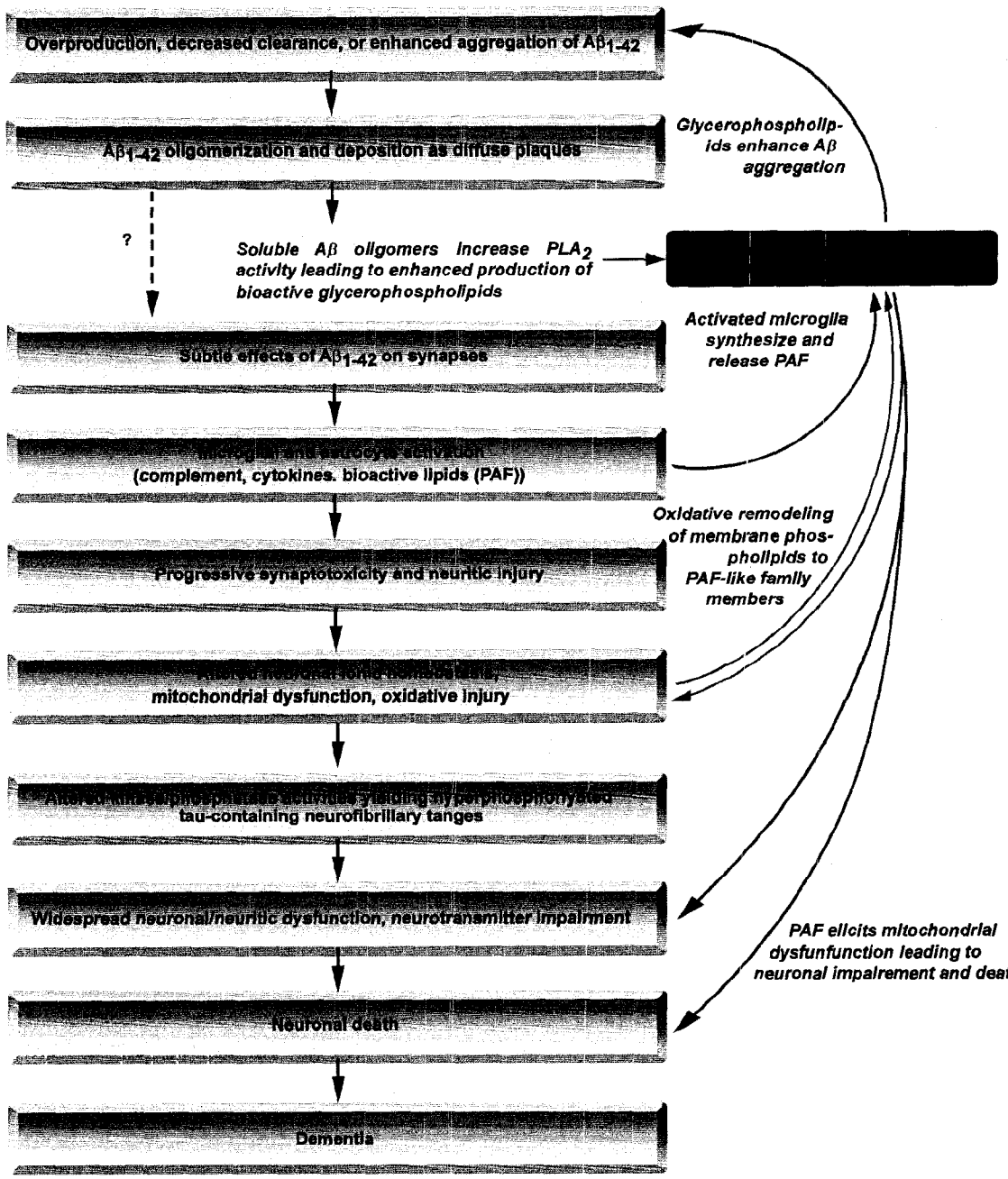
Overproduction, decreased clearance, or enhanced aggregation of Aβ1-42
Aβ1-42 oligomerization and deposition as diffuse plaques
?
Soluble Aβ oligomers increase PLA2 activity leading to enhanced production of bioactive glycerophospholipids
Subtle effects of Aβ1-42 on synapses
(complement, cytokines, bioactive lipids (PAF))
Progressive synaptotoxicity and neuritic injury
mitochondrial dysfunction, oxidative injury
tau-containing neurofibrillary tanges
Widespread neuronal/neuritic dysfunction, neurotransmitter impairment
Neuronal death
Dementia
Glycerophospholip-ids enhance Aβ aggregation
Activated microglia synthesize and release PAF
Oxidative remodeling of membrane phos-pholipids to PAF-like family members
PAF elicits mitochondrial dysfunfunction leading to neuronal impairement and death

Figure 1.7

lated glycerophospholipid imbalances suggests that PAF may mediate, in part, Aβ toxicity (Bate et al., 2004b; Farooqui and Horrocks, 2001; Kanfer et al., 1998; Sweet et al., 2002). PAF antagonists block prion- and Aβ-induced cell death in vitro, and protect neurons from activated microglia (Bate et al., 2006; Bate et al., 2004a; Bate et al., 2004b). Furthermore, studies of microglia response to PAF show a decrease in calcium signal transduction, suggesting more PAF is required to activate AD microglia than is necessary to activate healthy microglia (McLarnon et al., 2005). Finally, the omega-3 polyunsaturated fatty acid docosahexaenoic acid (DHA) suppresses PAF synthesis through the remodelling pathway (Watanabe et al., 2001). Dietary consumption of DHA reduces dendritic pathology in AD transgenic mouse models (Calon et al., 2004). The Framingham cohort study has demonstrated that humans that consume a diet high in DHA have a decreased probability of developing Alzheimer's dementia (Johnson and Schaefer, 2006; Schaefer et al., 2006), further implicating PAF synthesis in AD progression. Collectively, these observations provide strong support for the role of cytotoxic PAF signalling in AD pathology.

1.6.2 PAF in ischemia

Ischemia reperfusion injury is known to result in canonical PAF synthesis through the remodelling pathway by PLA_2 activation and generate oxidized lipids of which bioactive PAF-related mediators are a major component (Domingo et al., 1994; Francescangeli et al., 1996a; Francescangeli et al., 1996b; Nishida and Markey, 1996). Causal evidence for PAF-mediated neurotoxicity following ischemia was first elucidated through PAF an-

tagonist studies demonstrating that the ginkgolide BN 52021, a non-competitive PAFR antagonist, reduced neuronal loss following ischemia in rat cortex and hippocampus (Oberpichler et al., 1990; Prehn and Krieglstein, 1993). Genetically engineered mouse models confirmed the role of bioactive lipids in neuronal injury as both cytosolic PLA_2 knockout mice and PAF-AH II transgenic mice, exhibited less cerebral injury following transient focal ischemia (Bonventre et al., 1997; Umemura et al., 2007).

The most potent PAF species produced are believed to be C_{16}-PAF (1-O-hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine) and C_{18}-PAF (1-O-octadecyl-2-acetyl-*sn*-glycero-3-phosphocholine) (Nishida and Markey, 1996) with the former isoform having an order of magnitude greater affinity for PAFR than the latter isoform (Carolan and Casale, 1990; Hwang et al., 1989; Ishii et al., 1997). Following ischemia, while PAF levels in brain tissue have been shown to increase, the expression levels of PAFR have been shown to decrease (Domingo et al., 1994; Zhang et al., 2007). This is of critical importance as the majority of PAF effects are transduced by PAFR activation (Ishii and Shimizu, 2000). PAFR activation, however, has been found to be pro- and anti-apoptotic depending on cell type and death ligand (Brewer et al., 2002; Li et al., 2003; Southall et al., 2001). Taken together this suggests both extrinsic PAFR-mediated and intrinsic PAFR-independent PAF signalling mechanisms are likely involved in neuronal loss following cerebral ischemia.

1.6.3 PAF in HIV-1 associated dementia

HIV-1 associated dementia, also known as the AIDS dementia complex, is prevalent among 15-25 % of those infected with HIV worldwide (Sacktor et al., 2007). It is a

hyper-inflammatory condition induced by viral infection of microglia and perivascular macrophages (Gonzalez-Scarano and Martin-Garcia, 2005). Although neurons are not infected by HIV, progressive neuronal loss in patients affected by AIDS dementia underlies the cognitive impairment (Kaul and Lipton, 2006a, b; Kaul et al., 2005). It was hypothesized that enhanced secretion of pro-inflammatory mediators and neurotoxins by hyperactive microglia and infiltrating macrophages triggered neuronal loss characteristic of AIDS dementia (Gendelman and Tardieu, 1994). PAF was first characterized as being among those neurotoxins secreted by infected microglia along with the proinflammatory cytokines tumor necrosis factor alpha and interleukin 1β as well as arachidonic acid metabolites (Genis et al., 1992). PAF lipids were subsequently identified as the primary neurotoxin in AIDS dementia (Gelbard et al., 1994). Direct *in vitro* evidence demonstrated, that while conditioned media from HIV infected macrophages was toxic to neurons, it could be rendered benign by treatment with either recombinant PAF-AH II or PAF antagonists (Perry et al., 1998a). To this end, PAF antagonists have been met with success in clinical studies of their impact on cognitive function in HIV patients (Schifitto et al., 1999).

Furthermore, a structure-activity relationship exists between PAF antagonism and HIV-1 replication (Serradji et al., 2000; Serradji et al., 2004). The PAF antagonist PMS 601, displays both PAF-antagonism and anti-viral abilities although it is unclear whether anti-HIV effects are dependent on PAF antagonism. Conversely, our group has shown that, HIV-1 protease inhibitors, such as nelfinavir, can inhibit viral replication as well as prevent PAF-mediated apoptosis by blocking mitochondrial permeability transition (Weaver et al., 2005). While further investigation is required to show a causal link, these

data suggest that viral replication in AIDS-dementia may be linked to PAF production. In support of this argument, the HIV-1 transcription factor TAT is known to associate with the PAF-AH I regulatory protein LIS1 (Epie et al., 2005). This interaction facilitates enhanced viral gene transcription (Epie et al., 2006). Sequestration of LIS1 from PAF-AH I may have the added benefit of decreasing PAF hydrolysis, a sophisticated viral response should PAF signalling itself promote viral replication.

1.7 Rationale, Hypothesis and Objectives

The objective of this thesis was to elucidate underlying mechanisms of bioactive lipid-mediated neurodegeneration with specific focus on PAF and PAFR. PAF has been implicated in the regulation of synaptic plasticity, cerebral development, and programmed cell death (Aihara et al., 2000; Chen and Bazan, 2005). PAF lipids are potent inflammatory mediators central to activation of multiple death signals. PAF metabolism influences production and degradation of a multitude of bioactive lipids making it a promising point of intervention for development of a non-inflammatory lipid profile intervention in the diseased brain. In this thesis, I hypothesized that PAF lipids induce apoptosis in neurons devoid of PAFR. To test this hypothesis I proposed three specific aims:

1) Determine whether PAF triggers neuronal apoptosis in neurons devoid of PAFR (Chapters 2 and 5).

2) Determine whether PAFR expression protects neurons from PAF toxicity (Chapters 3 and 4).

3) Determine whether PAF-induced neuronal loss can be inhibited by blocking PAFR-independent death without perturbing PAFR-mediated neuroprotection (Chapters 3 and 4).

1.8 References

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Chapter 2: PAF triggers caspase-dependent neuronal apoptosis independently of its receptor[1]

2.1 Objective of this study

The first step in testing my hypothesis, that PAF lipids induce apoptosis in neurons devoid of PAFR, was to determine whether C_{16}-PAF signalling was apoptogenic. This was evaluated using the PAFR-negative PC12 cell model.

2.2 Statement of author contributions

SDR, FB and SALB conceived and designed the experiments. SDR and FB equally contributed to the experimental work. SDR wrote the manuscript with SALB. FM and LM performed western blots in Figure 5F. JL performed RNAi assay in Figure 8B and C. DJF co-supervised FB and provided guidance for microscopy analysis. HA provided antibodies to PAF-AH I α_1 and PAF-AH I α_2.

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2.3 Anti-apoptotic actions of the platelet activating factor acetylhydrolase I α_2 catalytic subunit

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Running title: Anti-apoptotic actions of PAF-AH I α_2

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2.4 Summary

Platelet activating factor (PAF) is an important mediator of cell loss following diverse pathophysiological challenges but the manner in which PAF transduces death is not clear. Both PAF receptor-dependent and independent pathways are implicated. In this study, we show that extracellular PAF can be internalized through PAF receptor-independent mechanisms and can initiate caspase-3-dependent apoptosis when cytosolic concentrations are elevated by approximately 15 pM/cell for 60 min. Reducing cytsolic PAF to less than 10 pM/cell terminates apoptotic signaling. By pharmacological inhibition of PAF acetylhydrolase I and II (PAF-AH) activity and downregulation of PAF-AH I catalytic subunits by RNA interference, we show that the PAF receptor-independent death pathway is regulated by PAF-AH I and, to a lesser extent, by PAF-AH II. Moreover, the anti-apoptotic actions of PAF-AH I are subunit-specific. PAF-AH I α_1 regulates intracellular PAF concentrations under normal physiological conditions but expression is not sufficient to reduce an acute rise in intracellular PAF levels. PAF-AH I α_2 expression is induced when cells are deprived of serum or exposed to apoptogenic PAF concentrations limiting the duration of pathological cytosolic PAF accumulation. To block the PAF receptor-death pathway, we screened a panel of PAF antagonists (CV-3988, CV-6209, BN 52021, and FR-49175). BN 52021 and FR-49175 accelerated PAF hydrolysis and inhibited PAF-mediated caspase 3 activation. Both antagonists act indirectly to promote PAF-AH I α_2 homodimer activity by reducing PAF-AH α_1 expression. These findings identify PAF-AH I α_2 as a potent anti-apoptotic protein and describe a new means of pharmacologically targeting PAF-AH I to inhibit PAF-mediated cell death.

2.5 Introduction

Platelet-activating factor (PAF[2]: 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) is a key mediator of neuronal death in ischemia, encephalitis, epileptic seizure, meningitis, and human immunodeficiency virus-1 dementia *in vivo* and participates in etoposide-, prion-, and β-amyloid-induced cell death *in vitro* (Bate et al., 2004a; Bate et al., 2004b; Bazan, 1998; Birkle et al., 1998; Darst et al., 2004; Li et al., 2003; Perry et al., 1998). In the periphery, pathological increases in PAF concentrations underlie cytotoxicity in chronic inflammatory dermatoses and lethality in systemic anaphylaxis (Fukuda et al., 2000; Grandel et al., 1985). Although the majority of PAF effects are understood to be transduced by its G-protein coupled receptor (PAFR) (Ishii and Shimizu, 2000), PAFR signaling has been shown to be both pro- and anti-apoptotic. Ectopic PAFR expression exacerbates cell death induced by etoposide and mitomycin C but protects cells from tumour necrosis factor α (TNFα), TRAIL, and extracellular PAF (Brewer et al., 2002; Darst et al., 2004; Li et al., 2003; Southall et al., 2001). These opposing effects likely depend upon the relative ratio of NFκB-dependent pro- and anti-apoptotic gene products elicited in different cell types in response to the combination of an external apoptotic inducer and PAF (Li et al., 2003).

Accumulating evidence points to additional PAF signaling pathways transduced independently of PAFR (Bratton, 1993; Bratton et al., 1992; Brewer et al., 2002; Marche-

[2] Abbreviations: B-PAF, Bodipy-platelet activating factor; B-*lyso*-PAF, Bodipy *lyso*-PAF; BSA, bovine serum albumin; GAPDH, glyceraldehydes phosphate dehydrogenase; DFP, diisopropyl fluorophosphates; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid; mc-PAF, methyl-carbamyl platelet activating factor; PAF, platelet activating factor; PAF-AH, PAF acetylhydrolase; PAFR, platelet activating factor receptor; PARP, poly ADP-ribose polymerase; PBS, phosphate buffered saline; siRNA, small interfering RNA; RT-PCR, reverse transcriptase-polymerase chain reaction; $t_{1/2}$, time to reach half maximal concentrations; TLC, thin-layer chromatography; TNFα, tumour necrosis factor α; TUNEL, terminal deoxytransferase dUTP nick-end labeling.

selli et al., 1990; Sapir et al., 1997; Tokuoka et al., 2003). PAFR-negative cells undergo apoptosis when extracellular PAF concentrations reach 100 nM and necrosis when PAF levels exceed the critical micelle concentration of 3 μM (Brewer et al., 2002). Little is known about how PAF signals cell death in the absence of PAFR. PAF activates NF-κB, glycogen synthase kinase 3β, and caspase 3 and triggers mitochondrial release of cytochrome C (Hostettler et al., 2002; Li et al., 2003; Ma and Bazan, 2001; Tong et al., 2001). Whether or not these effects are dependent on PAFR activation is not clear.

One approach to intervening in both PAFR-dependent and independent cell death lies in reducing pathological increases in PAF. PAF is hydrolyzed by a unique family of serine esterases or PAF acetylhydrolases (PAF-AHs) that cleave the biologically *sn*-2 active side-chain generating *lyso*-PAF. Three PAF-AH enzymes have been identified. Cytosolic PAF-AH I cleaves the acetyl group at the *sn*-2 position of PAF and PAF-like lipids with other phosphate head groups (Manya et al., 1999). The enzymatic complex is a G-protein-like trimer composed of two 29 kDa α_1 and α_2 catalytic subunits. The α subunits form homodimers or heterodimers that complex with a non-catalytic 45 kDa regulatory β□ subunit LIS1. Mutations in the *LIS1* gene are the genetic determinant of Miller Dieker Syndrome, a developmental brain disorder defined by type 1 lissencephaly (Hattori et al., 1994). PAF-AH II is a single 40 kDa polypeptide (Hattori et al., 1996). This isoenzyme recognizes both PAF and acyl analogs of PAF with moderate length *sn*-2 chains as well as short-chain diacylglycerols, triacylglycerols, and acetylated alkanols (Min et al., 2001). Ectopic expression reduces cell death triggered by oxidative stress (Marques et al., 2002; Matsuzawa et al., 1997). Plasma PAF-AH is a 45 kDa monomer secreted into circulation by endothelial and hematopoietic cells (Asano et al., 1999;

Tjoelker et al., 1995). The enzyme recognizes PAF and PAF analogs with short to medium *sn*-2 chains including oxidatively cleaved long-chain polyunsaturated acyl chains (Min et al., 2001). *In vitro*, recombinant plasma PAF-AH or ectopic expression protects cells from excitotoxicity or hypercholesterolemia (Chen et al., 2003; Hirashima et al., 2000; Ogden et al., 1998). *In vivo*, intravenous injection of human plasma PAF-AH reduces lethality in experimental models of anaphylactic shock (Fukuda et al., 2000). These findings provide compelling evidence that PAF-AH activity regulates PAF-mediated apoptosis. It remains to be determined whether these enzymes can, in fact, be targeted to inhibit PAF-mediated degeneration and disease.

In this study, we used the PC12 cell model to investigate the anti-apoptotic actions of PAF-AH I and PAF-AH II in the PAFR-independent pathway. We show that PC12 cells express all three PAF-AH I proteins (α_1, α_2 and LIS1) as well as PAF-AH II but not plasma PAF-AH or PAFR. Expression of PAF-AH I α_2□ but not PAF-AH I α□ is induced when PC12 cells are deprived of serum. We found that this induction regulates the duration of apoptotic signaling initiated by PAF challenge. To enhance the endogenous anti-apoptotic activity of PAF-AH I α_2, we screened a panel of PAF antagonists and identified two compounds that blocked PAFR-independent death. Both compounds, the fungal derivate FR 49175 and the ginkgolide BN 52021, protected cells by accelerating PAF hydrolysis. Surprisingly, both inhibitors suppressed α_1 protein expression thereby promoting α_2/α_2 homodimer activity following PAF treatment. These findings point to a novel anti-apoptotic function for the α_2 subunit of PAF-AH I and a potential means of pharmacologically targeting PAF-AH enzymes to reduce PAF-mediated cell death.

2.6 Experimental Procedures

2.6.1 Cell culture: PC12-AC cells, a clonal derivate of the PC12 pheochromocytoma cell line (American Tissue Culture Collection), were cultured in complete media composed of RPMI 1640 containing 10% horse serum and 5% newborn calf serum at 37°C in a 5% CO_2/95% air atmosphere. Culture reagents were obtained from Invitrogen.

2.6.2 Reverse transcriptase polymerase chain reaction (RT-PCR) analyses: Rat brain RNA was prepared from Wistar rats approximately 3 months of age (Charles Rivers). Rodents were anesthetized with sodium pentobarbital and euthanized by decapitation. All manipulations were performed in compliance with approved institutional protocols and according to the strict ethical guidelines for animal experimentation established by the Canadian Council for Animal Care. Total RNA was isolated using Trizol reagent (Invitrogen) and treated with RQ1-DNAse1 (Promega) to eliminate residual genomic DNA. First strand cDNA synthesis was performed using random hexamer primers (Promega) and Superscript II RT (Invitrogen). Control reactions for residual genomic contamination were carried out in the absence of Superscript II RT. cDNA synthesis was performed using 5 U of *Taq* DNA polymerase (Invitrogen) in the presence of 1 mM $MgCl_2$ and 10 pmol per primer for glyceraldehyde phosphate dehydrogenase (GAPDH), 20 pmol per primer for PAF-AH II#2, PAF-AH II#3, and PAF-AH II#4, 25 pmol per primer for PAF-AH I α_1, PAF-AH I α_2, PAF-AH I LIS1, PAF-AH II#1, plasma PAF-AH and PAFR. Sequences are provided in Table 2.1. Primers were synthesized at the Biochemistry Research Institute, University of Ottawa. Reactions were amplified in a

Table 2. 1. Primer pairs used to detect cytosolic and plasma PAF-AH transcript by RT-PCR

Gene	Strand	Sequence (5'-3')	Amplicon size (bp)
GAPDH	Sense	TGGTGCTGAGTATGTCGTGGAGT	292
	Antisense	AGTCTTCTGAGTGGCAGTGATGG	
PAF-AH I α_1	Sense	GACGGACGCTGGATGTCTCT	587
	Antisense	AGACGAAGCAGCAAGGAGTG	
PAF-AH I α_2	Sense	TGCAGCAGTACGAGATATGG	418
	Antisense	AACATGTCGTGGCAGGAGAT	
PAF-AH I LIS1	Sense	CTGCTTCAGAGGATGCTACA	373
	Antisense	ATCAGAGTGCCGTCCTGATT	
PAF-AH II #1	Sense	GGATGTGATGGAGGGTC	1017
	Antisense	TGCTTCTGCAGGAAGGCCAA	
PAF-AH II #2	Sense	CAGCTGGTGATGAGATGG	224
	Antisense	GCGCTTGTTATACTGCAGGT	
PAF-AH II #3	Sense	TGAGCCGAGTGGCTGTGATG	472
	Antisense	CCCTTGGATGAGCGATGGTC	
PAF-AH II #4	Sense	CAGCTGGTGATGAGATGG	1250
	Antisense	CAACCTAGAGGCTGGACAGA	
Plasma PAF-AH	Sense	GGGGCATTCTTTTGGAGGAG	413
	Antisense	GACAGTCCCACTGATCAAAGTC	
PAFR	Sense	CACTTATAACCGCTACCAGGCAG	381
	Antisense	AAGACAGTGCAGACCATCCACAG	

GeneAmp PCR System 2400 (Applied Biosystems): 94°C for 5 minutes, 30-35 cycles of 94°C for 30 seconds, 55°C for 60 seconds, and 72°C for 2 minutes, followed by a final incubation at 72°C for 7 minutes.

2.6.3 Western analysis: Rat brain protein was prepared from Wistar rat pups on postnatal day 10. Rodents were anesthetized with sodium pentobarbital and euthanized by decapitation. Brains were removed and placed in a 10 cm dia. tissue culture plate containing artificial cerebrospinal fluid (26 mM $NaHCO_3$, 124 mM NaCl, 5mM KCl, 2mM $CaCl_2$, 1.3 mM $MgCl_2$, 10 mM D-glucose, 100 units/ml penicillin, 100 µg/ml streptomycin, pH 7.3) and homogenized using a Tissue Tearor (Fisher). Protein was isolated using Trizol reagent (Invitrogen). Proteins from PC12 cells were isolated in RIPA buffer (10 mM PBS, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 30 µl/ml aprotinin, 10 mM Na orthovanadate, 100 µl/ml PMSF). Protein samples (30 µg) were separated by SDS-PAGE under reducing conditions. Antibodies were diluted in 1% heat-denaturated casein in 10 mM phosphate buffered saline (PBS: 10 mM sodium phosphate, 2.7 mM KCl, 4.3 mM NaCl, pH 7.5). Western analyses were performed using polyclonal anti-LIS1 (1:500, Chemicon), monoclonal anti-α_1 (1:1000, kind gift of Dr H. Arai, University of Tokyo, Tokyo, Japan), monoclonal anti-□α_2 (1:1000, Dr H. Arai), poly-ADP-ribose polymerase (PARP, 1:10 000, Clontech), and actin (1:1000, Sigma). Secondary antibodies were horseradish peroxidase-conjugated or biotin-conjugated anti-mouse IgG (1:2000, Jackson Immunolabs; 1:10 000, Sigma) or anti-rabbit IgG antibodies (1:5000, Jackson Immunolabs) and tertiary reagents were extravidin alkaline phosphatase (1:300 000, Sigma) as appropriate. Immunoreactive bands were visualized using SuperSignal West Pico (MJS

BioLynx Inc) or NBT/BCIP (Sigma). Densitometry was performed using ImageJ analysis software (NIH) standardized to actin loading controls.

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

2.6.5 Internalization assay (live cell imaging): PC12-AC cells ($5x10^4$ cells/well) were plated in complete media overnight in 24-well plates (VWR) coated with 0.1% gelatin. Cells were washed in 10 mM PBS and incubated with 1 μM Bodipy FL C_{11}-PAF (B-PAF) in RPMI 1640 containing 0.1% bovine serum albumin (BSA, Sigma). B-PAF was

custom synthesized for our laboratory by Molecular Probes. At each time point (0,10, 20, 30, 40, 50, 60, 80, 90, 100, 110, 120 min), incubation media was removed, cells were washed with 10 mM PBS. Live cell imaging under phase and fluorescence of identical cell fields was performed using a DMR inverted microscope (Leica) equipped with a QI-CAM digital camera (Quorum Technologies) and captured using OpenLab software v3.17 (Improvision). Following time-lapse imaging, the incubation media was replaced and internalization allowed to continue. Quantitation of fluorescence intensity of individual cells was performed using the Advanced Measurement Module of OpenLab v3,17.

2.6.6 Lipid extraction and thin layer chromatography (TLC): PC12-AC cells seeded at 1 x 10^5 cells onto 10 cm plates were maintained in complete media at 37°C in 5% CO_2 for 72 h. Cultures were incubated at 37°C with 1 μM B-PAF in RPMI 1640 containing 0.1% BSA, for 0, 5, 15, 30, 45, 60, and 75 minutes. At each time point, 4 plates/condition were removed from the incubator and were placed on ice. One ml of methanol acidified with 2% acetic acid was added to each plate and the extracellular fraction was collected. This fraction contained B-PAF in the culture media and uninternalized B-PAF bound to cell surface proteins or associated with the plasma membrane. The remaining monolayer of cells was collected in acidified methanol by scraping the plate with a cell lifter (Fisher). Lipids were extracted from the extracellular milieu and cytosolic fractions by the Bligh and Dyer method (Bligh and Dyer, 1959) and developed on TLC plates (20 x 20 cm Silica gel 60 (Fisher) in a solvent system of chloroform/methanol/acetic acid/water (50:30:8:5, v/v). B-PAF and B-*lyso*-PAF (Molecular Probes) were used as authentic markers. Fluorescent lipids were visualized under UV light using AlphaImager-1220

software (Alpha Innotech Corporation). Fluorescence intensity corresponding to lipid yield was determined by densitometry using the Advanced Measurement Module of OpenLab v3.17. Concentrations of cytosolic B-PAF were estimated in comparison to a B-PAF standard curve resolved in parallel. Data are expressed as pM/PAF-responsive cell following standardization to the number of cells per culture.

2.6.7 Cell death assays: PC12-AC cells (8800 cells/cm^2) were plated overnight in complete media in 6 cm dia. tissue culture plates (VWR). Cells were treated in serum-free RPMI media containing 0.025% BSA (treatment media) for 24-72 h with EtOH (0.1%), PAF (10 nM-1 μM, Biomol Research Laboratories), methyl-carbamyl PAF (mc-PAF; 100 nM-1 μM, Biomol Research Laboratories), or *lyso*-PAF (10 nM-1 μM, Biomol Research Laboratories). In some cases, cells were pretreated with BN 52021 (1-100 μM, Biomol Research Laboratories), CV-3988 (0.2-2 μM, Biomol Research Laboratories), CV-6209 (1-10 μM, Biomol Research Laboratories), FR-49175 (0.5-50 μM, Biomol Research Laboratories), DFP (0.1 mM or 1 mM, Sigma), DTNB (1 mM, Sigma) or combinations thereof for 15 min followed by addition of PAF (1 μM) for 24 h. Cell survival was assessed by hemocytometer cell counts of Trypan Blue-excluding cells. Metabolic activity was assessed based on the ability of mitochondrial dehydrogenases, active in viable cells, to reduce the formazan salt, WST-1 (measured at A_{450}-A_{690} nm, Roche). DNA fragmentation was determined by terminal deoxytransferase dUTP nick-end labeling (TUNEL, Roche) of cultures fixed for 20 min in 4% paraformaldehyde in 10 mM as described in (Bennett et al., 1998). Cells, processed for TUNEL, were double-labeled with Hoechst 33258 (0.2 μg/ml) for 20 min at room temperature for additional morphological

evidence of apoptotic loss.

2.6.8 RNA interference transfection: To suppress expression of the PAF-AH I α_2 subunit, we designed a double-stranded short interfering RNAs (siRNA) to the α_2 sequence (AATAAACATGCTTGTCACTCCC/CTGTCTC) and a negative control scrambled sequence (AATGCATAGGAGTTGGAGAGGC/CTGTCTC). Oligonucleotides were obtained from the Biotechnology Research Institute at the University of Ottawa. siRNA duplexes were generated using the Silencer siRNA Construction Kit (Ambion). Transfection of siRNA was performed with Lipofectamine 2000 (Invitrogen) and optimized to yield maximal transfection efficiency according to manufacturer's protocol. Briefly, 2 µl of Lipofectamine 2000 was diluted in 198 µl of RPMI-1640 media for 5 minutes at room temperature. siRNA duplexes (PAF-AH α_2 or scrambled) were suspended in 100 µl of RPMI-1640 media. PC12-AC cells grown in complete media to 30% confluence in 4-well gelatin-coated Labtek wells were treated with 100 µl of the Lipofectamine/siRNA complex and incubated for 72 hours at 37°C. Transfection efficiency was determined in separate cultures by counting EGFP-positive cells upon transfection with pEGFP-C1 as well as morphological changes using Ambion's β-actin siRNA positive control. Because we were unable to obtain >20% transfection efficiency, we co-transfected PAF-AH α_2 or scrambled siRNAs with 0.028 µg/µl of pEGFP-C1 to identify silenced cells. Cells were then exposed to 1 µM PAF in treatment media (RPMI + 0.025% BSA) for either 45 minutes or 24 hours. Data were standardized to the number of EGFP-positive cells in vehicle-treated cultures transfected with pEGFP-C1 only.

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

2.7 Results

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

We have previously demonstrated that PC12-AC cells do not express PAFR but undergo apoptosis-associated DNA fragmentation 24 h after treatment with $\geq$ 100 nM PAF and necrotic lysis when treated with > 3 μM PAF (Brewer et al., 2002). In this study, we addressed the kinetics and underlying signaling mechanism of PAF-induced apoptosis in the absence of PAFR (Fig 2.1A, *inset*). To determine whether cell death is mediated by PAF or downstream PAF metabolites, PC12-AC cells were treated with PAF (1-*O*-hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine), mc-PAF (1-*O*-hexadecyl-2-N-methyl-carbamyl-*sn*-glycero-3-phosphocholine), the PAF-AH-resistant synthetic PAF analog (O'Flaherty et al., 1987), or *lyso*-PAF (1-*O*-hexadecyl-2-*lyso*-*sn*-glycero-3-phosphocholine), the immediate PAF metabolite. Both mc-PAF and PAF triggered comparable concentration-dependent cell death 24 h after treatment (Fig 2.1A). *Lyso*-PAF had no significant effect on cell viability (Fig 2.1A). To establish the kinetics of PAF-mediated cytotoxicity, cell survival was assessed at various periods of time after

Figure 2.1: The kinetics of PAF-mediated apoptosis initiated independently of PAFR depend upon sustained exposure to active ligand. (A) PC12-AC cells were treated for 24 h with PAF (0.01-1 μM), mc-PAF (0.01-1 μM), or *lyso*-PAF (0.01-1 μM) in serum free treatment media. A dose-dependent decrease in cell number relative vehicle (0.1% ETOH)-treated cells was observed after 24 h of treatment with 1 μM PAF or mc-PAF (**$p<0.01$). RT-PCR analysis of PC12-AC cultures (inset) confirmed that both PC12-AC cells and PC12-AC cells differentiated to a neuronal phenotype for 7 days with nerve growth factor (PC12+NGF) do not express PAFR mRNA. The positive control was RNA isolated from PC12 cells stably transfected with PAFR. cDNA template integrity was confirmed using GAPDH as an internal standard (GAPDH). (B) PAF (1 μM) treatment resulted in significant cell loss within 24 h of treatment (*$p<0.05$) after which no additional reductions in cell number were observed. mc-PAF (1 μM) elicited incremental cell loss for up to 48 h after exposure (*$p<0.05$, **$p<0.01$). Culture in serum-free treatment media in the presence of vehicle (EtOH, 0.1%) did not affect cell viability until 48 h after treatment. Arrows indicates the time of PAF adminstration. (C) Repeated PAF (1 μM) administration incrementally decreased the metabolic activity of cells for up to 72 h as defined by the ability of mitochondrial dehydrogenases to reduce the formazan salt WST (**$p<0.01$). Arrows indicate the when PAF (1 μM) or vehicle (0.1% EtOH) was replenished in fresh media. (D) Incremental cell loss was observed for up to 48 h following chronic PAF (1 μM) treatment. (*$p<0.05$, **$p<0.01$, ANOVA, post-*hoc* Dunnett's *t* test). As in (C), media was replaced and PAF (1 μM) or vehicle (0.1% EtOH) was added every 24 h (arrows). In (A), data are expressed as percent survival of vehicle-treated cultures and, in (B,C,D) data are standardized to untreated cells cultured in treatment media to present the effect of vehicle treatment on cell survival. Results are reported as mean$\pm$ SEM of n=5-26 cultures per data point.

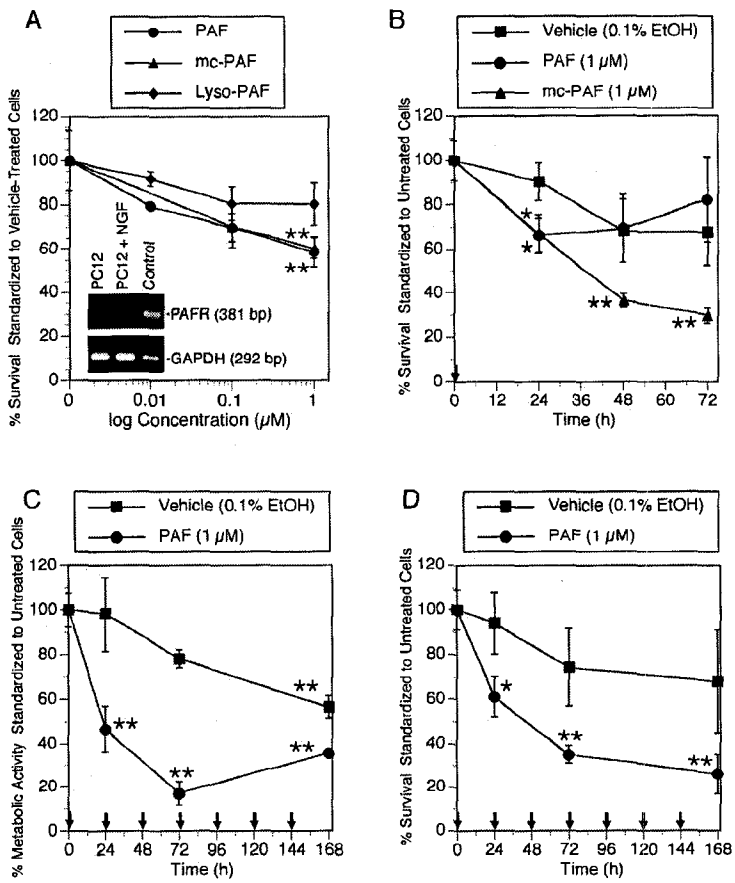
A
PAF
mc-PAF
Lyso-PAF
% Survival Standardized to Vehicle-Treated Cells
log Concentration (μM)
PC12
PC12 + NGF
Control
PAFR (381 bp)
GAPDH (292 bp)
B
Vehicle (0.1% EtOH)
PAF (1 μM)
mc-PAF (1 μM)
% Survival Standardized to Untreated Cells
Time (h)
C
Vehicle (0.1% EtOH)
PAF (1 μM)
% Metabolic Activity Standardized to Untreated Cells
Time (h)
D
Vehicle (0.1% EtOH)
PAF (1 μM)
% Survival Standardized to Untreated Cells
Time (h)

Figure 2.1

phospholipid administration. PAF (1 μM) elicited significant cell loss within 24 h of treatment; no further reductions in cell number were detected at 48 h or 72 h relative to vehicle controls (Fig 2.1B). mc-PAF (1 μM) elicited incremental cell loss for up to 72 after treatment (Fig 2.1B). Comparable kinetics were observed if PAF (1 μM) was replenished in fresh media at 24 h intervals with cells repeatedly treated with PAF exhibiting sustained impairment of cellular metabolic activity (Fig 2.1C) and an incremental reduction in cell survival (Fig 2.1D).

To determine whether PAF activates caspases in the absence of PAFR, we examined cleavage of the caspase 3 substrate, PARP. Caspase 3-mediated PARP cleavage from 116 kD to 85 kD was observed 45 min after a single PAF treatment (Fig 2.2A). Cleavage markedly increased 3 h after phospholipid administration and was detected for up to 24 h after treatment (Fig 2.2A) but not at later time points (data not shown). TUNEL-positive cells, evidence of terminal DNA fragmentation, were first observed 24 h after PAF exposure with no evidence additional death 48 h after exposure (Fig 2.2B).

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

Figure 2.2: PAF triggers a caspase-dependent apoptotic cascade within 45 minutes of exposure to active ligand. (A) PC12-AC cells were exposed to PAF (1 μM) for 24 h and assayed for caspase 3 activity. PARP cleavage to 85 kD was first detected 45 min after PAF treatment. Blots were re-probed with actin as a loading control. (B) TUNEL analysis detected apoptosis-associated DNA cleavage (arrows, upper panel) and DNA condensation (arrows, lower panel) 24 h after PAF treatment. No additional cell loss was observed 48 h after treatment. Scale bar, 10 μm.

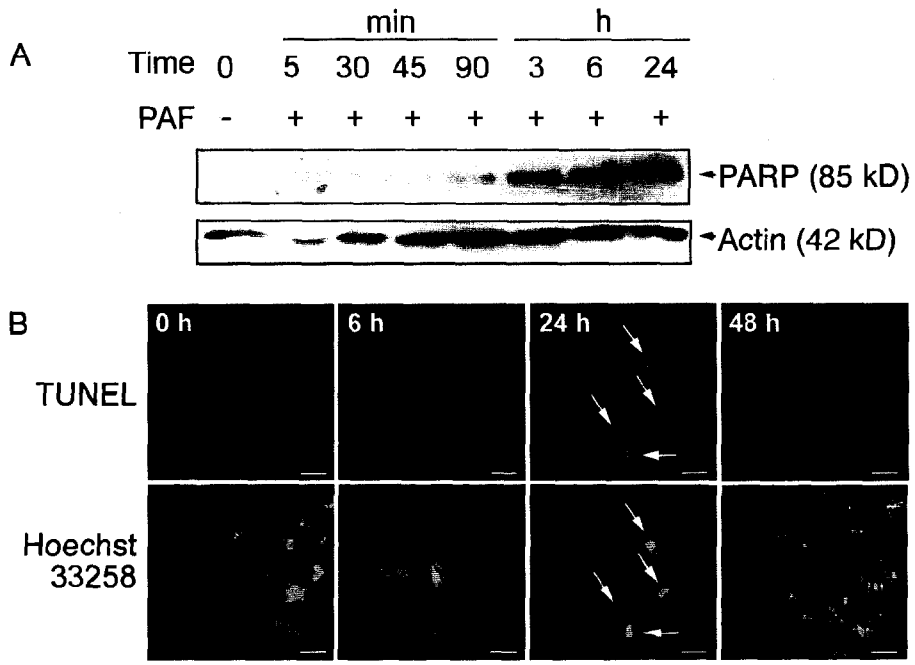
A
min
h
Time 0 5 30 45 90 3 6 24
PAF - + + + + + + +
PARP (85 kD)
Actin (42 kD)
B
0 h
6 h
24 h
48 h
TUNEL
Hoechst 33258

Figure 2.2

2.7.2 Cytosolic PAF-AH enzymes limit the duration of PAF-induced apoptotic signaling

These kinetics implicate PAF degradation in the control of apoptotic signaling. To test this hypothesis, we first identified *LIS1*, PAF-AH I α_1, PAH-AH I α_2, and PAF-AH II but not plasma PAF-AH transcripts in PC12 cells by RT-PCR (Fig 2.3A-F). Amplicon integrity of the PAF-AH I subunits was verified by sequencing on both strands. We then confirmed protein expression by Western analysis. α_1 protein was constitutively expressed in the presence or absence of PAF (Fig 2.3G). α_2 protein was present at extremely low levels (Fig 2.3H,I) with marked induction following exposure to PAF (Fig 2.3H,I). Closer examination revealed that $\alpha_{2\square}$ protein also increased when cells were cultured in serum-free treatment media suggesting a response to serum-withdrawal rather than PAF-specific induction (Fig 2.3I). LIS1 was constitutively expressed by PC12-AC cells (Fig 2.3J).

Lacking an antibody to PAF-AH II to verify protein expression, we sequenced the full-length transcript and assayed functional PAF-AH activity in the presence of cytosolic PAF-AH I and II inhibitors. Full-length transcript was amplified from Wistar rat brain and PC12-AC cells using a series of primer pairs homologous to conserved sequences in murine, bovine, and human genes (Table 2.1). A 1173 bp open reading frame was identified in both PC12-AC cells and Wistar rat brain with no base-pair mismatches between the sequences[3]. In functional assays, total cytosolic PAF-AH activity in cell lysates was comparable to enzymatic activity in adult mouse brain homogenates (Table 2.2). PAF hydrolytic activity was not detected in treatment media harvested from cells after 24 h of culture confirming the RT-PCR analysis that PC12-AC cells do not express

[3] The rat PAF-AH II cDNA cloned from adult Wistar rat brain was deposited under GenBank accession number AY225592.

Figure 2.3: Expression patterns of PAF-AH I, PAF-AH II, and plasma PAF-AH in PC12-AC cells. RT-PCR was performed for (A) LIS1, (B) PAF-AH I α_1, (C) PAF-AH I α_2, and (D) PAF-AH II. Plasma PAF-AH mRNA was not detected (E). The absence of genomic DNA contamination or reagent contamination was confirmed by the control reactions: no RT during the RT reaction, no template during the PCR reaction, and no primers during the PCR reaction. Rat brain RNA was reverse-transcribed as a same-species positive control (Rat Brain lane). Template integrity of the random-primed PC12-AC RT product was verified using GAPDH (F). Equal amounts of protein (30 μg) from cells cultured in complete media (0) or cells treated for 60 min with 1 μM PAF in treatment media (60) were separated under reducing conditions and immunoblotted for α_1 (G), α_2 (H,I), or LIS1 (J). Protein lysates from neonatal rat brain were used as a same-species positive control. Representative immunoblots of replicate experiments are depicted. PAF-AH α_1 was constitutively expressed (G). Significant PAF-AH α_2 protein was not detected until cells were treated with PAF (1 μM) (H). Longer exposure times (overnight) indicated that PC12-AC cells expressed low levels of protein and that serum deprivation was sufficient to induce α_2 protein expression (I). LIS1 was constituitively expressed (J). The major species was a 45 kD protein although multiple bands were detected in untreated PC12-AC cells consistent with phosphorylated LIS1 (arrows). An additional non-specific high molecular weight band (~70 kDa) was also detected (asterisk). Blots were probed for actin as a loading control (G-J).

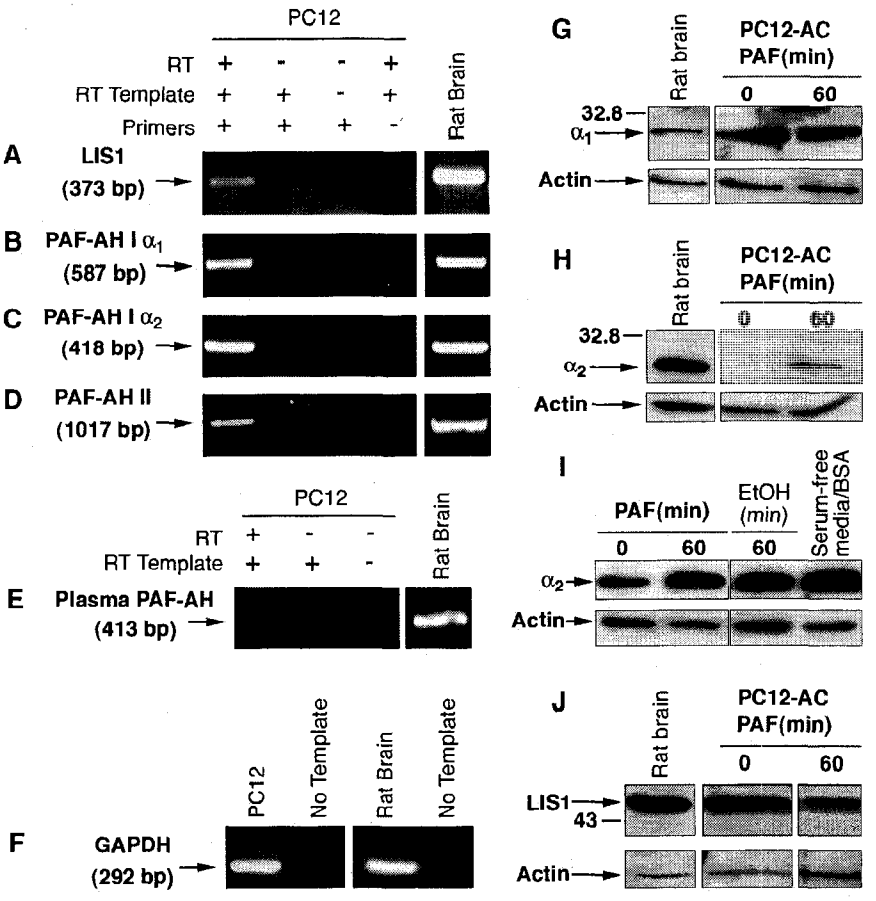
PC12
RT + - - +
RT Template + + - +
Primers + + + -
Rat Brain
A LIS1
(373 bp)
B PAF-AH I α1
(587 bp)
C PAF-AH I α2
(418 bp)
D PAF-AH II
(1017 bp)
PC12
RT + - -
RT Template + + -
Rat Brain
E Plasma PAF-AH
(413 bp)
PC12
No Template
Rat Brain
No Template
F GAPDH
(292 bp)
G
Rat brain
PC12-AC
PAF(min)
0 60
32.8
α1
Actin
H
Rat brain
PC12-AC
PAF(min)
0 60
32.8
α2
Actin
I
PAF(min)
EtOH
(min)
Serum-free media/BSA
0 60 60
α2
Actin
J
Rat brain
PC12-AC
PAF(min)
0 60
LIS1
43
Actin

Figure 2.3

Table 2. 2. PAF-AH activity in PC12-AC cells, mouse brain lysate, and tissue culture media

PAF-AH activity[1]	Mouse brain lysate	PC12-AC Lysates				Cell-free media		
			% inhibition[2]					
	Positive control	Untreated Lysates	Time	0.1 mM DFP	1 mM DFP	Complete Media	Serum-Free Treatment Media	PC12-AC conditioned Treatment Media
nmol/min/μg or % inhibition	1.97 ± 0.44 n=8	1.37 ± 0.19 n=23	15 min	68% n=6	75% n=5	3.36 ± 0.10 n=2	0.01 ± 0.00 n=2	0.00 ± 0.00 n=3
			30 min	70% n=3	74% n=3			

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

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

the secreted PAF-AH isoenzyme (Table 2.2). To distinguish between PAF-AH I and PAF-AH II activity, cell lysates were treated with the active serine blocking agent DFP. 0.1 mM DFP inhibits PAF-AH I α_1/α_1 or α_1/α_2 dimer activity while 1 mM DFP inhibits PAF-AH I α_1/α_1, PAF-AH I α_1/α_2, and PAF-AH II activity. PAF- AH I $\alpha_2\square\alpha_2$ dimers are DFP-resistant (Hattori et al., 1996; Hattori et al., 1995; Manya et al., 1999). 68-70% of the total cytosolic PAF-AH activity was blocked by 0.1 mM DFP indicative of PAFAH I α_1/α_1 or$\square\alpha_1/\alpha_2$ activity (Table 2.2). Increasing the DFP concentration to 1 mM blocked an additional 4-7% of PAF-AH activity attributed to PAF-AH II. DFP-resistant PAF-AH I α_2/α_2 homodimer activity comprised 25% of cytosolic PAF-AH activity in lysates extracted from cells cultured in complete media.

The kinetics of PAF hydrolysis by PAF-AH I and II were established using the fluorescent PAF substrate, Bodipy FL C_{11}-PAF (B-PAF). Cells were treated with 1 μM B-PAF to initiate apoptotic signaling. B-PAF and its metabolites were extracted at various time points using a modified Bligh and Dyer procedure, identified by TLC, and fluorescent intensity quantified by comparison to resolved fluorescent standards. Cytosolic and extracellular lipids were fractionated from B-PAF-treated cultures with an acidified methanol wash separating phospholipids bound to proteins on plasma membrane from internalized lipids (Ohshima et al., 2002). We found that 1 μM B-PAF was stable in cell-free, serum-free treatment media at 37°C for at least 60 min; no degradation to B-*lyso*-PAF was observed in the absence of cells (Fig 2.4A). B-PAF was rapidly internalized by PC12-AC cells (Fig 2.4B, Extracellular) with concentrations reaching approximately 16-20 pM/cell within 5 min of incubation (Fig 2.4B, Cytosolic). Internalized B-PAF levels remained constant for 60 min dropping to 6-8 pM/cell by 75

Figure 2.4: Internalization, kinetic analysis, and metabolic fate of B-PAF and B-*lyso*-PAF in PC12-AC cells. PC12 cells were incubated with 1 μM B-PAF at 37°C for 0, 5, 15, 30, 45, 60, and 75 minutes. At each time point, lipids were extracted from the extracellular and cytosolic fractions, and were separated by TLC. (A-C) Data represent relative fluorescence of B-PAF in each fraction expressed as a percentage of the total fluorescent lipids recovered. Data are the mean of 2-4 independent experiments conducted in replicate. (A) B-PAF was not degraded by treatment media in the absence of PC12 cells. (B) In cell cultures, B-PAF was rapidly internalized within 5 min (Extracellular fraction) after which, levels remained stable until 60 min and dropped by 75 min (Cytosolic fraction). (C) Conversion of B-PAF to B-*lyso*-PAF was monitored to determine the degree of PAF hydrolysis in the presence or absence of the PAF-AH α_1 and PAF-AH II inhibitor DFP. Cells were pre-treated for 15 min in treatment media with Vehicle (PBS) or DFP before addition of B-PAF. (D) The fate of the B-PAF metabolite B-*lyso*-PAF was followed by fractionation. (E) To determine whether B-*lyso*-PAF secreted from cells remains loosely associated with the lipid bilayer or bound to membrane proteins, cell-associated fluorescence was tracked by live-cell imaging. The B-PAF containing media was removed every 10 min and cells were washed with PBS + 1% BSA to remove phospholipids at the extracellular face of the plasma membrane but not lipids associated with membrane proteins. Data are reported as the mean Increase in fluorescence intensity standardized to background fluorescence in untreated cells $\pm$ SEM (n= 16-25 per data point). Cell-associated fluorescence increased 3-fold over the first 75 min of exposure and remained constant between 75 and 120 min despite repeated washes indicating that the secreted material removed by the acidified methanol wash in (D, Extracellular) was likely bound to proteins at the plasma membrane (E). Scale bars, 5 μm.

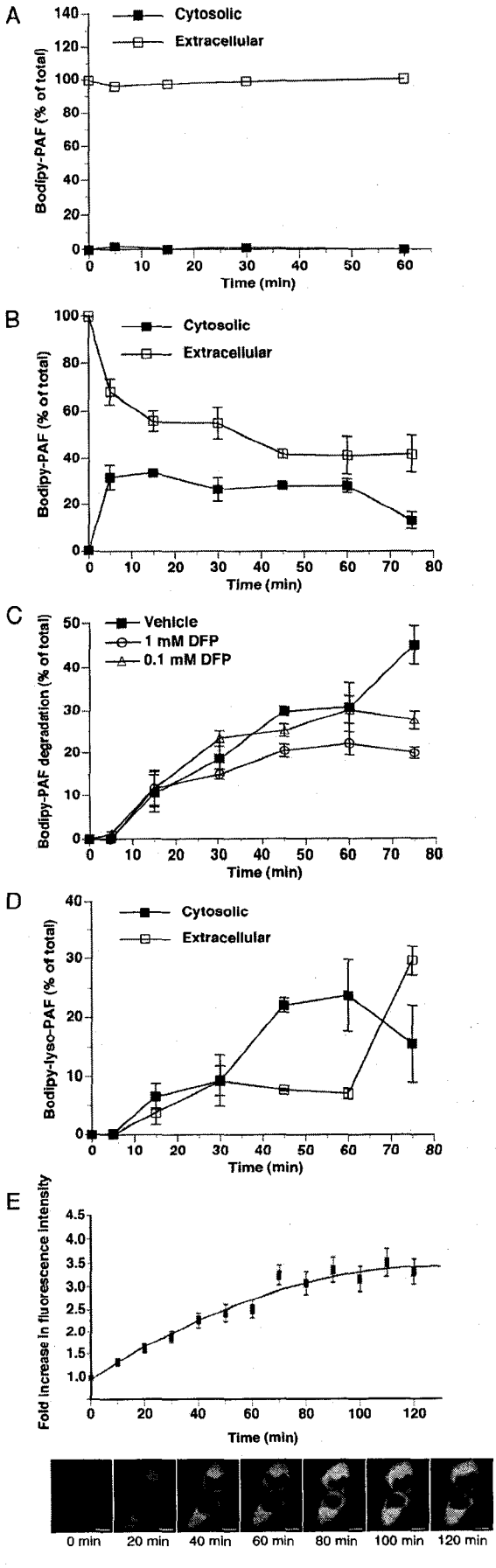
A
Cytosolic
Extracellular
Bodipy-PAF (% of total)
Time (min)
B
Cytosolic
Extracellular
Bodipy-PAF (% of total)
Time (min)
C
Vehicle
1 mM DFP
0.1 mM DFP
Bodipy-PAF degradation (% of total)
Time (min)
D
Cytosolic
Extracellular
Bodipy-lyso-PAF (% of total)
Time (min)
E
Fold increase in fluorescence intensity
Time (min)
0 min
20 min
40 min
60 min
80 min
100 min
120 min

Figure 2.4

min (Fig 2.4B, Cytosolic). B-PAF degradation was detected within 15 min of internalization (Fig 2.4C, Vehicle). A linear rate of metabolism (r^2=0.97) was observed for up to 75 min (Fig 2.4C, Vehicle). Closer examination of the fate of B-PAF metabolite by fractionation revealed that B-*lyso*-PAF was released from cells in two stages (Fig 2.4D). Between 5 and 15 min of incubation, 50% of internalized B-PAF was converted to B-*lyso*-PAF (Fig 2.4D, Cytosolic) and secreted from cells (Fig 2.4D, Extracellular). Between 30 and 60 min, B-*lyso*-PAF was not released and accumulated in the cell cytosol (Fig 2.4D, Cytosolic). A delayed phase of secretion was observed between 60 and 75 min (Fig 2.4D, Extracellular).

To track the fate of secreted B-*lyso*-PAF, we performed quantitative time-lapse fluorescence microscopy (Fig 2.4E). B-PAF-containing media was removed and cells were washed with PBS containing 1% BSA at various time points to remove free lipids loosely associated with the plasma membrane but not phospholipids bound to membrane proteins. Cells were photographed and the B-PAF containing media was replaced. A linear increase in cell-associated fluorescence was observed over the first 70 min of exposure (r^2=0.97) followed by a plateau in cell-associated B-PAF (Fig 2.4E).

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

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

The kinetics of PAF-induced apoptosis (Fig 2.1, 2) and PAF degradation (Fig 2.4)

suggest that cytosolic PAF concentrations must remain elevated for at least 60 min to elicit apoptosis. This hypothesis predicts that compounds capable of inhibiting PAF internalization or increasing cytosolic PAF-AH activity will block PAF-mediated death triggered independently of PAFR. To test this hypothesis, we evaluated the anti-apoptotic actions of four PAF antagonists (CV-3988, CV-6209, BN 52021, and FR-49175). These compounds were chosen because of their affinities for different PAF binding sites identified pharmacologically. CV-3988 and CV-6209 are competitive PAF antagonists that preferentially interact with synaptosomal and microsomal PAF binding sites (Marcheselli et al., 1990). CV-3988 competes for PAF at the plasma membrane, likely PAFR, as well as interacting with intracellular microsomal binding sites, likely internalized PAFR (Marcheselli et al., 1990). CV-6209 preferentially interacts with a single binding site in microsomal membranes (Marcheselli et al., 1990). BN 52021 (also known as ginkgolide B) is a non-competitive PAF antagonist with affinity for three discrete PAF binding sites (Marcheselli et al., 1990) as well as potent antioxidant activity (Pietri et al., 1997). FR-49175 is a fungal metabolite derivative that inhibits PAF-induced biological activity through unknown mechanisms (Okamoto et al., 1986; Yoshida et al., 1988). Treatment of PC12-AC cells with CV-3988, BN 52021, or FR-49175 had no effect on cell survival (Fig 2.5A-C) however CV-6209 was toxic to cells at concentrations above 1 μM (Fig 2.5D). We observed significant concentration-dependent anti-apoptotic activity when cells were pre-incubated for 15 min with BN 52021 (1-100 μM) or FR-49175 (0.5-50 μM) and then exposed to PAF (Fig 2.5E). Both BN 52021 and FR 49175 inhibited PAF-mediated caspase 3 activation as assessed by PARP cleavage (Fig 2.5F). CV-3988 (0.1-10 μM) and CV-6209 (0.01-1 μM) did not

Figure 2.5: Anti-apoptotic actions of the PAF antagonists BN 52021 and FR-49175 but not CV 6209 or CV 3988 in PAFR-negative cells. PC12-AC cells were pre-treated for 15 min with 0.1% DMSO (Vehicle) or different concentrations of PAF antagonists and exposed to 0.1% EtOH (A-D) or PAF 1 μM (E,F) for 24 h. In A-E, data are expressed as percent survival of vehicle-treated cultures. Results are reported as Mean± SEM of n=5-47 cultures per data point. BN 52021, FR-49175, and CV 3988 had no effect on the viability of vehicle-treated cultures. CV 6209 (10 μM) was toxic (*$p<0.05$). BN 52021 and FR-49175 but not CV 3988 or CV 6209 dose-dependently inhibited PAF-mediated death († $p<0.05$, †† $p<0.01$). (F) BN 52021 and FR-49175 inhibited PAF-mediated caspase 3 activation as assessed by PARP cleavage.

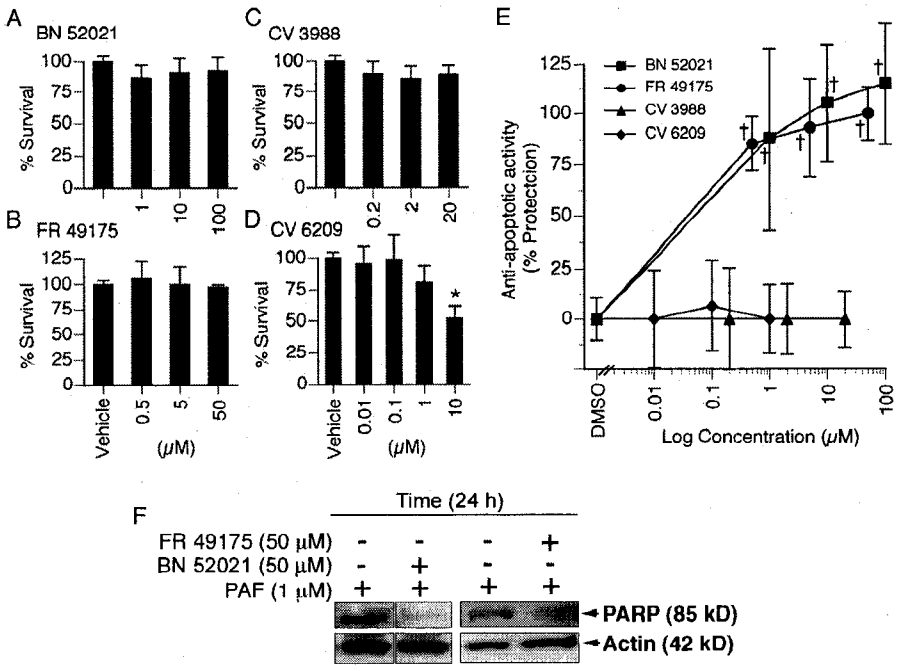
A
BN 52021
% Survival
100
75
50
25
0
1
10
100
B
FR 49175
% Survival
125
100
75
50
25
0
Vehicle
0.5
5
50
(μM)
C
CV 3988
% Survival
100
75
50
25
0
1
0.2
2
20
D
CV 6209
% Survival
100
75
50
25
0
*
Vehicle
0.01
0.1
1
10
(μM)
E
BN 52021
FR 49175
CV 3988
CV 6209
Anti-apoptotic activity
(% Protectcion)
125
100
75
50
25
0
†
DMSO
0.01
0.1
1
10
100
Log Concentration (μM)
F
Time (24 h)
FR 49175 (50 μM) - - - +
BN 52021 (50 μM) - + - -
PAF (1 μM) + + + +
PARP (85 kD)
Actin (42 kD)

Figure 2.5

exhibit anti-apoptotic activity (Fig 2.5E).

We next tracked B-PAF fate by lipid extraction and TLC in the presence or absence of BN 52021 and FR-49175 to establish whether these antagonists affect phospholipid internalization or PAF-AH activity. FR-49175 and BN 52021 did not alter the rate or extent of B-PAF internalization (Fig 2.6A,D, Extracellular). In both antagonist- and vehicle-treated cultures, maximal cytosolic B-PAF levels were attained within 5 min of B-PAF exposure (Fig 2.6A,D Cytosolic 5 min). We did not observe an increase in total B-PAF hydrolysis. Cytosolic B-PAF concentrations, estimated at 8 pM/cell, in BN 52021- and FR-49175-treated cultures were comparable to vehicle-treated cells by the end of the 75 min test period (Fig 2.6A,D Cytosolic, 75 min). Significantly, we found that FR-49175 and BN 52021 accelerated both the kinetics of B-PAF degradation (Fig 2.6A,D, 45 min) and the release of B-*lyso*-PAF into the media (compare Fig 2.6B,E Cytosolic 45 min and Fig 2.6C,F Extracellular 45 min). By 45 min, 39% (FR-49175) or 41% (BN 52021) of exogenous B-PAF had been converted to B-*lyso*-PAF compared to 29% in vehicle-treated cells. This acceleration dropped cytosolic B-PAF levels more rapidly in antagonist-treated cultures from 16 pM/cell (5 min) to 10 pM/cell (45 min) in the presence of FR-49175 or 8 pM/cell (45 min) in the presence of BN 52021 (Fig 2.6B,E). Maximal release of B-*lyso*-PAF from cells was observed within 45 min in antagonist-treated cultures compared to 75 min in vehicle-treated cultures (Fig 2.6C,F). 50% of the released metabolite diffused or was transported back into the cells within 75 min in FR-49175 treated cultures; 32% of B-*lyso*-PAF re-entered cells in BN 52021-treated cultures (Fig 2.6B-F).

Figure 2.6: FR-49175 and BN 52021 accelerate the kinetics of PAF-AH activity. PC12-AC cells were pre-treated with FR-49175 (10 μM), BN 52021 (50 μM), or PBS (vehicle) for 15 minutes and then incubated with 1 μM B-PAF at 37°C. Lipids were extracted from the extracellular and cytosolic fractions and separated by TLC. The relative fluorescence of B-PAF or B-*lyso*-PAF in each fraction is expressed as the percentage of the total fluorescent lipids recovered. Data represent the mean ± SEM of n=4 (FR-49175) or n=8 (BN 52021) independent experiments conducted in replicate. (A) FR-49175 and (D) BN 52021 had no effect on B-PAF internalization (Extracellular) but cytosolic B-PAF levels were reduced within 45 min of exposure (Cytosolic). (B,E) The kinetics of B-PAF degradation and (C,F) release were accelerated in FR-49175- (B,C) and BN 52021- (E,F) treated cells.

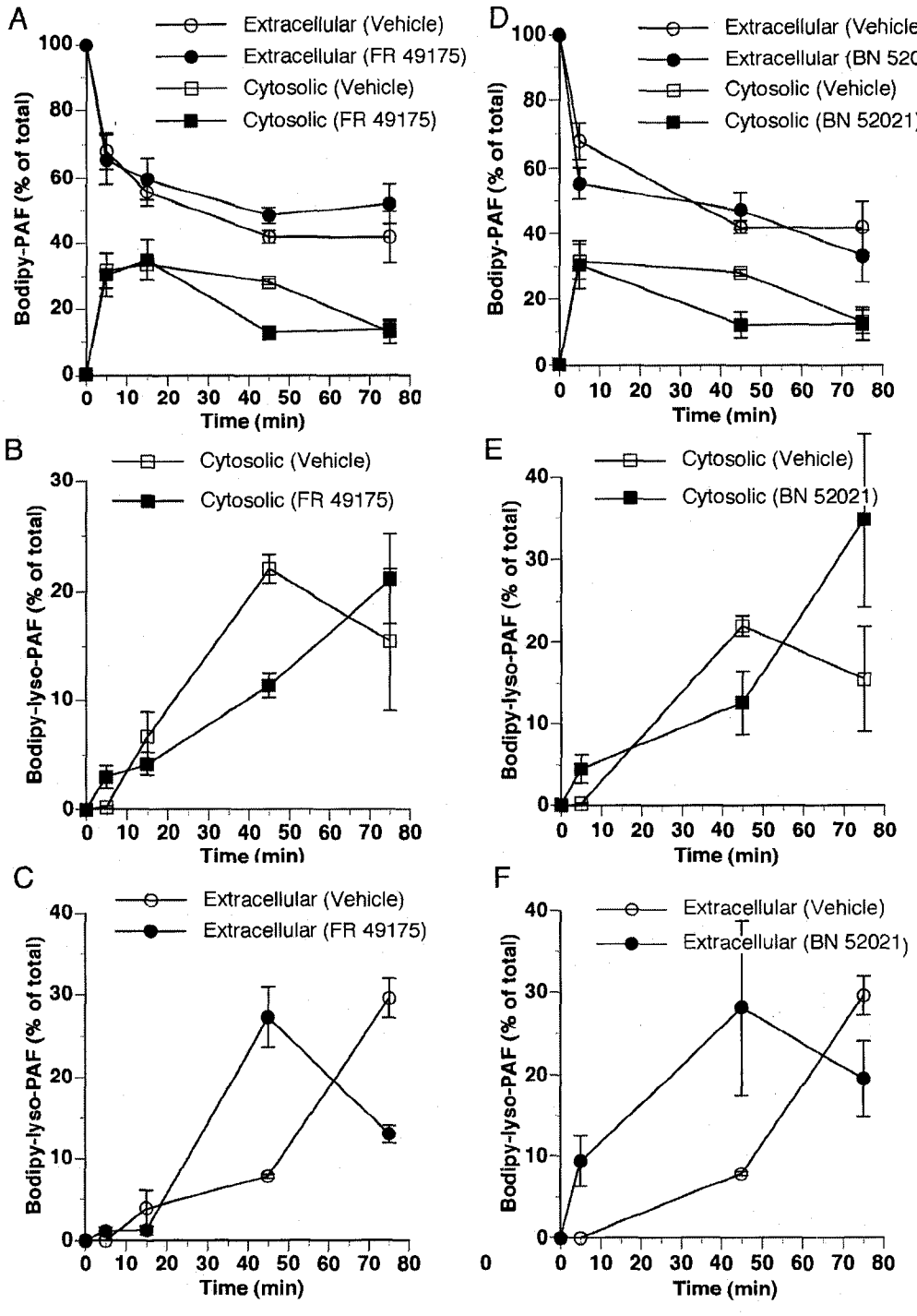
A
Extracellular (Vehicle)
Extracellular (FR 49175)
Cytosolic (Vehicle)
Cytosolic (FR 49175)
Bodipy-PAF (% of total)
Time (min)
D
Extracellular (Vehicle)
Extracellular (BN 52021)
Cytosolic (Vehicle)
Cytosolic (BN 52021)
Bodipy-PAF (% of total)
Time (min)
B
Cytosolic (Vehicle)
Cytosolic (FR 49175)
Bodipy-lyso-PAF (% of total)
Time (min)
E
Cytosolic (Vehicle)
Cytosolic (BN 52021)
Bodipy-lyso-PAF (% of total)
Time (min)
C
Extracellular (Vehicle)
Extracellular (FR 49175)
Bodipy-lyso-PAF (% of total)
Time (min)
F
Extracellular (Vehicle)
Extracellular (BN 52021)
Bodipy-lyso-PAF (% of total)
Time (min)

Figure 2.6

2.7.4 PAF-AH I α_2/α_2 activity is anti-apoptotic

To determine how BN 52021 and FR-49175 accelerate PAF-AH hydrolysis, we examined PAF-AH I α_1 and α_2 expression. Cells were preincubated in serum-free treatment media with vehicle (DMSO), BN52021, or FR-49175 for 15 min before addition of PAF (Fig 2.7). We found PAF-AH α_1 expression remained constant whether cells were cultured in complete media, serum-free treatment media, pretreated with DMSO, or exposed to PAF (Fig 2.7A, PAF). Surprisingly, PAF-AH α_1 protein levels decreased progressively over the first 30 min of PAF treatment and remained low until the end of the 90 min test period (Fig 2.7A, BN52021). Comparable results were observed in FR-49175-treated cultures (Fig 2.7A, FR 49175). α_2 expression was not altered by BN 52021 or FR-49175 in that protein expression increased dramatically during the 15 min preincubation period in serum-free treatment media regardless of antagonist or vehicle administration and remained elevated over the 90 min PAF test period (Fig 2.7B).

The finding that both compounds accelerate PAF degradation by reducing PAF-AH I subunit α_1 expression was unexpected. Two alternative, but not mutually exclusive, explanations lie in the possibility that this reduction in α_1 expression promotes formation of PAF-AH I α_2/α_2 homodimers and/or that PAF-AH II activity is increased. Following exposure to PAF in serum-free media, the catalytic composition of PAF-AH I would be expected to change from a predominance of α_1/α_1 homodimers in control cultures (Table 2.1) to α_1/α_2 heterodimers and α_2/α_2 homodimers given the 8-10 fold increase in α_2 protein expression (Fig 2.3, 2.7). In cultures pre-treated with BN 52021 or FR-49175, suppression of α_1 protein expression likely favours a more a rapid transition from α_1/α_1 to

Figure 2.7: BN 52021 and FR-49175 increase the relative ratio of PAF-AH I α_2 to α_1 expression following PAF challenge. PC12 cells were treated with PAF (1 μM) following a 15 min pre-incubation in the presence or absence of BN 52021 (50 μM), FR-49175 (50 μM), or vehicle (0.1% DMSO). Immunoblotting was performed for α_1 (A) or α_2 (B). BN 52021 and FR-49175 reduced α_1 expression (A) without affecting the α_2 induction (B). Blots were reprobed for actin as a loading controls (A,B). α_1 (A) and α_2 (B) protein levels were standardized to actin and expressed as a percent of the basal expression in cells cultured in complete media. (C) The relative ratio of α_2 to α_1 protein following BN-52021 or FR-49175 revealed a stochiometric increase in the levels of α_2 compared to α_1 relative to cells exposed to PAF in the absence of antagonist.

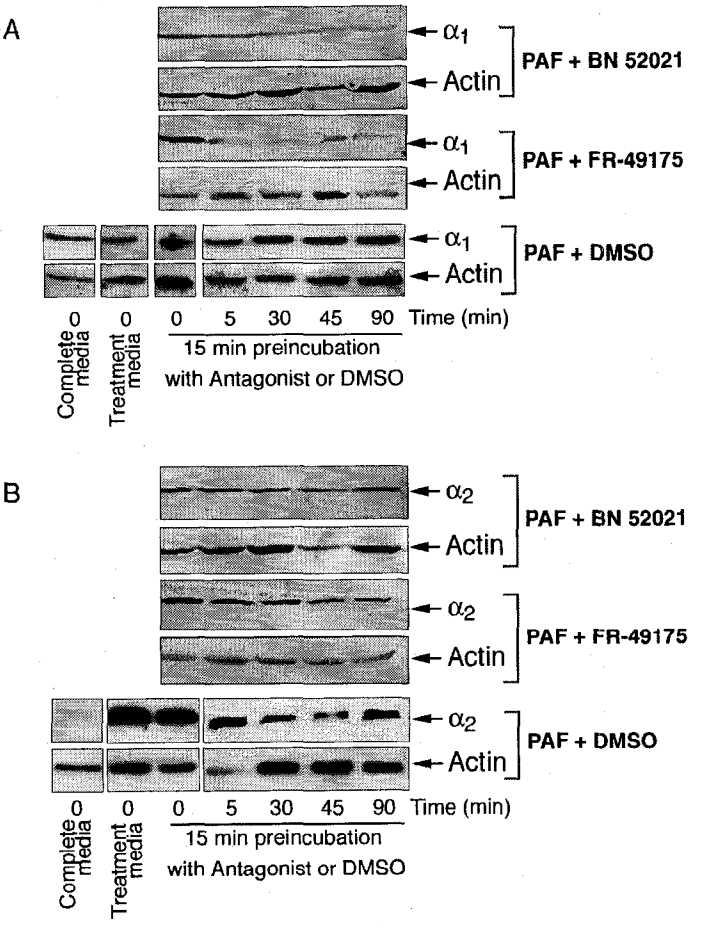
A
α1
Actin
PAF + BN 52021
α1
Actin
PAF + FR-49175
α1
Actin
PAF + DMSO
0 0 0 5 30 45 90 Time (min)
Complete media
Treatment media
15 min preincubation
with Antagonist or DMSO
B
α2
Actin
PAF + BN 52021
α2
Actin
PAF + FR-49175
α2
Actin
PAF + DMSO
0 0 0 5 30 45 90 Time (min)
Complete media
Treatment media
15 min preincubation
with Antagonist or DMSO
C
α2/α1 relative ratio
50
45
30
25
20
15
10
5
0
0 min
5 min
30 min
45 min
90 min
PAF + BN 52021
PAF + FR-49175
PAF + DMSO

Figure 2.7

α_2/α_2 homodimers following PAF exposure. This hypothesis is supported by the dramatic increase in the α_2 to α_1 ratio observed 30-90 min after PAF exposure in BN 52021 and FR-49175-treated cultures relative to vehicle (Fig 2.7C). Alternatively, in addition to effects on PAF-AH α_1, BN 52021 and/or FR-49175 may alter PAF-AH II activity, an enzyme with proven anti-apoptotic properties (Marques et al., 2002).

To address these possibilities, we performed three loss of function studies. First, we treated cells with 0.1 mM DFP to inhibit PAF-AH I α_1/α_1 and α_1/α_2 catalytic activity without changing the relative ratio of α_1 to α_2 protein expression (Hattori et al., 1996; Hattori et al., 1995; Manya et al., 1999). In the unlikely event that the anti-apoptotic effects of BN 52021 and FR-49175 are mediated by a loss of α_1 catalytic activity, then 0.1 mM DFP should be cytoprotective. Cells were exposed for 15 min (Fig 2.4C, Fig 2.8A) before addition of B-PAF. DFP did not affect B-PAF internalization (data not shown) but reduced B-PAF degradation by 38% 75 min after B-PAF exposure (Fig 2.4C, 0.1 mM DFP, 75 min). To ensure intracellular concentrations of DFP reached 0.1 mM by the time cells were exposed to PAF, we extended the preincubation period from 15-30 min with no additional effect on the kinetics of PAF-AH inhibition (data not shown). Inhibition of PAF-AH I α_1/α_1 and α_1/α_2 catalytic activity did not alter survival of cells treated for 24 h with vehicle (Fig 2.8A, Vehicle (0.1% EtOH)) and did not intensify PAF-mediated cell death (Fig 2.8A, PAF). These data suggest that PAF-AH I α_1 does not play a significant role in regulating PAF-induced apoptosis.

Second, to determine the role of PAF-AH II in control of PAF-mediated cell death, we treated cultures with PAF (1 μM) or PAF vehicle (0.1% EtOH) in the presence of DFP, the sulfhydryl blocking reagent (DTNB), or vehicle (PBS) (Fig 2.4C, 8A). Cells

Figure 2.8: PAF-AH α_2 activity is anti-apoptotic. (A) PC12 cells were treated with PAF (1 μM) or ethanol (0.1%) at 37°C for 24 h following 15 min pre-incubation with DFP (0.1 mM), DFP (1 mM), DTNB (1mM), or DFP (1 mM) + DTNB (1 mM) to abolish PAF-AH α_1 and PAF-AH II activity. Cell survival was assessed as described in Fig 2.5. Inhibition of PAF-AH II but not PAF-AH α_1 activity moderately intensified PAF-mediated death. (ANOVA, *post hoc* Tukey tests, * $p<0.05$, ** $p<0.01$). (B) Short interfering RNA (siRNA) oligonucleotides were employed to silence PAF-AH α_2. Western analysis for α_2 was performed at various time points after transfection following a 45 min treatment with PAF (1 μM) in serum-free treatment media. A partial knockdown in α_2 protein levels, consistent with the maximal 20% transfection efficiency, was detected 72 h after transfection (α_2 RNAi) compared to non-transfected cells (Control) or cells that had been transfected with nonspecific scrambled RNAi (Scrambled). β-actin blot was performed as a loading control. (C) PC12 cells were transfected with pEGFP-C1 (Control) or cotransfected with pEGFP-C1 and α_2 RNAi (α_2 RNAi) or scrambled RNAi (Scrambled). 72 h after transfection, cells were treated with PAF (1 μM) or vehicle (0.1% EtOH) for 24 h. Data are expressed as % survival of EGFP-positive cells standardized to vehicle-treated controls. Survival was reduced to 28% in cells transfected with α_2 RNAi compared to 70% in cells transfected with EGFP alone able to upregulate PAF-AH I α_2 (Fig 2.1). Asterisks indicate statistically significant differences by Student's *t* test.

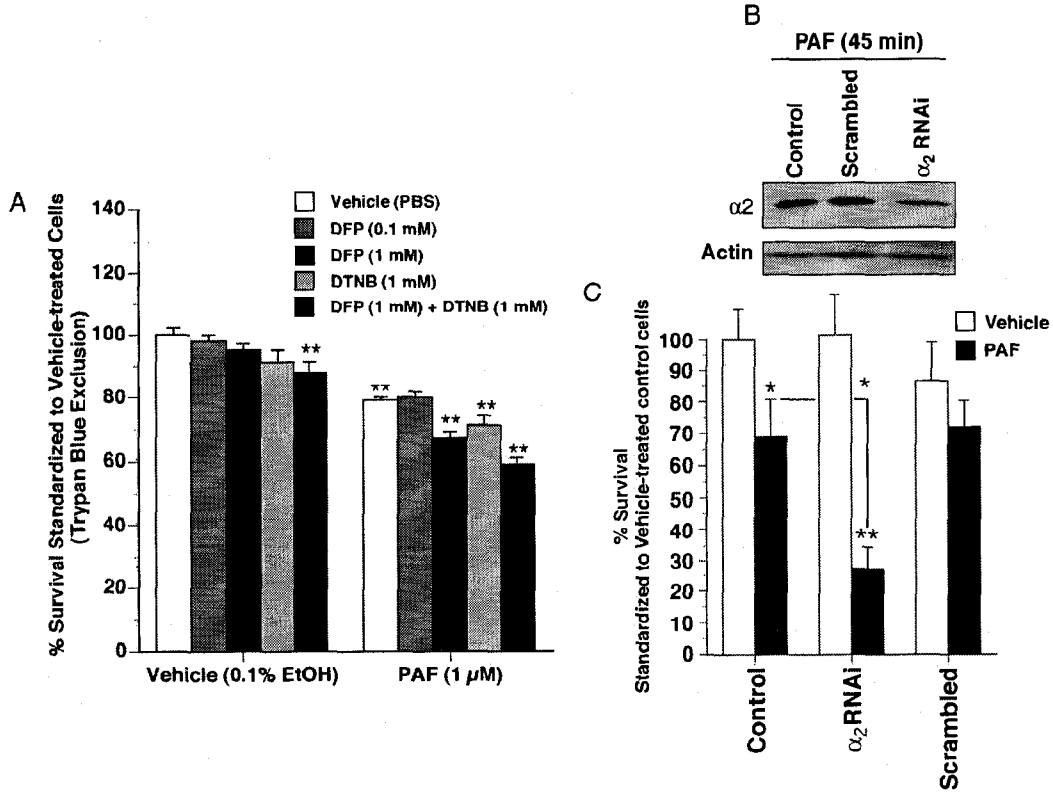
A
% Survival Standardized to Vehicle-treated Cells (Trypan Blue Exclusion)
140
120
100
80
60
40
20
0
Vehicle (PBS)
DFP (0.1 mM)
DFP (1 mM)
DTNB (1 mM)
DFP (1 mM) + DTNB (1 mM)
**
Vehicle (0.1% EtOH)
PAF (1 μM)
B
PAF (45 min)
Control
Scrambled
α2 RNAi
α2
Actin
C
% Survival Standardized to Vehicle-treated control cells
100
90
80
70
60
50
40
30
20
10
0
Vehicle
PAF
*
**
Control
α2RNAi
Scrambled

Figure 2.8

were pre-exposed to 1 mM DFP or 1 mM DTNB for 15 min (Fig 2.4C, 8A) or 30 min (data not shown) before treatment with PAF or vehicle. Individually DFP and DTNB were found to have no effect on the survival of vehicle-treated cells (Fig 2.8A, Vehicle (0.1% EtOH)). Increasing the DFP concentration from 0.1 mM to 1 mM inhibited 55% of B-PAF degradation within 75 min indicating that 17% of DFP-sensitive PAF-AH activity was PAF-AH II (Fig 2.4C, 1 mM DFP, 75 min). Despite this substantial inhibition of PAF hydrolysis (55%), PAF-mediated cell loss was intensified by only 8% (Fig 2.8A, 1 mM DFP). To confirm these results, we used a pharmacological agent that, unlike DFP, inhibits PAF-AH II but not PAF-AH I through different mechanisms. Exposure to 1 mM DTNB elicited the same results as 1 mM DFP (Fig 2.8A, DTNB). To ensure complete inhibition of PAF-AH II, we exposed cells to both DFP and DTNB. Combination treatment reportedly abolishes activity of purified PAF-AH II (Hattori et al., 1995). Preincubation in DFP and DNTB followed by 24 h exposure to EtOH decreased vehicle-treated cell survival by 12% (Fig 2.8A, Vehicle (0.1% EtOH)). Combination treatment reduced PAF-treated cell survival by 20% likely the cumulative results of inhibitor toxicity (12%) and PAF-AH II inhibition (8%) (Fig 2.8A, PAF). These findings suggest that a) PAF-AH I α_1 activity is not anti-apoptotic and b) that while PAF-AH II activity is cytoprotective, PAF-AH II plays a secondary role to PAF-AH I α_2 in regulating PAF-mediated apoptosis.

The data point to PAF-AH I α_2 as a potential therapeutic target to reduce apoptogenic PAF concentrations under diverse pathophysiological conditions. To directly confirm this role, we acutely suppressed α_2 expression using a RNAi strategy. PAF-AH I α_2-specific RNAi but not the scrambled control or transfection reagent alone reduced α_2 pro-

tein expression (Fig 2.8B). Because we were unable to achieve > 20% transfection efficiency, we could not knockdown PAF-AH I α_2 expression in all cells. To identify transfected cells, we co-transfected cultures with EGFP in combination with α_2-specific or scrambled siRNAs (Fig 2.8C). To control for possible cytotoxic effects of siRNAs, parallel experiments were performed in which cultures were transfected with EGFP alone and survival was expressed relative to EGFP-positive vehicle-treated cells. Importantly, PAF-AH I α_2 RNAi downregulation significantly enhanced PAF-mediated cytotoxicity (Fig 2.8D).

2.8 Discussion

PAF-like lipids have been implicated as key apoptotic second messengers induced by a variety of pathological stressors (Barber et al., 1998; Bennett et al., 1998; Hirashima et al., 2000; Li et al., 2003; Ma and Bazan, 2001; Marques et al., 2002; Matsuzawa et al., 1997; Ogden et al., 1998; Tong et al., 2001). Converging evidence points to PAFR activation of the conserved mitochondrial death pathway (Hostettler et al., 2002; Li et al., 2003; Lu et al., 2004; Ma and Bazan, 2001; Southall et al., 2001; Tong et al., 2001). Our previous work has demonstrated that PAF can also transduce cell death independently of its G-protein coupled receptor (Brewer et al., 2002) but it is not known how cell death is regulated in the absence of PAFR. The importance of this apoptotic cascade is underlined by the fact that neurons express low levels to no PAFR (Bennett et al., 1998; Mori et al., 1996) and yet PAF is a principle mediator of neuronal loss in ischemia, encephalitis, epileptic seizure, meningitis, and human immunodeficiency virus-1 dementia (Bate et al., 2004a; Bate et al., 2004b; Bazan, 1998; Birkle et al., 1998; Perry et al., 1998). To

provide mechanistic insight into this PAFR-independent pathway, we show that: 1) PAF can initiate caspase-dependent cell death in the absence of it G-protein coupled receptor; 2) The duration of PAF apoptogenic signaling is regulated by the α_2 subunit of PAF-AH I and, to a lesser extent, by PAF-AH II; 3) PAFR-independent cell death can by inhibited by two PAF antagonists: the gingkolide BN 52021 and the fungal derivate FR-49175, but not by CV-3988 or CV-6209 that share PAF's glycerophospholipid; 4) Both BN 52021 and FR-49175 protect cells from PAF-induced cell death by reducing PAF-AH I α_1 protein levels indirectly promoting PAF-AH I α_2 homodimer activity.

2.8.1 Apoptotic PAF signal transduction in the absence of PAFR

To provide mechanistic insight into how PAF transduces cell death in the absence of PAFR, we followed the internalization and metabolic fate of Bodipy fluorophore-conjugated PAF in PAF-sensitive cells (Brewer et al., 2002; Kornecki and Ehrlich, 1988). PC12 cells express all of the components of both cytosolic PAF-AH enzymes (I and II) but not plasma PAF-AH or PAFR. Extracellular PAF was internalized by PC12 cells with a $t_{1/2}$ of approximately 5 min. Downstream activation of caspase 3 was initiated when cytosolic PAF concentrations were elevated by approximately 15-20 pM/cell. These findings complement previous work demonstrating that exposure to oxidative stressors triggers apoptogenic remodeling of membrane phospholipids into PAF-like lipids (Marques et al., 2002) and provides strong evidence that cytosolic accumulation of PAF and PAF-like lipids can trigger apoptotic death independently of PAFR. In fact, internalization of PAF mediated by PAFR endocytosis (Ohshima et al., 2002) may protect cells from this cell death cascade. Endocytosis of the PAF/PAFR complex triggers re-

lease of plasma PAF-AH from macrophages thereby reducing extracellular ligand concentrations (Ohshima et al., 2002).

While we have yet to identify the effector proteins responsible for transducing increases in intracellular PAF concentration into downstream caspase activation, our data indicate that the temporal kinetics of PAF accumulation regulate the duration of apoptotic signaling and that PAFR-independent cell death is triggered by PAF and not by its immediate metabolite *lyso*-PAF. We found that intracellular PAF levels must remain elevated by approximately for 60 min to elicit significant apoptotic death. Decreasing cytosolic concentrations to less than 10 pM/cell of exogenous PAF stops the cell death signalling. *Lyso*-PAF, the immediate PAF metabolite, does not transduce downstream apoptotic induction given that equimolar concentrations of *lyso*-PAF are not cytotoxic and that the endogenous metabolite remains cell-associated without detectable effect. Accelerating the kinetics of PAF degradation with BN 52021 or FR-49175 reduces PAF concentration to sub-apoptotic levels (~ > 8 pM/cell) within 45 min and prevents PAF-induced caspase activation. The PAFR-specific antagonists CV-3988 and CV-6209 have no effect on PAF-mediated PAFR-independent cell death when administered at concentrations up to 20 times that of their reported IC 50 antagonist activities (Nunez et al., 1986; Terashita et al., 1987; Valone, 1985). Interestingly, the anti-cell death activities of the antagonists tested in this study (FR-49175 > BN 52021 > CV-3988 or CV-6209) are in direct opposition to their PAF antagonist potency in other biological assays (CV-6209 > CV-3988 > BN 52021 > FR 49175) (Dupré et al., 2001; Nunez et al., 1986; Terashita et al., 1987; Valone, 1985; Yoshida et al., 1988) suggesting that their PAFR-independent anti-apoptotic actions are inversely proportional to their affinity for PAFR.

We do not know how PAF is internalized by PC12 cells in the absence of its G-protein coupled receptor. In addition to endocytosis as a PAF/PAFR complex, active trafficking of PAF across the plasma membrane is accomplished by a PAF-specific trans-glutaminase and by interaction with low affinity binding sites yet to be identified at the molecular level (Bratton, 1993; Lachachi et al., 1985). Passive PAF internalization is regulated by transbilayer movement (flipping) across the plasma membrane occurring as a result of physicochemical changes in membrane properties accompanying cellular activation (Bratton et al., 1992). It has been suggested that PAF internalization in the absence of PAFR is not rapid enough to elicit acute biological activity in hematopoietic cells (Gerard and Gerard, 1994; Ohshima et al., 2002). In this study, we present evidence that PAFR-independent PAF internalization by non-hematopoietic cells is indeed sufficient to trigger apoptotic cell loss.

2.8.2 PAF-AH α_2 activity is anti-apoptotic

Administration of recombinant PAF-AH II or plasma PAF-AH and ectopic over-expression protects cells from death elicited by low-density lipoprotein, glutamate, or oxidative stress (Chen et al., 2003; Hirashima et al., 2000; Marques et al., 2002; Matsuzawa et al., 1997; Ogden et al., 1998). In this study, we show that PAF-AH I exerts similar cytoprotective effects with three important distinctions. First, the anti-apoptotic actions of PAF-AH I are subunit specific. Under normal culture conditions, we found PAF hydrolysis in PC12 cells to be primarily mediated by the PAF-AH I α_1 subunit and to a lesser extent by PAF-AH II. Following serum deprivation and exposure to pathological PAF concentrations, PAF-AH I α_2, but not PAF-AH I α_1, protein expression is acutely

upregulated. By pharmacological inhibition using DFP and DTNB and by RNA interference, we found that PAF-AH I α_2 but not PAF-AH I α_1 activity reduces the duration of PAF-mediated apoptotic signaling. When cells were cultured under normal conditions, we found that cytosolic PAF-AH activity is PAF-AH 1 α_1/α_1 and α_1/α_2 (~70%) > PAF-AH I α_2/α_2 (~25%) > PAF-AH II (5%). When cells were deprived or serum and challenged with PAF, this activity profile shifted in favour of PAF-AH I α_2/α_2 (45%) > PAF-AH α_1/α_1 or α_1/α_2 (38%) > PAF-AH II (17%). Surprisingly, pharmacological inhibition of α_1 enzymatic activity had no effect on PAF-mediated cell death while suppression of α_2 induction by RNA interference significantly enhanced cell loss providing strong evidence for anti-apoptotic subunit specificity.

Second, PAF-AH I α_2 is mobilized as part of an endogenous cell survival response. These findings complement studies documenting the anti-apoptotic actions of plasma PAF-AH and PAF-AH II (Chen et al., 2003; Fukuda et al., 2000; Hirashima et al., 2000; Marques et al., 2002; Matsuzawa et al., 1997; Ogden et al., 1998). Moreover, we find that PAF-AH II is not able to compensate for a loss of PAF-AH I α_2 function. In fact, we were surprised to find that a 55% reduction in PAF hydrolysis observed 75 min after PAF challenge following DFP treatment only moderately enhanced PAF-mediated cell death. The kinetics of PAF-AH activation may explain the lack of significant PAF-AH II protection. PAF-AH α_2 is mobilized acutely in response to serum withdrawal but an increase in DFP- or DTNB-sensitive PAF-AH II activity is only observed after 30-60 min of PAF exposure. Only PAF-AH I α_2 was found to reduce apoptogenic concentrations of cytosolic PAF within the first 30 min of exposure. PAF-AH II is mobilized as part of a delayed cell survival program possibly to ensure that "sub-apoptotic" PAF con-

centrations are maintained.

Third and perhaps most importantly, the anti-apoptotic response afforded by shifting PAF-AH I α_1 subunit expression in favour of α_2 can be enhanced by PAF antagonists. Pharmacological suppression of α_1 by BN 52021 or FR-49175 accelerates the kinetics of PAF deacetylation to *lyso*-PAF and protects cells from PAF-mediated apoptosis. While it would at first appear counterintuitive that a reduction in PAF-AH subunit expression enhances PAF hydrolysis, these data are consistent with previous reports that BN 52021 inhibits PAF degradation under non-apoptotic culture conditions (Lachachi et al., 1985; Lamant et al., 1987). PAF-AH I α_2/α_2 is known to hydrolyze PAF more efficiently than α_1/α_1 homodimers and it is likely that reducing PAF-AH α_1 coincident with PAF-AH α_2 induction would promote more rapid formation of α_2/α_2 homodimers, enhance PAF hydrolysis, and increase cell survival. Interestingly, this phenomenon models the graded reduction in α_1 observed over the course of cerebral development changing the predominant PAF-AH I catalytic composition from α_1/α_2 heterodimers in embryonic central nervous system to α_2/α_2 homodimers in adult brain (Manya et al., 1998). Our data suggest that this shift may render adult brain more resistant to PAF challenge than embryonic brain.

2.8.3 Summary

In summary, this study provides mechanistic evidence that sustained exposure to elevated intracellular PAF concentrations is sufficient to elicit apoptosis through a PAFR-independent cell death pathway. Previous studies have demonstrated that oxidative modification of membrane lipids during atherosclerotic injury, treatment with chemo-

therapeutic agents, ultraviolet B exposure, or excitoxicity is sufficient to produce PAF-like lipids (Barber et al., 1998; Chen et al., 2003; Li et al., 2003; Ma and Bazan, 2001; Ogden et al., 1998). Our data suggest that these effects can occur in the absence of PAFR. To intervene in PAF-mediated death, we describe a novel anti-apoptotic function for PAF-AH I α_2 and II and identify two PAF antagonists that accelerate cytosolic PAF-AH I activity to inhibit PAF-mediated apoptosis.

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Chapter 3: A neuroprotective role for PAFR[4]

3.1 Objective of this study

Having determined that PAF triggers apoptosis in PC12 cells devoid of PAFR, I then proceeded to determine whether apoptogenic C_{16}-PAF signalling was restricted to PAFR-negative neurons. In addition, I sought to evaluate whether PAFR protected neurons from cytotoxic C_{16}-PAF challenge.

3.2 Statement of author contributions

SDR, CH and SALB conceived and designed the experiments. SDR and CH equally contributed to the experimental work and wrote the manuscript with SALB. FM performed Caspatag assay in WT condition in Figure 3 and assisted with PC12 cell screen of benzoates in Figure 5 A and B. HL designed the adenoviral vector used in Figure 2 under the guidance of STH. PSH and JTA supplied benzoates for screening and NGB provided $PAFR^{-/-}$ mice.

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

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Abbreviations[5]

[5]BSA, bovine serum albumin; CGN, cerebellar granule neuron; DMSO, dimethylsulfoxide; EGFP, enhanced green fluorescent protein; EMEM, Eagle's minimum essential medium; ET, ethidium homodimer; FBS, fetal bovine serum; L-NAME, N-nitro-L-arginine methyl ester; NOS, nitric oxide synthase; NMDA, N-methyl-D-aspartate; PAF, platelet activating factor; PAF-AH, PAF-acetylhydrolase; PAFR, platelet activating factor receptor; PARP, poly(ADP ribose) polymerase; RPMI 1640, Roswell Park Memorial Institute medium; SE, standard error

3.4 Summary

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 NMDA receptor signalling given that L-NAME 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.

Running title: Orsellinic acid inhibits PAF neurotoxicity

Key Words: platelet activating factor, platelet activating factor receptor, neurotoxicity, apoptosis, benzoic acids, orsellinic acid

3.5 Introduction

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 dementia (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 signalled 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 tumour necrosis factor α, 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 cytotoxic signalling 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. Here, we show that PAF elicits caspase-dependent neuronal apop-

tosis 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 (Marcheselli et al. 1990). 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 signalling and a novel pharmacological means of inhibiting PAF-induced neuronal apoptosis.

3.6 Materials and Methods

3.6.1 Reagents: All cell culture reagents were obtained from Invitrogen (Burlington, ON Canada) and all chemicals were purchased through Sigma-Aldrich (St Louis, 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 JT Arnason (University of Ottawa, Canada). Cultures were treated with a final concentration of 1 μg/ml based on previous dose-response studies (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 μM), N-nitro-L-arginine methyl ester (L-NAME, 5 mM, Biomol

Research Laboratories, Plymouth Meeting, 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.

3.6.2 Congenic $PAFR^{-/-}$ and $PAFR^{+/+}$ Mice: Breeding pairs of $PAFR^{-/-}$ mice in a hybrid C57BL/6J x 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, Canada) for 10 generations (N10). Congenic N10 $PAFR^{+/+}$ and $PAFR^{-/-}$ colonies were established and maintained from $PAFR^{+/-}$ matings. Animals were genotyped as described by (Nagase et al. 1999).

3.6.3 Primary murine cell culture: CGNs were cultured from postnatal day 7 to 10 mice as previously described (Cregan et al. 2000). Briefly, cerebella were dissected, meninges removed, and tissue 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 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 re-

suspended in dissection solution containing 0.75 mg/ml DNase I (Roche, Mississauga, Canada) and triturated. $CaCl_2$ was added to a final concentration of 15 μM. Following centrifugation, cells were plated in Eagle's minimum essential medium (EMEM) containing 25 mM glucose, 10% fetal bovine serum (FBS), 1% gentamycin, 2mM L-glutamine, and 20 mM KCl at 37°C in a 5% CO_2/95% air atmosphere. CGNs were seeded at a density of 2 x 10^5 cells/cm^2 in 96 well plates coated with laminin (20 μg/ml) and poly-D-lysine (100 mg/ml, Sigma-Aldrich, St Louis, USA) 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).

3.6.4 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 (EMEM containing 25 mM glucose, 0.025% BSA, 1% gentamycin, 2 mM L-glutamine, and 20 mM KCl). CGNs were treated for 24 h with 1 μM PAF, 1 μM *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 acetylhydrolase (PAF-AH). Where treatment with L-NAME, MK-801, test componds 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, Canada) equipped with a QICAM digital camera (Quorum Technologies, Guelph, Canada) and captured using OpenLab software v5.05 (Improvision, Lexington, USA). Percent survival was calculated as (viable cell number$^{(calcein^+ - calcein^+/ET^+)}$/ mean number of viable cells in vehicle control$^{(calcein^+ - calcein^+/ET^+)}$ x 100.

3.6.5 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, USA) and titred 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 (MOI) 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 μM) treatment was calculated as $EGFP^+$ cell number following treatment / mean $EGFP^+$ cell number in the vehicle control x 100.

3.6.6 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 medium (RPMI 1640) 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 x 10^4 cells/well) for treatment. After plating, cells were treated for 24 h with PAF (1 μM, 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 RPMI 1640 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, Mississauga, Canada) was added to each well at the end of the treatment period and incubated for 60 minutes 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$^{(\text{vehicle control})}$ x 100.

3.6.7 Caspase activation: Executioner caspase activation in CGNs was determined using CaspaTag (caspase-3/7) assay (Chemicon, Temecula, 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 μM) or vehicle (treatment media). Cells were imaged using a DMIR epifluorescent inverted microscope (Leica, Richmond Hill, Canada) 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 RIPA buffer (10 mM PBS, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 30 μl/ml aprotinin, 10 μl/ml Na orthovanadate, 10 μl/ml PMSF 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) and monoclonal anti-actin (1:1000, Sigma). Secondary antibodies were horseradish peroxidase-conjugated anti-mouse IgG (1:2000, Jackson Immunolabs). Immunoreactive bands were visualized using SuperSignal West Pico (MJS BioLynx Inc).

3.6.8 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 **).

3.7 Results

3.7.1 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 μM 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 3.1a,c). In $PAFR^{-/-}$ neurons, PAF reduced cell survival by 40% compared to vehicle controls (Fig 3.1c,d). To establish PAF specificity, CGNs were treated with the immediate PAF

Figure 3.1: 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 μM) or *lyso*-PAF (1 μM). 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 ± standard error (SE). Scale bars, 20 μm.

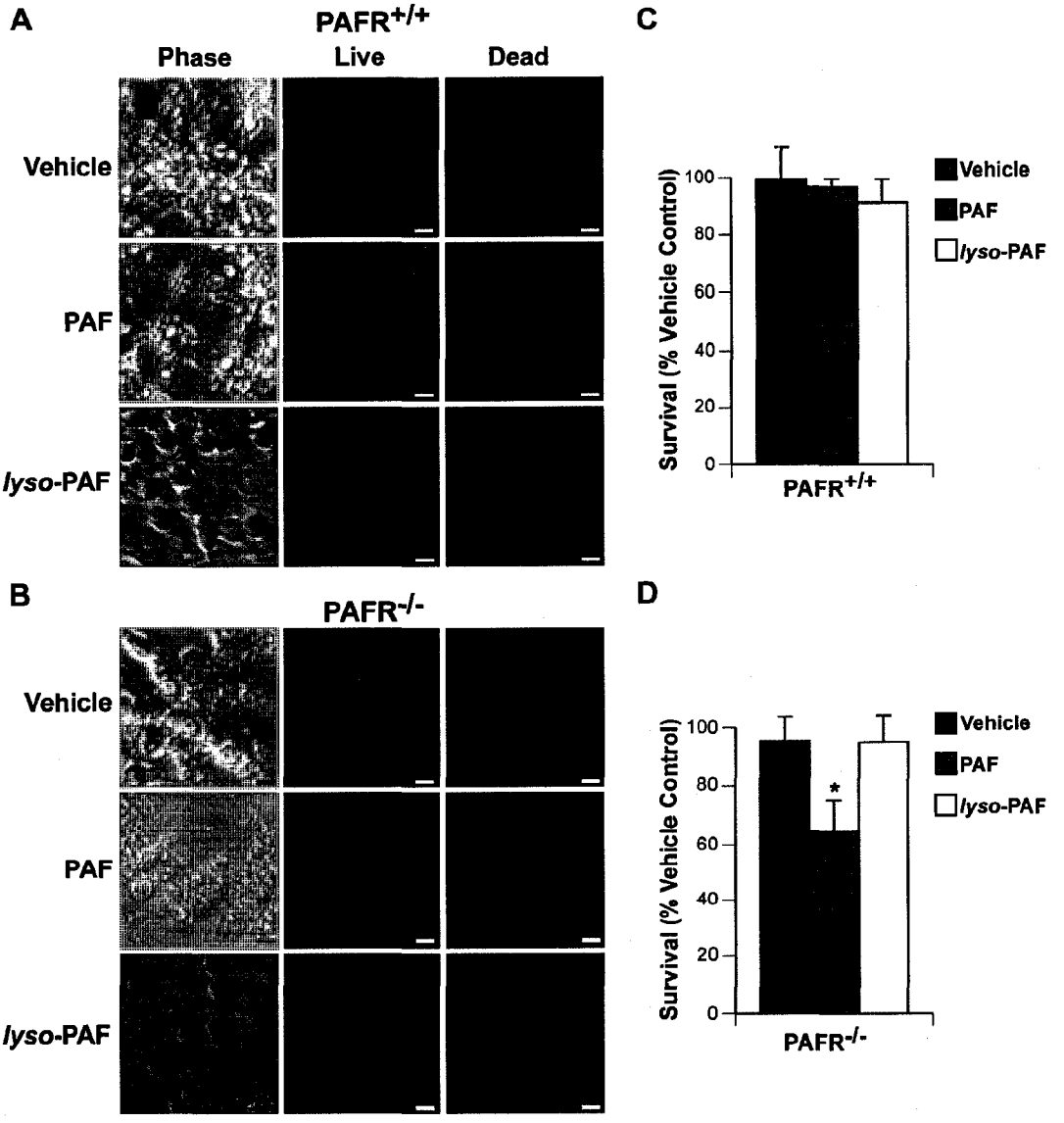
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Figure 3.1

metabolite, *lyso*-PAF. *Lyso*-PAF (1 μM) did not alter $PAFR^{+/+}$ or $PAFR^{-/-}$ neuronal viability demonstrating that PAF and not its metabolite was responsible for the observed effects (Fig 3.1). 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 3.2a,c) whereas ectopic expression of PAFR inhibited PAF-induced cell death (Fig 3.2b,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 3.3a,c). By contrast, in $PAFR^{-/-}$ neurons, a significant increase in caspase activation was observed after 24 h exposure to PAF (Fig 3.3b,d).

PAF-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 μM) in the presence of the NO synthase (NOS) inhibitor L-NAME (5 mM) or the NMDA receptor (NMDAR) antagonist MK-801 (10 μM). 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 3.4).

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

To identify novel compounds capable of inhibiting PAFR-independent neuronal

Figure 3.2: 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 μM) 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

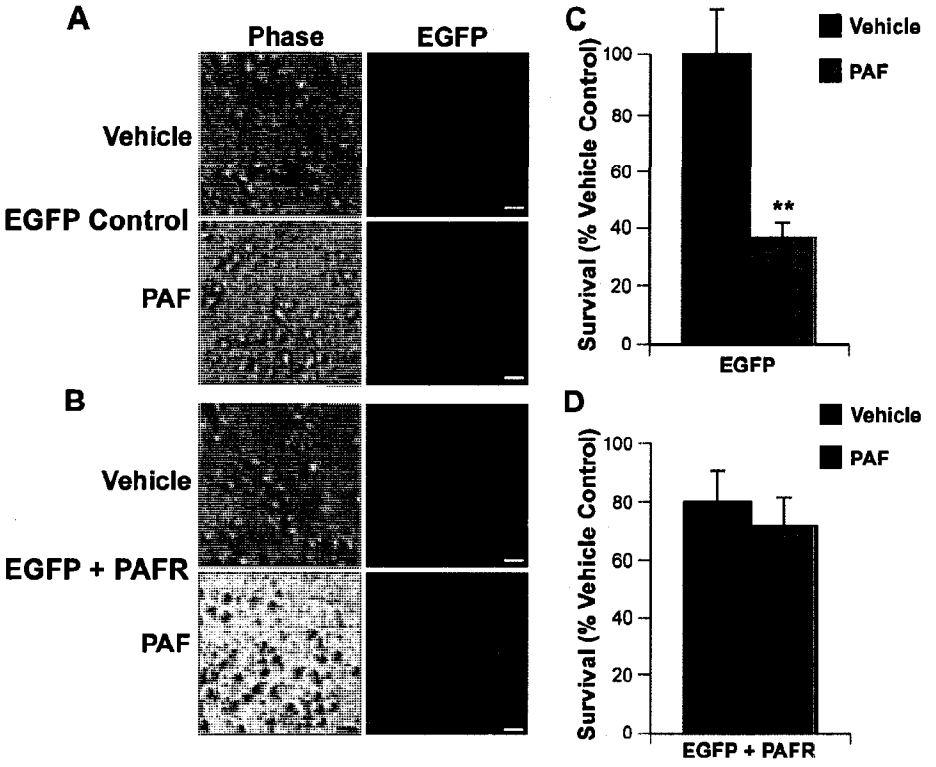
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Figure 3.2

Figure 3.3: PAF induces a caspase 3/7-dependent apoptotic pathway in PAFR$^{-/-}$ neurons. PAFR$^{+/+}$ and PAFR$^{-/-}$ CGNs were treated with vehicle or PAF (1 μM) 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.

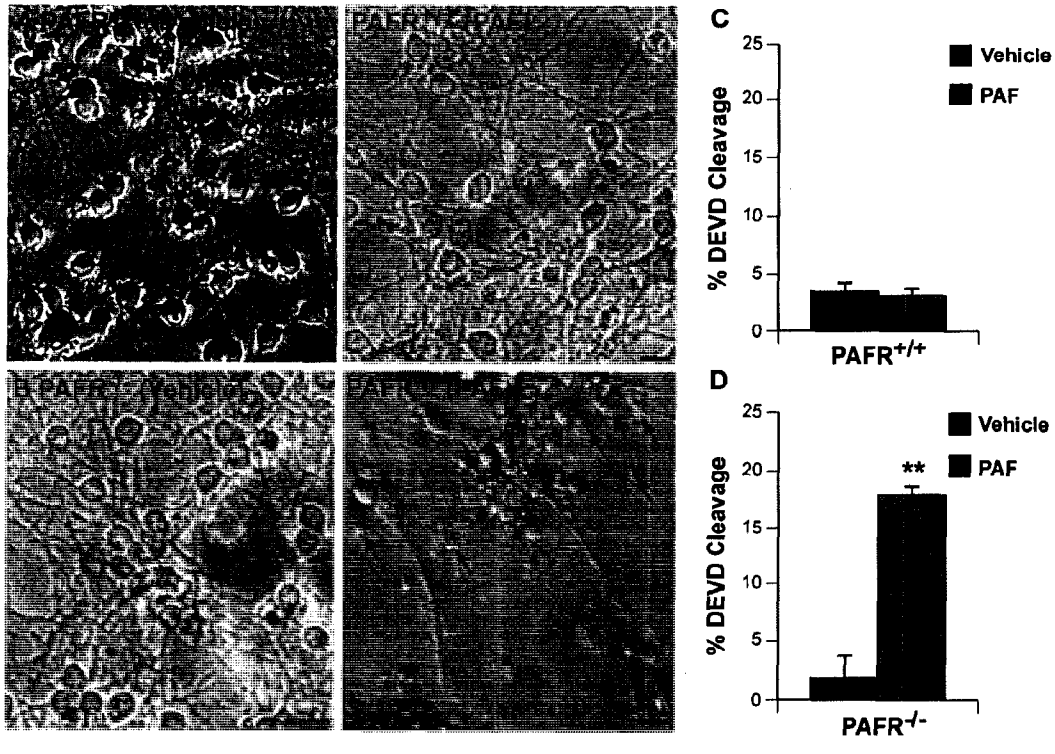
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Figure 3.3

Figure 3.4: $PAFR^{-/-}$ neurons are not protected by NOS inhibition or NMDA receptor blockade. Cell viability was assessed by LIVE/DEAD Viability/Cytotoxicity Kit. The effect of L-NAME (5 mM) and MK 801 (10 μM) 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.

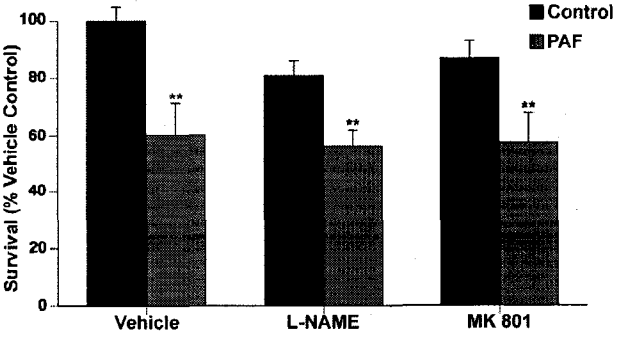
Control
PAF
Survival (% Vehicle Control)
100
80
60
40
20
0
**
**
**
Vehicle
L-NAME
MK 801

Figure 3.4

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 published studies demonstrating that (a) benzoic acid derivatives can alter PAF catabolism and thereby PAF-mediated biological activity and (b) 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 ten benzoic acid derivatives (Table 3.1) present in our library that enhanced PC12-AC cell viability following etoposide challenge (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 signalling 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 3.5a,b). CV 3988 had no significant effect on cell viability in the presence or absence of PAF (Fig 3.5a,b). Five benzoic acids (gentisic acid, veratric acid, gallic acid, syringic acid, salicylic acid) increased viable cell number in both vehicle- and PAF-treated cultures (Fig 3.5a,b) likely indicative of mitogenic effects in growth factor-deprived treatment media. Two benzoates (resorcyclic acid and *m*-digallic acid)

Table 3. 1. Names and molecular weights of benzoic acids screened for PAF inhibitor activity

Common Name	*IUPAC Name*	*Mol. Wt.*
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

Figure 3.5: 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.

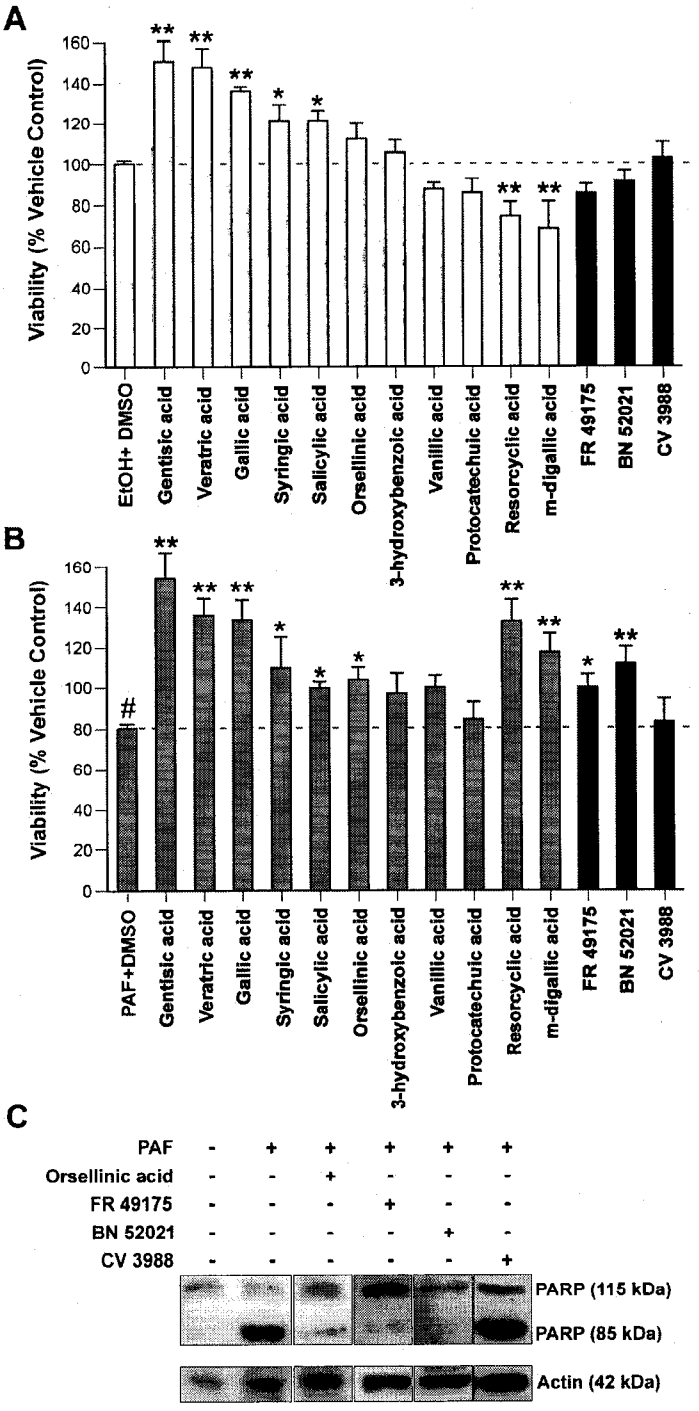
A
Viability (% Vehicle Control)
EtOH+ DMSO
Gentisic acid
Veratric acid
Gallic acid
Syringic acid
Salicylic acid
Orsellinic acid
3-hydroxybenzoic acid
Vanillic acid
Protocatechuic acid
Resorcyclic acid
m-digallic acid
FR 49175
BN 52021
CV 3988
B
Viability (% Vehicle Control)
PAF+DMSO
Gentisic acid
Veratric acid
Gallic acid
Syringic acid
Salicylic acid
Orsellinic acid
3-hydroxybenzoic acid
Vanillic acid
Protocatechuic acid
Resorcyclic acid
m-digallic acid
FR 49175
BN 52021
CV 3988
C
PAF
Orsellinic acid
FR 49175
BN 52021
CV 3988
PARP (115 kDa)
PARP (85 kDa)
Actin (42 kDa)

Figure 3.5

decreased viable cell number in vehicle-treated cultures (Fig 3.5a). Interestingly, both compounds significantly increased viable cell number in PAF-treated cultures (Fig 3.5b). Of the benzoic acids tested, only orsellinic acid significantly inhibited PAF-mediated death without affecting viability of vehicle-treated cells (Fig 3.5a,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 3.5c).

3.7.3 Orsellinic acid inhibits PAF-mediated neuronal apoptosis

PC12-AC cells are tumour 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 μM) in the presence or absence of CV3988 (1 μg/ml), BN 52021 (21 μg/ml), FR 49175 (4 μg/ml), or orsellinic acid (1 μg/ml) (Fig 3.6). 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 compounds will likely not impede PAFR-mediated neuroprotection (Fig 3.6a). Likewise, test compounds had no effect on the viability of vehicle-treated $PAFR^{-/-}$ CGNs (Fig 3.6b). Only BN 52021 and orsellinic acid increased neuronal survival (Fig 3.6b) and inhibited caspase 3/7 activation following treatment of

Figure 3.6: 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.

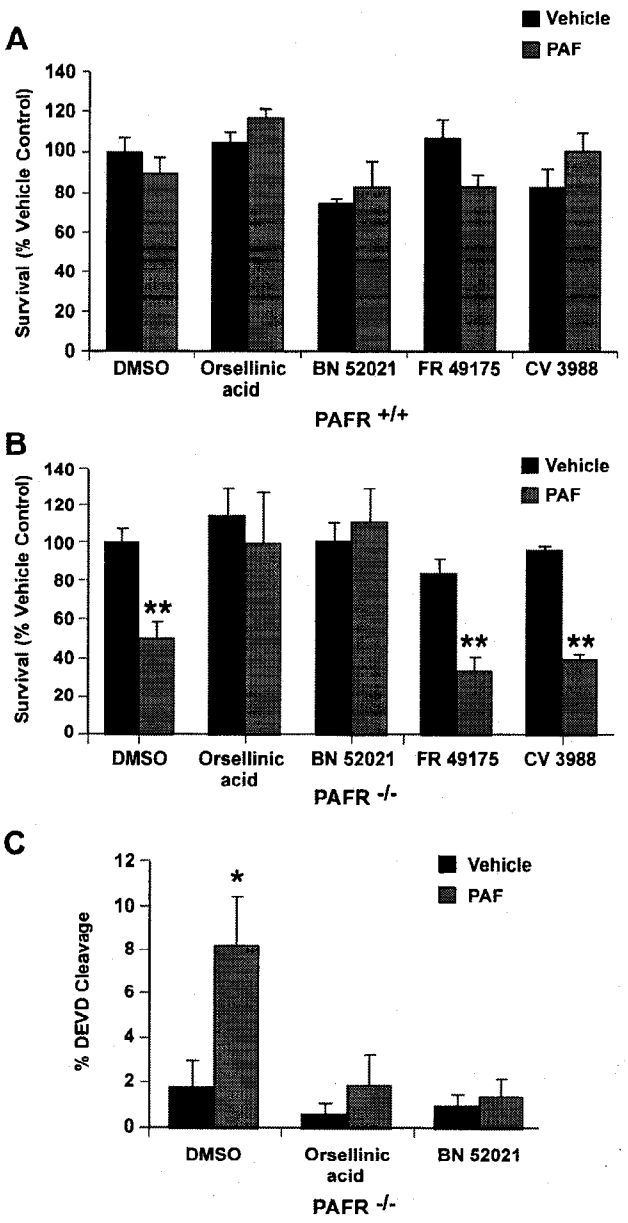
A
Vehicle
PAF
Survival (% Vehicle Control)
140
120
100
80
60
40
20
0
DMSO
Orsellinic acid
BN 52021
FR 49175
CV 3988
PAFR +/+
B
Vehicle
PAF
Survival (% Vehicle Control)
**
**
**
DMSO
Orsellinic acid
BN 52021
FR 49175
CV 3988
PAFR -/-
C
Vehicle
PAF
% DEVD Cleavage
12
10
8
6
4
2
0
*
DMSO
Orsellinic acid
BN 52021
PAFR -/-

Figure 3.6

PAFR$^{-/-}$ cultures with PAF (Fig 3.6c).

3.8 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 NMDAR 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. 2004b; Bate et al. 2004a; Farooqui et al. 2006), yet therapeutic strategies targeting PAF signalling have met with limited success (Le Bars et al. 1997; Le Bars et al. 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; Mori et al. 1997;

Bennett et al. 1998). Earlier studies have hinted at the possibility that PAFR-independent signalling 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 (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.

3.9 Acknowledgments

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Chapter 4: PAF mediated neuronal apoptosis is isoform specific[6]

4.1 Objective of this study

Having established that the C_{16}-PAF isoform was toxic I then asked whether apoptogenic PAF signalling was restricted to the C_{16}-PAF isoform or was common to other major PAF family members. In addition, I sought to determine whether PAFR-mediated neuroprotection was conserved following challenge by the predominant PAF isofrom C_{18}-PAF.

4.2 Statement of author contributions

SDR and SALB conceived and designed the experiments. SDR performed the bulk of the experimental work and wrote the manuscript with SALB. CH performed cell counts for PC12-HPAFR in Fig 2B and assisted with LIVE/Dead assay for C_{16}-PAF treatments in Fig 3A and B.

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

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Running title: Isoform specific actions of platelet activating factor

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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; EGFP, enhanced green fluorescent protein; EMEM, Eagle's minimum essential medium; PAF, platelet activating factor; PAFR, platelet activating factor G-protein coupled receptor; PARP, poly(ADP-ribose) polymerase; WT, wild-type

4.4 Summary

The platelet activating factor (PAF) family of glycerophospholipids accumulate 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). Our data indicate that the carbon chain length of *sn*-1 fatty acid regulates PAF pro- and anti-apoptotic signaling in primary neurons. We find that C_{16}-PAF triggers caspase-dependent apoptosis in PAF receptor-deficient neurons. C_{18}-PAF triggers caspase-independent death in PAF receptor-deficient neurons. We further show that heterogeneity in *sn*-1 fatty acid determines whether PAF receptor activation is neuroprotective or neurotoxic. C_{16}-PAF stimulation of the PAF receptor inhibits caspase-dependent death and protects neurons from PAF challenge. C_{18}-PAF stimulation of the PAF receptor initiates caspase-dependent death. To intervene in these pathways, we show that the non-competitive PAF antagonist BN 52021 blocks PAF neurotoxic signaling regardless of PAF receptor status. The competitive PAF antagonist CV 3988 inhibits the PAF receptor-dependent apoptosis but not PAF-receptor independent death pathways. 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.

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

4.5 Introduction

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 (LipidMaps Consortium, 2007; Prescott et al., 2000) (Fig 4.1). PAF species are synthesized through two enzymatic pathways (remodelling and *de novo*, Fig 4.1). PAF-like lipids with longer *sn*-2 carbon chains or polar head substitutions can also be produced by oxidation of structural membrane glycerophosphocholines or enzymatic modification of glycerophosphoethanolamines (Fig 4.1). All three routes generate molecular species that differ in carbon chain length and degree of unsaturation at the *sn*-1 position (Tokumura et al., 1989). 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 ubiquitously expressed by multiple peripheral cell types but regionally restricted to neuronal subpopulations in the central nervous system (Bennett et al., 1998; Ishii and Shimizu, 2000; Mori et al., 1996). PAFR recognizes glycerophospholipids with an ether bond at the *sn*-1 position, an *sn*-2 acetyl moiety, and an *sn*-3 phosphocholine group (O'Flaherty et al., 1994; Prescott et al., 2000). The *sn*-1 ether linkage is required for receptor interaction (Marathe et al., 1999). The length of *sn*-2 carbon chain dictates PAFR affinity. Species with an *sn*-2 acetyl moiety exhibit the highest affinity (Carolan and Casale, 1990; Hwang et al., 1989; Ishii et al., 1997a). Substituting phosphoethanolamine for phosphocholine substantively reduces binding (O'Flaherty et al., 1994). The few studies that have examined the role of the *sn*-1 fatty

Figure 4.1: 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). Non-enzymatic oxidation of membrane lipids (green) generates PAF-like family members with longer chain fatty acids (up to 8 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-alky-2-arachidonyl-glycerophosphocholine parental lipid is depicted in this schematic.

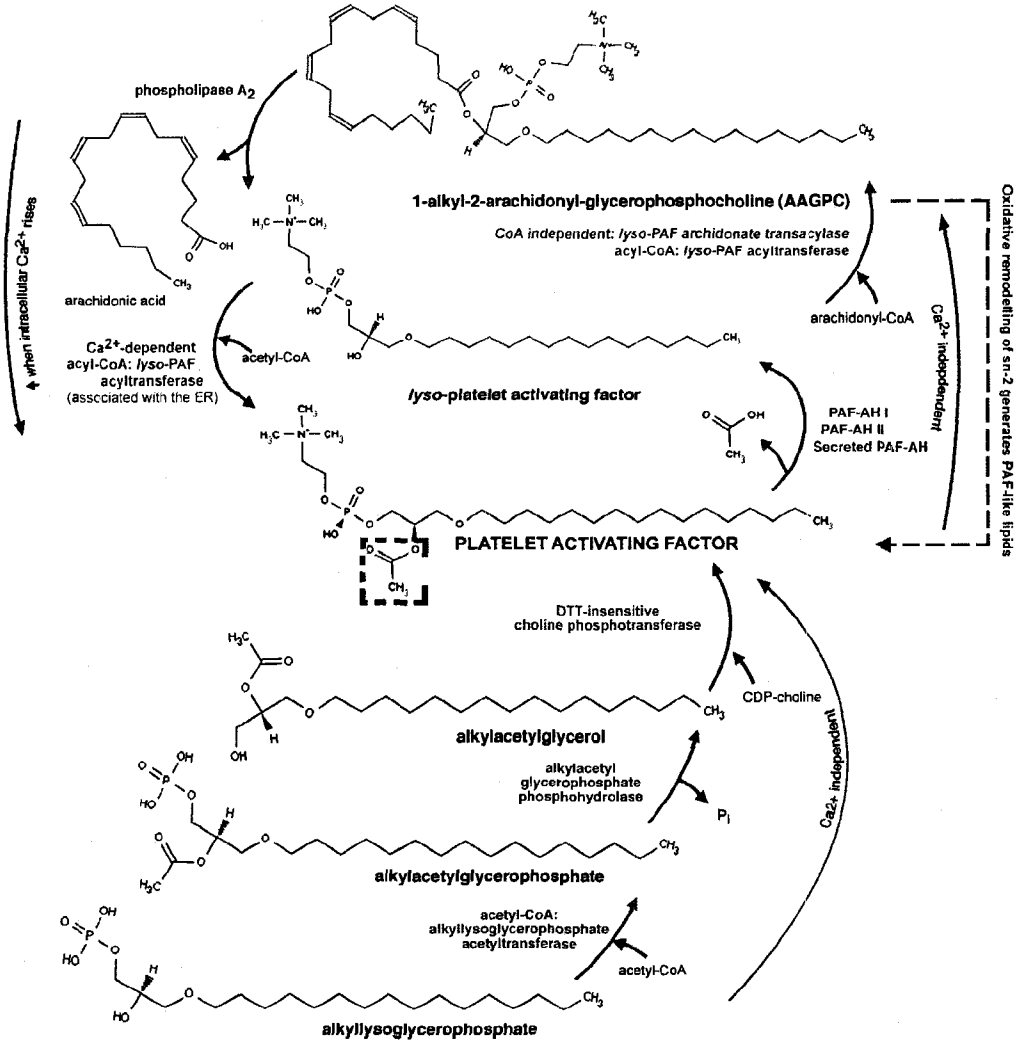
phospholipase A2
1-alkyl-2-arachidonyl-glycerophosphocholine (AAGPC)
CoA independent: lyso-PAF archidonate transacylase
acyl-CoA: lyso-PAF acyltransferase
arachidonic acid
arachidonyl-CoA
Ca2+-dependent
acyl-CoA: lyso-PAF
acyltransferase
(asscciated with the ER)
acetyl-CoA
lyso-platelet activating factor
PAF-AH I
PAF-AH II
Secreted PAF-AH
PLATELET ACTIVATING FACTOR
DTT-insensitive
choline phosphotransferase
CDP-choline
alkylacetylglycerol
alkylacetyl
glycerophosphate
phosphohydrolase
Pi
alkylacetylglycerophosphate
acetyl-CoA:
alkyllysoglycerophosphate
acetyltransferase
acetyl-CoA
alkyllysoglycerophosphate
when intracellular Ca2+ rises
Ca2+ independent
Ca2+ independent
Oxidative remodelling of sn-2 generates PAF-like lipids

Figure 4.1

acid in PAF signaling implicate carbon chain length and degree of unsaturation in PAFR signaling (Carolan and Casale, 1990; Erger and Casale, 1996; Handa et al., 1991; Snyder, 1987; Stoddart et al., 2001). 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) (Bonin et al., 2004b; Brewer et al., 2002; Li et al., 2003; Southall et al., 2001).

The PAF family is implicated in both synaptic plasticity and neuronal death. Under physiological conditions, PAF lipids act as retrograde neurotransmitters contributing to hippocampal CA1 long-term potentiation by enhancing excitatory synaptic transmission (Chen et al., 2001; Heusler and Boehmer, 2007). When concentrations remain elevated, PAF elicits caspase-dependent neurotoxicity (Bazan et al., 2002; Perry et al., 1998; Ryan et al., 2007). This latter role has been identified as a primary mediator of neuronal apoptosis in HIV-1-associated dementia, encephalitis, epileptic seizure, and ischemia (Bazan et al., 2002; Birkle et al., 1998; Perry et al., 1998). In progressive neurodegenerative disorders, PAF concentrations increase as a result of enhanced phospholipase A_2 activity (Farooqui and Horrocks, 2006; Kanfer et al., 1998; Klein, 2000; Sweet et al., 2002). Inhibition of the PAF remodelling pathway (Fig 4.1) through dietary consumption of the omega-3 polyunsaturated fatty acid docosahexaenoic acid (Watanabe et al., 2001) reduces dendritic pathology in transgenic models of Alzheimer Disease (Calon et al., 2004). These changes may also 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 (Igbavboa et al., 2002). In relapsing-remitting multiple sclerosis, however, increases in both 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 (Callea et al., 1999).

To address whether changes in the *sn*-1 PAF fatty acid alter neuronal viability, we compared bioactivity of C_{16}-PAF and C_{18}-PAF at sub-micellar concentrations in the presence or absence of PAFR. Here, we show that the carbon chain length at the *sn*-1 position determines whether neurons undergo caspase-dependent apoptosis, caspase-independent neurodegeneration, or are protected from PAF toxicity. In PAFR-deficient neurons, our data indicate that C_{16}-PAF triggers caspase-dependent apoptosis whereas C_{18}-PAF signals caspase-independent degeneration. We further show that these pathways are inhibited by PAFR activation with neuroprotective or neurotoxic effects dependent upon the identity of the *sn*-1 carbon chain. PAFR activation by C_{16}-PAF inhibits caspase-dependent apoptosis protecting cells from PAF challenge whereas PAFR activation by C_{18}-PAF inhibits caspase-independent neurodegeneration but promotes caspase-dependent apoptosis. 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.

4.6 Materials and Methods

4.6.1 Reagents: All cell culture reagents were obtained from Invitrogen (Burlington, ON) and all chemicals were purchased through Sigma-Aldrich (St Louis, USA) unless other-

wise stated. Cultures were treated with 1 μM 1-O-hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine (C_{16}-PAF) or 1-O-octadecyl-2-acetyl-*sn*-glycero-3-phosphocholine (C_{18}-PAF) or PAF vehicle (0.1% EtOH or treatment media). In some experiments, cultures were treated with the PAF antagonists BN 52021 (50 μM), CV 3988 (2 μM), or antagonist vehicle (0.1% DMSO). Concentrations employed were based on previous reports (Bonin et al., 2004b; Brewer et al., 2002; Ryan et al., 2007). PAF species and antagonists were obtained from Biomol Research Laboratories, Plymouth Meeting, USA. All treatments were performed in serum-free media to ensure that exogenous PAF was not hydrolyzed by serum-derived PAF acetylhydrolases. Serum-free media was supplemented with delipidated 0.025% BSA as a PAF carrier as previously described (Ammitt and O'Neill, 1997; Ryan et al., 2007).

4.6.2 PC12 cell culture and treatment: A clonal derivative of the PC12 pheochromocytoma cell line and PC12 cells stably expressing ectopic human V5-tagged PAFR were treated with PAF isoforms in the presence or absence of PAF antagonists. These lines have been previously characterized (Bonin et al., 2004b; Brewer et al., 2002). Cells were cultured in Roswell Park Memorial Institute medium (RPMI 1640) supplemented with 10% horse serum and 5% newborn calf serum and maintained at 37°C and 5% atmospheric CO_2. Cells were seeded in 35mm plates (5×10^4 cells/plate) for treatment. After plating, cultures were treated in RPMI 1640 supplemented with 0.025% BSA. Negative controls included cultures incubated in treatment media only. Percent viability was determined by hemocytometer counts of trypan blue excluding cells.

4.6.3 Primary murine cell culture and treatment: Breeding pairs of $PAFR^{-/-}$ mice generated in the laboratory of Dr. Takao Shimizu (University of Tokyo, Japan) (Ishii et al., 1998) were kindly provided by Dr Shimizu and Dr Nicolas Bazan (Louisiana State University, USA). Mice were backbred into a C57BL/6 background for 10 generations (N10) and cerebellar granule neurons (CGNs) extracted from congenic $PAFR^{-/-}$ and $PAFR^{+/+}$ mice as previously described (Ryan et al., 2007). Briefly, cerebella from postnatal day 7-10 pups were removed. The meninges were dissected and tissue 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, St Louis, USA) 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, Canada) 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_2/95% air 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 before being exposed to PAF in serum-free treatment media (EMEM, 25 mM glucose, 1% gentamycin, 2mM L-glutamine, 20 mM KCl, 0.025% BSA). 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 (Policy 31).

4.6.4 Cell survival assay: C_{16}-PAF and C_{18}-PAF (1 μM) were dissolved in treatment media. CGNs were treated for 24 h with 1 μM C_{16}-PAF, C_{18}-PAF, or vehicle (treatment media alone). All treatments were performed in serum-free media containing 0.025% BSA to ensure that cultures were not exposed to exogenous serum-derived PAF acetylhydrolases. Where treatment with PAF antagonists CV 3988, BN 52021, or antagonist vehicle (0.1% DMSO) are indicated, cultures were pre-treated for 15 min with the test compounds prior to addition of PAF. Test compounds were maintained in the media throughout the PAF treatment. CGN survival was assessed by Live/Dead viability/cytotoxicity assay (Invitrogen, Burlington Canada). 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 as a result of loss of membrane integrity. Cells were imaged using a DMIR epifluorescent inverted microscope (Leica, Richmond Hill, Canada) equipped with a QICAM digital camera (Quorum Technologies, Guelph, Canada) and captured using OpenLab software v5.05 (Improvision, Lexington, USA). Percent survival was calculated as (viable cell number$^{(calcein^+ - calcein^+/ET^+)}$/ mean number of viable cells in vehicle control$^{(calcein^+ - calcein^+/ET^+)}$ x 100.

4.6.5 Recombinant adenovirus infection: Recombinant adenovirus preparation, characterization, and infection were performed as previously described (Ryan et al., 2007). Briefly, recombinant adenovirus carrying enhanced green fluorescent protein (EGFP) and PAFR under separate cytomegalovirus promoters or EGFP alone were added to cell suspensions at the time of plating. All experiments were performed at a multiplicity of in-

fection 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 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 x 100.

4.6.6 Assessment of caspase activation: Executioner caspase activation in CGNs was determined using CaspaTag (caspase 3/7) assay (Chemicon, Temecula, USA) according to the manufacturer's protocol and as we have previously described (Ryan et al., 2007). 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 pre-treatment and exposure to PAF in the presence of the caspase 3/7 inhibitor Z-DEVD-FMK (50 μM). Caspase 3/7 activity was determined in PAF-treated PC12 cultures by Western analysis of poly(ADP-ribose) polymerase (PARP) cleavage. 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 phenylmethylsulfonyl fluoride). 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 monoclonal anti-PARP, (1:10000, Clontech, Mississauga Canada) detecting the p85 PARP caspase cleavage product and actin (1:1000, Sigma). Secondary antibodies were horseradish peroxidase- conjugated anti-mouse IgG (1:2000, Jackson ImmunoResearch, Burlington Canada). Immunoreactive bands were visualized

using SuperSignal West Pico (MJS BioLynx Inc.).

4.6.7 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 were considered highly significant (shown as **).

4.7 Results

4.7.1 PAFR expression protects PC12 cells and primary CGNs from the toxic effects of C_{16}-PAF but not C_{18}-PAF

To assess cytotoxicity, wild-type (WT) PC12 cells and PC12 transfectants stably expressing human PAFR (hPAFR) were treated with 1 μM C_{16}-PAF, C_{18}-PAF, or 0.1% vehicle alone for 24 h in serum-free media. WT PC12 cells do not express PAFR (Bonin et al., 2004b; Brewer et al., 2002). Both PAF species were toxic to PAFR-deficient WT cultures (Fig 4.2A). Ectopic expression of PAFR protected PC12 cells from C_{16}-PAF but not C_{18}-PAF (Fig 4.2B).

To determine if these isoform-specific effects were conserved in neurons, we compared primary neuronal cultures endogenously expressing PAFR with cultures derived from null-mutant mice. CGNs from $PAFR^{-/-}$ and $PAFR^{+/+}$ mice were treated with 1 μM C_{16}-PAF, C_{18}-PAF, or vehicle (treatment media) for 24 h in serum-free media. Cell survival was assessed by Live/Dead assay. C_{16}-PAF and C_{18}-PAF significantly reduced

Figure 4.2: Ectopic PAFR expression protects PC12-AC cells from C_{16}-PAF but not C_{18}-PAF challenge. (A) PC12 cells and (B) PC12 cells stably transfected with human PAFR were treated with either C_{16}-PAF (1 μM), C_{18}-PAF (1 μM), or vehicle (0.1% DMSO) for 24 h in serum-free treatment media. Viability was assessed by hemocytometer counts of trypan blue-excluding cells. Data are expressed as mean ± SEM. (A) Both C_{16}-PAF and C_{18}-PAF were toxic to PC12 cells. (B) Ectopic PAFR expression protected PC12 cells from C_{16}-PAF but not C_{18}-PAF-induced death (*$p<0.05$, ANOVA, *post hoc* Dunnett's *t*-test of PAF treatment vs. vehicle treatment, n=6 cultures conducted in duplicate experiments).

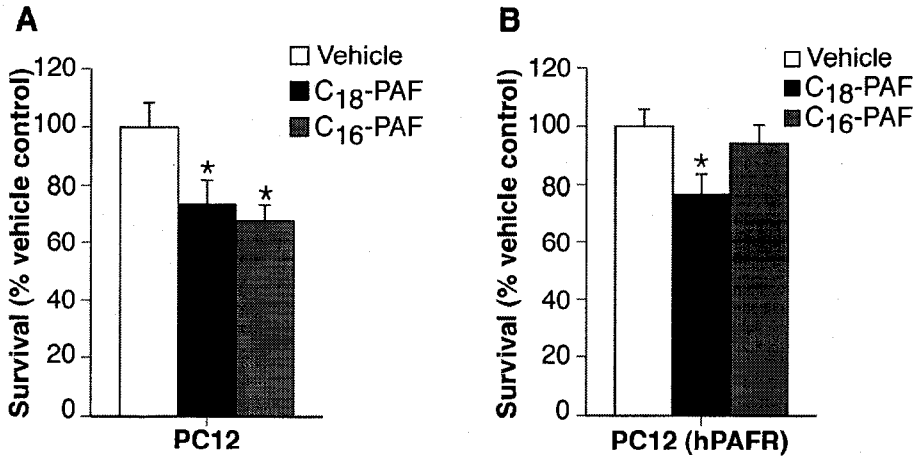
A
Vehicle
C_{18}-PAF
C_{16}-PAF
Survival (% vehicle control)
120
100
80
60
40
20
0
*
*
PC12
B
Vehicle
C_{18}-PAF
C_{16}-PAF
Survival (% vehicle control)
120
100
80
60
40
20
0
*
PC12 (hPAFR)

Figure 4.2

$PAFR^{-/-}$ CGN number (Fig 4.3A). In WT cultures, PAFR expression protected $PAFR^{+/+}$ neurons from C_{16}-PAF but not C_{18}-PAF (Fig 4.3B). In complementary gain of function experiments, $PAFR^{-/-}$ cultures were adenovirally infected with EGFP or EGFP and human PAFR. Both C_{16}-PAF and C_{18}-PAF triggered neuronal death in $PAFR^{-/-}$ cells infected with EGFP alone (Fig 4.3C). Adenoviral PAFR infection protected PAFR null-mutant neurons from C_{16}-PAF but not C_{18}-PAF (Fig 4.3D). Collectively these data show that C_{16}-PAF and C_{18}-PAF are both toxic to PC12 cells and primary neurons deficient in PAFR and that PAFR expression selectively protects primary neurons and PC12 cells from C_{16}-PAF but not C_{18}-PAF toxicity.

4.7.2 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 1μM C_{16}-PAF, C_{18}-PAF, or vehicle (treatment media). Executioner caspase 3/7 activity was determined by quantifying cleavage of FAM-DEVD-FMK. Despite comparable toxicity (Fig 4.3A,C), 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 4.4A). Consistent with the observed protection conferred by PAFR expression (Fig 4.4B,D), C_{16}-PAF did not activate caspase 3/7 in $PAFR^{+/+}$ CGNs (Fig 4.4B). The number of caspase-positive cells was comparable to vehicle-treated cultures (Fig 4.4B). Taken together, these data demonstrate that caspase-dependent apoptosis initiated by C_{16}-PAF in PAFR-deficient cells can be inhibited by PAFR expression. By contrast, a statistically significant

Figure 4.3: 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 (1 μM) and C_{18}-PAF (1 μM) were toxic to $PAFR^{-/-}$ CGNs. (B) WT $PAFR^{+/+}$ neurons were protected from C_{16}-PAF but not C_{18}-PAF. (C) Both C_{16}-PAF and C_{18}-PAF were toxic to $PAFR^{-/-}$ CGNs infected with pAdTrack-CMV adenoviral vector containing EGFP alone. Note that PAF-induced neuronal loss was greater in (C) compared to (A) as a result of adenoviral infection. (D) Ectopic expression of PAFR using pAdTrack-CMV adenoviral vector containing EGFP and hPAFR protected $PAFR^{-/-}$ CGNS from C_{16}-PAF but not C_{18}-PAF. (**$p<0.01$, ANOVA, *post hoc* Dunnett's *t*-test of PAF treatment vs. vehicle treatment, n=30-75 fields conducted in duplicate experiments).

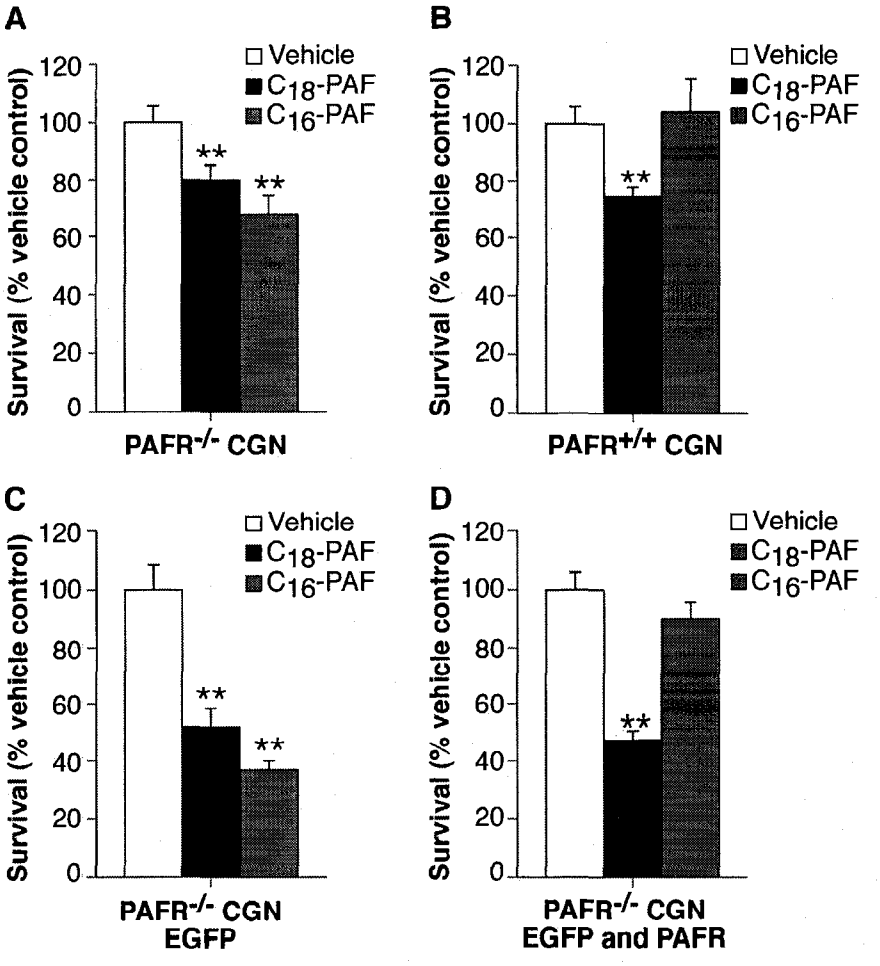
A
B
C
D
Vehicle
C18-PAF
C16-PAF
Survival (% vehicle control)
120
100
80
60
40
20
0
**
PAFR-/- CGN
PAFR+/+ CGN
PAFR-/- CGN EGFP
PAFR-/- CGN EGFP and PAFR

Figure 4.3

Figure 4.4: PAF isoforms differentially induce caspase-dependent death in the presence or absence of PAFR. Executioner caspase activation was determined using CaspaTag assay. The number of cells exhibiting caspase 3/7 activation 24 h after addition of PAF isoforms (1 μM) or vehicle (treatment media) was quantified and data expressed as the percentage of cells (mean ± SEM) exhibiting DEVD cleavage. (A) $PAFR^{-/-}$ CGNs exhibited a significant increase in caspase 3/7 activation following C_{16}-PAF but not C_{18}-PAF treatment. (B) C_{18}-PAF but not C_{16}-PAF elicited significant increase in caspase 3/7 activation in $PAFR^{+/+}$ CGNs. (C) Pre-treatment 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. (*$p<0.05$,**$p<0.01$, ANOVA, *post hoc* Dunnett's *t*-test of PAF treatment vs. vehicle treatment (A,B) or compound treatment vs. control (C,D), n=18-30 fields conducted in duplicate experiments). (E) To establish cell specificity, Western analysis for PARP cleavage was performed on PC12 cells treated with vehicle or PAF isoform (1 μM). C_{16}-PAF induced PARP cleavage in WT but not hPAFR transfectants. C_{18}-PAF primarily elicited caspase activation in hPAFR transfectants.

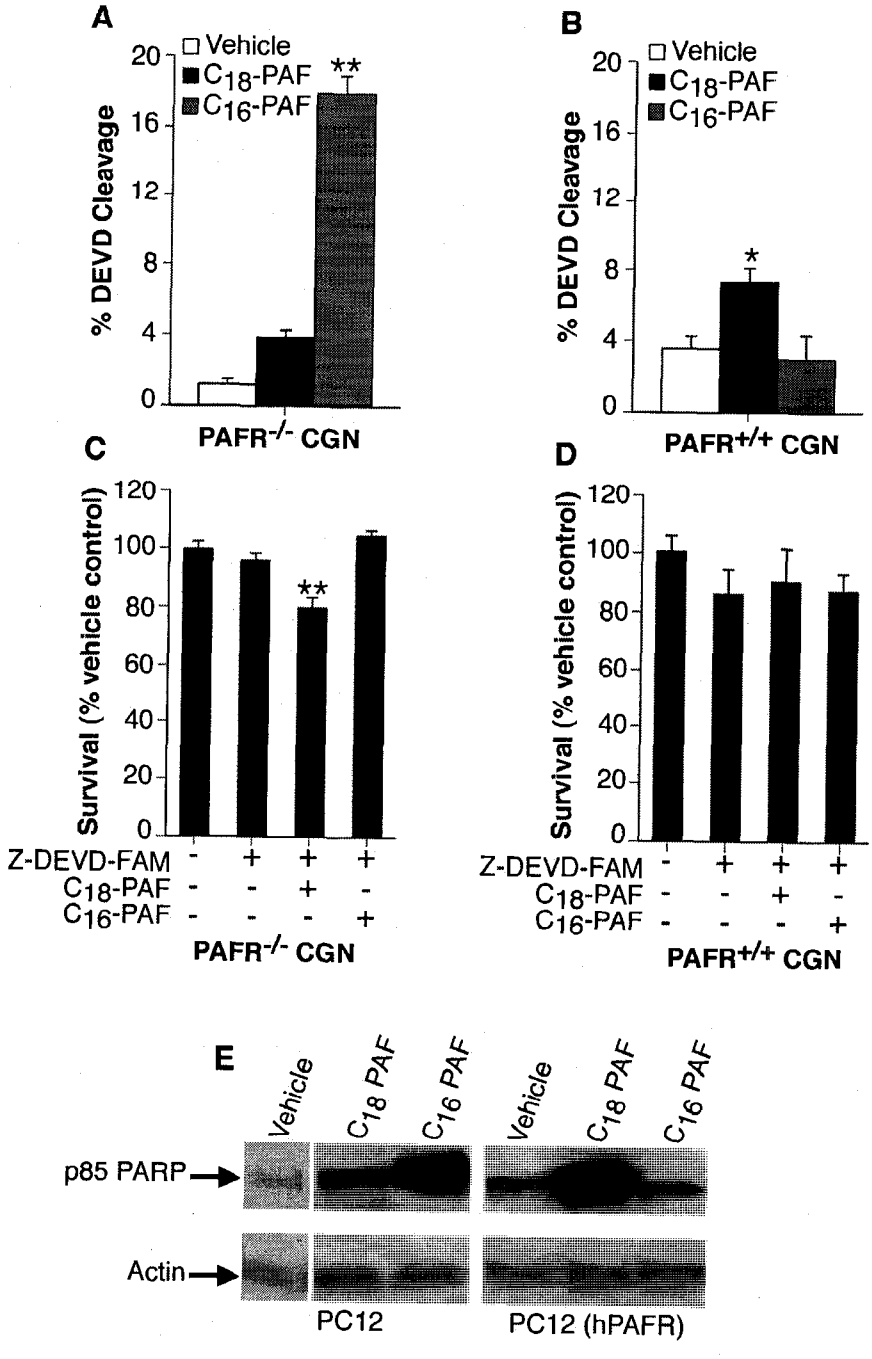
A
Vehicle
C18-PAF
C16-PAF
% DEVD Cleavage
PAFR-/- CGN
B
PAFR+/+ CGN
C
Survival (% vehicle control)
Z-DEVD-FAM
C18-PAF
C16-PAF
PAFR-/- CGN
D
PAFR+/+ CGN
E
Vehicle
C18 PAF
C16 PAF
p85 PARP
Actin
PC12
PC12 (hPAFR)

Figure 4.4

increase in caspase 3/7 activation was detected following C_{18}-PAF treatment of $PAFR^{+/+}$ CGNs (Fig 4.4B) indicating 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 then maintained in media for the duration of PAF challenge. Preventing caspase 3/7 activation protected $PAFR^{-/-}$ CGNs from C_{16}-PAF-induced apoptosis but not C_{18}-PAF caspase-independent death (Fig 4.4C). In $PAFR^{+/+}$ neurons, caspase inhibition effectively blocked the C_{18}-PAF-induced caspase-dependent apoptosis (Fig 4.4D) without impacting on neuroprotection initiated by C_{16}-PAF activation of PAFR (Fig 4.4D).

To establish cell specificity, we treated PC12 cells and PC12 cells transfected with human PAFR with C_{16}-PAF and C_{18}-PAF for 24 h and assessed cleavage of the caspase 3/7 substrate PARP (Fig 4.4E). In PAFR-deficient PC12 cells, caspase-dependent PARP cleavage to p85 PARP increased dramatically following C_{16}-PAF treatment and moderately following C_{18}-PAF exposure (Fig 4.4E, left panel). In PAFR transfectants, only C_{18}-PAF increased PARP cleavage compared to control cultures. Taken together, these data demonstrate that the ability of PAF isoforms to balance cell death and cell survival through modulation of caspase-dependent and caspase-independent pathways occurs in both primary neurons and non-neuronal cells.

4.7.3 PAFR-dependent and PAFR-independent death pathways initiated by discrete PAF isoforms can be targeted pharmacologically

To intervene in these cell death pathways, $PAFR^{-/-}$ and $PAFR^{+/+}$ CGNs were exposed to PAF isoforms (1 μM) in the presence of the non-competitive PAF inhibitor BN52021 or the competitive PAFR antagonist CV 3988 (Fig 4.5A). Control cultures were treated with antagonist vehicle (0.1% DMSO) and PAF vehicle (treatment media) (Fig 4.5A,B). Both C_{16}-PAF and C_{18}-PAF were toxic to $PAFR^{-/-}$ CGNs (Fig 4.5A, DMSO) but only C_{18}-PAF elicited neuronal loss in $PAFR^{+/+}$ CGNs (Fig 4.5B, DMSO). No significant neuronal toxicity was detected in the presence of BN 52021 regardless of PAF isoform or receptor status (Fig 4.5A,B, BN52021). CV 3988 only protected CGNs from C_{18}-PAF-induced PAFR-dependent apoptosis (Fig 4.5A, CV3988). These data show that PAF-mediated death pathways are distinct from one another and can be pharmacologically targeted.

4.8 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 PAF, C_{16}-PAF triggers caspase-dependent apoptosis whereas C_{18}-PAF-induces caspase-independent neurodegeneration. Neurotoxicity is further modulated by PAFR expression. Ectopic or endogenous PAFR expression protects neurons from C_{16}-PAF whereas PAFR expression shifts C_{18}-PAF-mediated neurotoxicity from a caspase-independent to a caspase-dependent signaling

Figure 4.5: Cell death pathways initiated by discrete PAF isoforms are inhibited by specific PAF antagonists. (A) $PAFR^{-/-}$ CGNs and (B) $PAFR^{+/+}$ CGNs 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), the non-competitive PAF antagonist BN 52021 (50 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). No significant toxic effects of C_{16}-PAF or C_{18}-PAF were detected in $PAFR^{-/-}$ neurons pre-treated with BN 52021 (A, BN 52021). BN 52021 also inhibited C_{18}-PAF-induced death in $PAFR^{+/+}$ cultures (B, BN 52021). 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 vs. PAF vehicle treated in the presence of DMSO (antagonist vehicle), n=25 fields conducted in duplicate experiments).

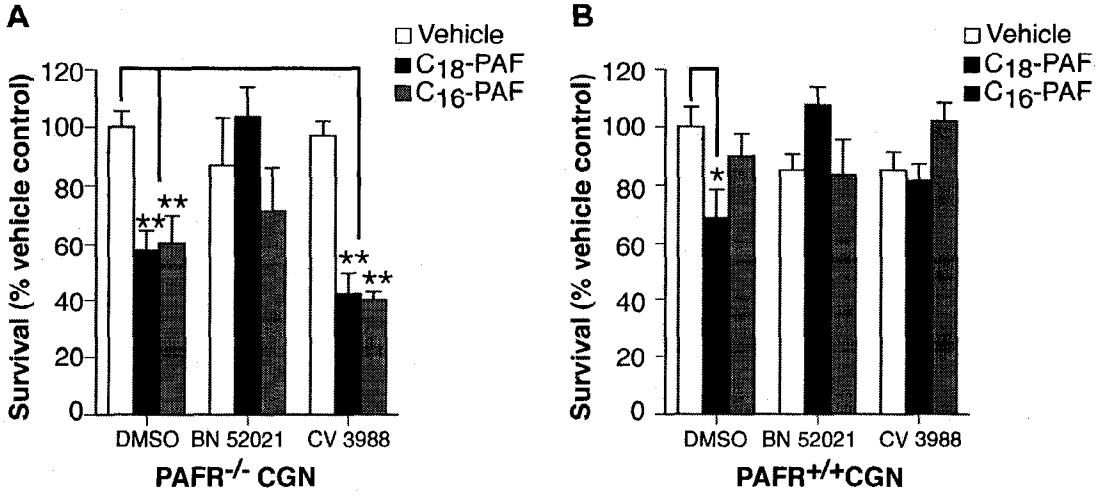
A
Vehicle
C18-PAF
C16-PAF
Survival (% vehicle control)
120
100
80
60
40
20
0
**
**
**
**
DMSO
BN 52021
CV 3988
PAFR-/- CGN
B
Vehicle
C18-PAF
C16-PAF
Survival (% vehicle control)
120
100
80
60
40
20
0
*
DMSO
BN 52021
CV 3988
PAFR+/+CGN

Figure 4.5

pathway. These data provide insight into the underlying cell death pathways initiated following PAF challenge that, in part, reconcile reports of both pro- and anti-apoptotic PAF signaling (Bellizzi et al., 2005; Brewer et al., 2002; Li et al., 2003; Ryan et al., 2007; Southall et al., 2001; Zhu et al., 2004).

Converging evidence suggests that regional variations in the synthesis of discrete PAF isoforms are associated with neurodegenerative disease (Callea et al., 1999; Calon et al., 2004; Farooqui and Horrocks, 2006; Igbavboa et al., 2002; Kanfer et al., 1998; Klein, 2000; Sweet et al., 2002; Watanabe et al., 2001). C_{16}-PAF and C_{18}-PAF are the predominant PAF species present in uninjured tissue (Okumura et al., 2006; Travers et al., 1997) but differentially accumulate over development or following injury. C_{16}-PAF concentrations increase during neuronal differentiation or following neuropathological challenge (Callea et al., 1999; Whitehead et al., 2007) whereas local C_{18}-PAF concentrations, to date, have only been shown to increase in injured CNS (Callea et al., 1999). The impact of these changes in the CNS has been attributed to activation of PAFR (Bazan et al., 2002; Birkle et al., 1998; Ishii and Shimizu, 2000; Perry et al., 1998). However, the widespread neuronal death observed after brain injury associated with PAF accumulation has been difficult to reconcile with the restricted pattern of neuronal PAFR expression (Bennett et al., 1998; Mori et al., 1996). Here, we show that PAF lipids do not require PAFR to signal neuronal loss. Both C_{16}-PAF and C_{18}-PAF trigger neuronal death in PAFR-deficient cells but through isoform-specific caspase-dependent or independent signaling pathways respectively. Surprisingly, C_{16}-PAF was not toxic when neurons express 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 disease likely impact on the efficacy of strategies to intervene in PAF synthesis in neurodegenerative disease (Watanabe et al., 2001).

In support of such a combinatorial approach, we find that existing PAF inhibitors differentially target isoform-specific signaling pathways. BN 52021 blocks both the caspase-independent and dependent pathways triggered by C_{16}-PAF and C_{18}-PAF in PAFR-deficient neurons and inhibits PAFR-dependent death triggered by C_{18}-PAF alone. BN 52021, also known as Ginkgolide B, displays non-competitive binding affinity for PAFR (Maclennan et al., 2002) and acts directly on the PAF acetylhydrolase 1b complex to enhance PAF hydrolysis (Bonin et al., 2004a). These dual actions likely work in concert to enhance PAF catabolism and inhibit PAFR-signal transduction in the face of PAF challenge. By contrast, CV 3988 is PAFR-specific competitive antagonist (Marcheselli et al., 1990). Here we show that CV 3988 is only able to protect neurons from PAFR-dependent neurotoxicity consistent with its PAFR specificity. These data may help explain the mixed success of single PAF antagonists in therapeutic intervention (Akisu et al., 2003; Brochet et al., 1995; DeKosky et al., 2006; Hirashima et al., 2001; Hirashima et al., 2002; Ponto and Schultz, 2003).

Our inhibitor studies also provide insight into the neuroprotection conferred by PAFR expression under C_{16}-PAF challenge. We found that non-competitive PAFR antagonists capable of accelerating cytosolic PAF degradation block PAFR-independent neuronal apoptosis. This mechanism may also underlie the PAFR-dependent neuroprotection. Previous studies have attributed PAF isoform-specific bioactivity in peripheral

tissue to differences in PAFR affinity (Carolan and Casale, 1990; Erger and Casale, 1996; Handa et al., 1991; Stoddart et al., 2001). C_{16}-PAF has a 10-fold higher affinity for PAFR than C_{18}-PAF (Carolan and Casale, 1990; Ishii et al., 1997b; Snyder, 1987). PAF stimulation of PAFR activation has been shown to elicit receptor/ligand internalization and rapid degradation of the internalized PAF to *lyso*-PAF (Chen et al., 2007; Ohshima et al., 2002; Svetlov et al., 1996). 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. The switch between caspase-independent to caspase-dependent neuronal loss elicited by C_{18}-PAF depending in the presence of PAFR in both primary neurons and PC12 cells is less clear. In non-neuronal 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 (Kravchenko et al., 1995; Li et al., 2003). It may be that the lower affinity of C_{18}-PAF for PAFR and thus potentially longer PAF half-life compared to 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 (Hughes et al., 2001; Okano et al., 2003). Clearly, validation of these mechanisms will require a direct comparison of the kinetics of C_{16}-PAF and C_{18}-PAF internalization and degradation in the presence and absence of PAFR.

In summary, these data illustrate that *sn*-1 identity of PAF isoforms dictates pro- or anti- apoptotic signaling dependent upon PAFR expression status. Although these pathways can be pharmacologically targeted, we show that PAFR-specific reagents are

not sufficient to inhibit all aspects of PAF neurotoxicity. 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 predominant PAF isoform present in degenerating tissue.

4.9 Acknowledgments

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Chapter 5: PAF initiates neuronal death through an ER-dependent stress mechanism[8]

5.1 Objective of this study

Experimental analysis to this point has established that the C_{16}-PAF isoform is the primary initiator of PAF-mediated apoptosis in neurons devoid of PAFR. The final objective of this thesis was to determine the mechanism of C_{16}-PAF-mediated apoptosis in human neurons.

5.2 Statement of author contributions

SDR and SALB conceived and designed the experiments and analyzed the data. SDR performed the majority of the experimental work and wrote the manuscript with SALB. TCM contributed data to Fig 5.2. JR and DQ performed the Cdk5 kinase assays with guidance in design and data analysis from DSP. PF contributed new reagents/analytical tools. SDR was ABs senior mentor who trained him in lipid extractions and supervised his lipidomic analysis. SDR, SNW, WH, LS, ME, AJGB performed and analyzed the LC-ESI-MS experiments with assistance from SALB and DF.

[8] A version of this chapter has been submitted to PLoS Biology

5.3 Aberrant lipid metabolism compromises neuronal viability in Alzheimer disease

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Running Head: Aberrant lipid metabolism signals neuronal death

5.4 Summary

Aberrant metabolism of choline-containing lipids in neural membranes is a defining characteristic of Alzheimer disease. Little is known about how downstream metabolites impact on neuronal function. A critical challenge lies in the identification of pathogenic lipid second messengers at the molecular level. To address this issue, we used nanoflow high-performance liquid chromatography electrospray ionization tandem mass spectrometry to quantify glycerophosphocholine metabolites in Alzheimer disease tissue. We find that 1-O-hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine (C_{16}-PAF) is elevated in the temporal cortex of Alzheimer patients. This accumulation is an early event in disease etiology as demonstrated using TgCRND8 transgenic mice overexpressing a double mutant form of the human amyloid precursor protein. We further show that intracellular concentrations of C_{16}-PAF increase in human hNT neurons exposed amyloid-$\beta_{1\text{-}42}$ oligomers. These rising concentrations activate endoplasmic reticulum stress responses initiating the unfolded protein response and successively activating m-calpain, cyclin-dependent kinase 5, and glycogen synthase kinase 3β. Kinase-mediated phosphorylation of microtubule-associated proteins, including tau and Nudel, facilitates the translocation of dynein-associated LIS1 to the α_2 subunit of the PAF-acetylhydrolase (PAF-AH) I complex. Translocation enhances PAF-AH I catalytic activity in human hNT neurons and accelerates the inactivation of C_{16}-PAF to C_{16}-*lyso*-PAF. However, when faced with sustained C_{16}-PAF challenge, sequestering of LIS1 from neuronal microtubules to the PAF-AH I complex ultimately contributes to the activation of caspases 2, 3, and 7 and signals neuronal apoptosis. Together, these data provide new mechanistic insight into how aberrant lipid metabolism compromises neuronal function in Alzheimer disease.

5.5 Introduction

Glycerophosphocholines are often overlooked as immediate response elements yet they exert fundamental control over cell viability. Aberrant metabolism of structural choline-containing membrane lipids in neurodegenerative disorders including Alzheimer disease produces key families of bioactive lipid second messengers including arachidonic acid, eicosanoids, and platelet activating factor (PAF) lipids (Farooqui and Horrocks, 2001; Klein, 2000; Sweet et al., 2002). Enhanced cytosolic phospholipase A_2 ($cPLA_2$) activity has been repeatedly linked to Alzheimer disease progression (Chen and Bazan, 2005; Farooqui and Horrocks, 2006; Kanfer et al., 1998) yet little is known about how early changes in cerebral glycerophosphocholine metabolism impact upon neuronal function. A major obstacle lies in the identification and quantification of pathogenic lipid second messengers within complex lipid extracts. To address this issue, we directed our attention to one glycerophosphocholine family repeatedly implicated in the etiology of neurodegenerative disease, the PAF family of lipid second messengers.

PAF lipids are members of the 1-alkyl,2-acylglycerophosphocholine subclass (GP0102) of glycerophosphocholines (GP01) (2008). Isoforms are 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. Non-enzymatic oxidation of structural glycerophosphocholines can also generate cytotoxic PAF-like lipids with up to nine carbons at the *sn*-2 position (Chen et al., 2007). Individual species vary in the length of their *sn*-1 carbon chain and degree of unsaturation (Tokumura et al., 1989). PAF family members mediate a wide variety of biological activities in brain, ranging from the enhancement of synaptic transmission to the induction of neuronal apoptosis (Chen and Bazan, 2005). PAF accumula-

tion in injured tissue is a primary mediator of neuronal death in ischemia, seizure, and AIDS dementia *in vivo* and has been implicated in amyloid-β toxicity *in vitro* (Bate et al., 2006; Bazan et al., 2002; Farooqui and Horrocks, 2006; McLarnon et al., 2005; Perry et al., 1998). Dietary consumption of the omega-3 polyunsaturated fatty acid docosahexaenoic acid suppresses PAF production and reduces dendritic pathology in transgenic mice expressing mutant forms of the human amyloid precursor protein (Calon et al., 2004; Watanabe et al., 2001). Overexpression or recombinant administration of PAF-acetylhydrolases (PAF-AHs) protects neurons from ischemic insult and inhibits neuronal damage associated with $cPLA_2$ activation (Kolko et al., 2003; Umemura et al., 2007). Two isoforms are endogenously expressed by neurons. Intracellular PAF-AH II is a single 40 kDa peptide expressed by some neurons and most glia. Cytosolic PAF-AH I is a neuron-specific complex composed of a 29 kDa α_1 and a 30 kDa α_2 catalytic subunit as well as two 45 kDa regulatory subunits made up of homodimers of the multifunctional LIS1 protein (Hattori et al., 1993; Prescott et al., 2000). PAF-AH I α_2 protein expression is induced following PAF challenge (Bonin et al., 2004). In cell-free systems, LIS1 association enhances the catalytic activity of PAF-AH I α_2 homodimers but not α_1/α_2 heterodimers or α_1 homodimers (Manya et al., 1999).

LIS1 is also a functional component of the microtubular dynein-associated motor complex. Through mutual association with Nudel, the dynein complex controls shuttling of endosomes and lysosomes from the endoplasmic reticulum (ER) to the Golgi (Harada et al., 1998; Hirokawa, 1998; Smith et al., 2000). Mutations in the LIS1 gene are the genetic determinant of Miller-Dieker lissencephaly, a cerebral developmental disorder characterized by defective neuronal migration resulting in a smooth cortical surface and

mental retardation (Hirotsune et al., 1998). Disease-associated mutations impair the microtubule-associated functions of LIS1 (Bai et al., 2008; Hirotsune et al., 1998). In mitotic cells, ectopic expression of cytosolic PAF-AH I α_2 competes for LIS1 at the microtubules eliciting their disorganization (Sapir et al., 1999; Sapir et al., 1997; Yamaguchi et al., 2007). *In vitro*, restoration of LIS1 reduces microtubule catastrophe events (Sapir et al., 1999; Sapir et al., 1997). While these gain of function studies conceptually link PAF signaling to compromised dynein function, it is not known whether the physiological induction of PAF-AH I α_2 expression following PAF challenge is sufficient to impact upon the subcellular localization of LIS1. To date, this link has only been demonstrated using superstoichiometric amounts of target proteins (Tarricone et al., 2004). Clearly, the potential for PAF-AH I activation to attenuate or transduce PAF neurotoxicity must be reconciled before PAF signaling can be targeted in neurodegenerative disease.

Here, we sought to provide mechanistic insight into how $cPLA_2$ glycerophosphocholine metabolites impair neuronal function in Alzheimer disease. Using nanoflow high-performance liquid chromatography electrospray ionization tandem mass spectrometry (LC-ESI-MS), we show that the 1-O-hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine PAF isoform (C_{16}-PAF) increases both *in vivo* in Alzheimer disease temporal cortex and *in vitro* in human neurons following exposure to soluble amyloid-β_{1-42} ($A\beta_{42}$) oligomers. This accumulation is an early event in Alzheimer disease pathogenesis demonstrated using TgCRND8 transgenic mice overexpressing a double mutant form of the human amyloid precursor protein. To provide mechanistic insight, we studied downstream signaling pathways by which C_{16}-PAF compromises neuronal function. We elucidate an ER-dependent signaling cascade leading to PAF-AH I activation, initial-

ly mobilized to protect human neurons from rising concentrations of C_{16}-PAF, but ultimately contributing to the impairment of ER and microtubule function leading to cell death under conditions of elevated lipid metabolism. These findings identify, for the first time, underlying molecular mechanisms by which aberrant glycerophosphocholine lipid second messengers in Alzheimer disease contributes to disease progression.

5.6 Results

5.6.1 C_{16}-PAF metabolism is altered in Alzheimer disease

Studies of glycerophospholipid changes in degenerating tissue have, for the most part, been restricted to "macro" analyses of subclasses (Bader Lange et al., 2007; Goodenowe et al., 2007; Sweet et al., 2002) defined by polar head groups (e.g., phosphocholine, phosphoethanolamine, phosphoserine) and linkage to the glycerol backbone (LipidMaps Consortium, 2008). While these studies provide strong evidence that choline-containing phospholipid metabolism is altered in Alzheimer disease brain (Chen and Bazan, 2005; Farooqui and Horrocks, 2001; Farooqui and Horrocks, 2006; Kanfer et al., 1998; Klein, 2000; Sweet et al., 2002), to our knowledge, no study has identified pathogenic isoforms at the molecular level. To address this question, we used a new nanoflow LC-ESI-MS protocol optimized for the identification of choline-containing phospholipids, specifically PAF lipid species (Whitehead et al., 2007). We focused our analysis on the most potent PAF isoform, 1-O-hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine (C_{16}-PAF) (Prescott et al., 2000).

Glycerophospholipids were extracted from the posterior temporal cortex of Alzheimer disease patients and age-matched controls (Fig 5.1A). Samples were spiked at the

Figure 5.1: C_{16}-PAF concentrations are elevated in the temporal cortex of Alzheimer disease patients. (A) Region of temporal lobe parahippocampal gyrus dissected for lipid extraction and ESI-LC-MS analysis. Posterior entorhinal cortex is indicated in blue with dissected regions delineated by the rectangular box. (B) Representative extracted ion chromatographs of species at m/z 524 (upper panel) or 528 (lower panel) with a phosphocholine product ion at m/z 184.0 detected by precursor ion scan in positive ion mode are presented. Peak retention times were determined using Analyst 1.4.1 with a $\pm$ 0.2 min shift in elution window between MS runs. The species eluting at 20.4 min is C_{18}-LPC. The species eluting at 19.1 min is C_{16}-PAF (upper panel) as confirmed by coelution with d_4-C_{16}-PAF (lower panel). (C) A significant increase in C_{16}-PAF concentrations was detected in Alzheimer disease tissue (*$p<0.05$, Student's *t* test, n=4 individuals/condition). Endogenous C_{16}-PAF concentrations were estimated in comparison to the peak intensity of 1.25 ng d_4-C_{16}-PAF. Data are expressed as pg/mg of tissue (wet weight). Each square represents an individual patient. Blue and red squares indicate the patients analyzed in (B). Mean and standard error of measurement (SEM) are presented in Table 1. Details are as described in Materials and Methods

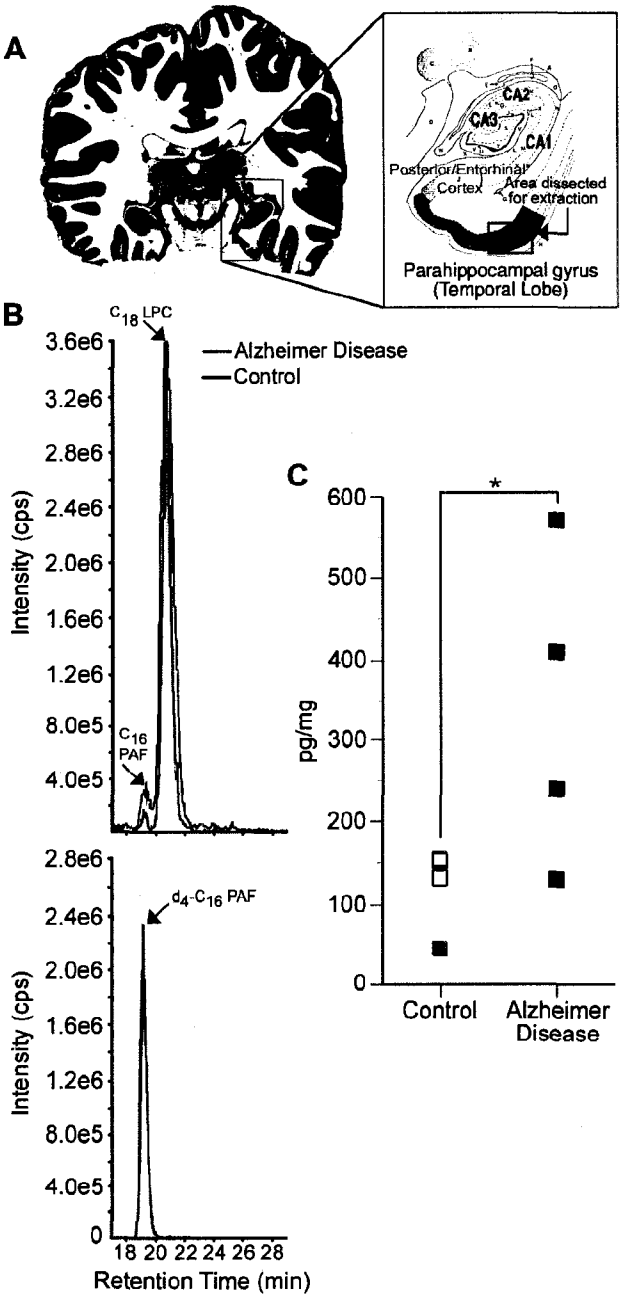

A
CA2
CA3
CA1
Posterior Entorhinal Cortex
Area dissected for extraction
Parahippocampal gyrus (Temporal Lobe)
B
C18 LPC
Alzheimer Disease
Control
C16 PAF
d4-C16 PAF
Intensity (cps)
3.6e6
3.2e6
2.8e6
2.4e6
2.0e6
1.6e6
1.2e6
8.0e5
4.0e5
0
18 20 22 24 26 28
Retention Time (min)
C
pg/mg
600
500
400
300
200
100
0
*
Control
Alzheimer Disease

Figure 5.1

time of extraction with C_{13}-lysophosphatidylcholine (LPC), a lipid not endogenously present in biological tissue, as an internal standard. Deuterated C_{16}-PAF (d_4-C_{16}-PAF) was added at the time of MS analysis to identify endogenous C_{16}-PAF and control for variations in peak intensities over MS runs. Glycerophosphocholines were characterized by MS scan for a protonated molecule at expected m/z followed by precursor ion scan in positive ion mode for a phosphocholine product ion at m/z 184 (Whitehead et al., 2007). Two isobaric species C_{16}-PAF, and C_{18}-LPC (Owen et al., 2005; Whitehead et al., 2007) within the expected m/z range for C_{16}-PAF were present in the extracted ion chromatographs (Fig 5.1B). The identity of endogenous C_{16}-PAF (m/z 524.7) was verified based on coelution with d_4-C_{16}-PAF (m/z 528.7) (Fig 5.1B). For quantitation, C_{16}-PAF peak intensities were standardized against that of C_{13}-LPC to control for extraction efficiency and concentrations estimated in comparison to known amounts of d_4-C_{16}-PAF. A significant 3-fold increase in C_{16}-PAF concentrations was detected in the posterior temporal cortex of Alzheimer disease patients (Fig 5.1C). Data are expressed as pg/mg (Fig 5.1C) with molar equivalents provided in Table 5.1.

While these data demonstrate that C_{16}-PAF concentrations are elevated at time of death, glycerophosphocholine species that accumulate early in Alzheimer disease etiology are predicted to have greater impact on neuronal function over the course of disease progression. To determine whether aberrant C_{16}-PAF metabolism is an early neurodegenerative event, lipids in the posterior temporal cortex were analyzed in four month old TgCRND8 mice and non-transgenic (NonTg) littermates. TgCRND8 mice express a double mutant form of the human amyloid precursor protein 695 (KM670/671NL+V717F) under the control of the prion protein promoter. Accelerated

Table 5. 1: C16-PAF concentrations in vivo and in vitro under control and pathological conditions

Sample and Condition		Tissue or intracellular neuronal concentration	
		pg/mg or pg/10^6 cells	pM*
Human cortex	Control	122±25 pg/mg	245 ± 51
	Alzheimer	338±97 pg/mg	678 ± 194
Mouse cortex	NonTg	52±12 pg/mg	105 ± 25
	TgCRND8	203±53 pg/mg	410 ± 105
Human neurons	Untreated	152 ± 96 pg/10^6 cells	130 ± 30
	Aβ (25 μM) 24 h	755 ± 410 pg/10^6 cells	620 ± 110
	PAF (1 μM) 5 min	1825 ±33 pg/10^6 cells	1520 ± 3
	PAF (1 μM) 45 min	710 ±93 pg/10^6 cells	616 ± 30

* Molar equivalents were calculated as described by Nelson et al.[47]. Tissue wet weight in grams was divided by the specific gravity of brain tissue (1.050) to obtain the volume in ml for molar calculations. For cultured neurons, pellets containing known cell number were weighed to obtain cell weight in grams. Data are expressed as mean ± SEM from a minimum of four human samples/condition, three murine samples/condition, or replicate neuronal cultures.

$A\beta_{42}$ biogenesis is first observed between three and four months of age correlating with detectable behavioral impairment in learning and memory (Chishti et al., 2001). As tissue availability was not limiting factor, we established absolute C_{16}-PAF concentrations by standard addition method (Whitehead et al., 2007). Analytical samples were spiked with 200, 400, 600, 800, and 1000 pg of synthetic C_{16}-PAF prior to MS analysis. Integrated peak areas were normalized to that of endogenous C_{16}-LPC (Whitehead et al., 2007). C_{16}-PAF was clearly identified from isobaric C_{18}-LPC in spiked samples (Fig 5.2A). Native PAF concentrations were determined from the x-intercept by linear regression analysis following n=15 MS analyses of triplicate samples (Fig 5.2B). A significant four-fold increase in C_{16}-PAF concentrations was detected in the cortex of TgCRND8 mice (Fig 5.2C). Data are expressed as pg/mg (Fig 5.2C) converted to molar equivalents in Table 5.1.

5.6.2 $A\beta_{42}$ elicits increases in intracellular C_{16}-PAF concentrations in human hNT neurons

Lipids extracted from brain tissue represent the sum total of species synthesized by multiple cell types or present in cerebral circulation at time of analysis. To provide direct evidence that neuronal C_{16}-PAF metabolism is affected in Alzheimer disease, we used the NT2/D1 teratocarcinoma cell line to generate post-mitotic human neurons (hNTs) (Pleasure et al., 1992). Following a 6 week differentiation protocol, NT2/D1 cells terminally differentiate into cholinergic, GABAergic, and dopaminergic neuronal subtypes referred to as hNT neurons sensitive to $A\beta_{42}$ (Guillemain et al., 2000; Novitskaya et al., 2007). Here, hNT neurons were treated for 24 h with vehicle (media) or $A\beta_{42}$ (25 μM) prepared

Figure 5.2: Elevated C_{16}-PAF concentrations are observed early in disease etiology in TgCRND8 mice. (A) Glycerophospholipids were extracted from posterior temporal cortex from 4 month old TgCRND8 and n=3 NonTg littermates (n=3 per group). Representative extracted ion chromatographs of m/z 524 from lipids extracted from a TgCRND8 (red) and NonTg (black) mouse spiked with 200 pg of exogenous C_{16}-PAF are presented. Peak retention times were determined using Analyst 1.4.1 with a $\pm$ 0.4 min shift in elution window over n=15 MS runs. Insets depict the same NonTg sample spiked with 200 pg (black) or 1000 pg (blue) distinguishing between C_{16}-PAF eluting at 12.8 min and isobaric C_{18}-LPC eluting at 13.2 min. Note that the addition of excess synthetic C_{16}-PAF to the lipid matrix did not alter C_{18}-LPC peak intensity indicating thus increases in one isobaric species will not significantly impact upon detection of the other (A, inset). These controls demonstrate that both C_{16}-PAF and C_{18}-LPC are elevated in 4 month old TgCRND8 compared to NonTg mice. (B) Because tissues availability was not a limiting factor, absolute quantitation of C_{16}-PAF species was performed by standard addition method as described in Materials and Methods. Extracts were spiked with 200, 400, 600, 800, or 1000 ng of synthetic C_{16}-PAF. Data are represented as mean area of peak intensity $\pm$ standard deviation (SD) following standardization as described in Materials and Methods. Each standard addition was repeated in triplicate (n=15 measurements/sample). Native C_{16}-PAF concentrations were calculated from the x-intercept using linear regression. (D) A significant increase in PAF was detected in TgCRND8 cortex (*$p<0.05$, Student's *t* test, n=3 mice/condition). Blue and red squares indicate the mice analyzed in (A,B).

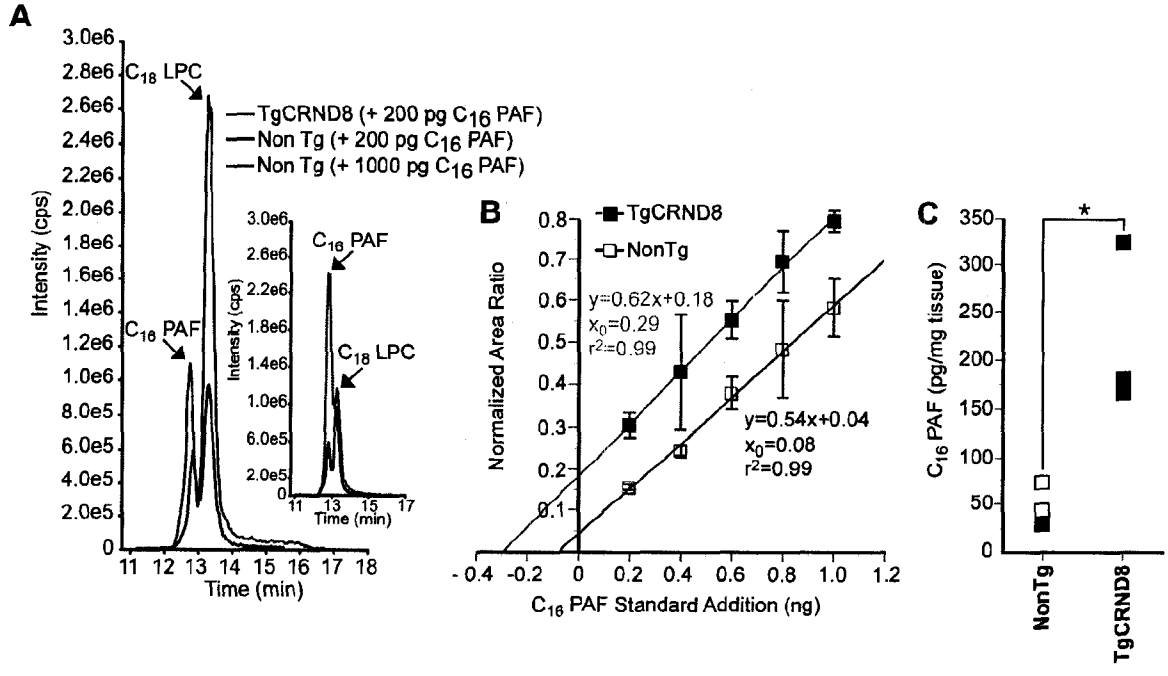

A
B
C
Intensity (cps)
Time (min)
C18 LPC
C16 PAF
TgCRND8 (+ 200 pg C16 PAF)
Non Tg (+ 200 pg C16 PAF)
Non Tg (+ 1000 pg C16 PAF)
TgCRND8
NonTg
y=0.62x+0.18
x0=0.29
r2=0.99
y=0.54x+0.04
x0=0.08
r2=0.99
Normalized Area Ratio
C16 PAF Standard Addition (ng)
C16 PAF (pg/mg tissue)
*
NonTg
TgCRND8

Figure 5.2

as toxic oligomers (Klein, 2002). All treatments were performed in serum-free media to ensure that PAF species would not be hydrolyzed by the secreted PAF-AH isoform present in serum. Neuronal viability was assessed by TUNEL. C_{16}-PAF concentrations were determined by ESI-LC-MS.

$A\beta_{42}$ exposure resulted in significant neuronal death (Table 5.2) coupled with a 6-fold increase in intracellular C_{16}-PAF concentrations (Table 5.1). These concentrations are comparable to those observed in post-mortem Alzheimer disease tissue (Table 5.1). Together, these data provide converging evidence that C_{16}-PAF accumulates in human neurons early in Alzheimer disease etiology as a result of $A\beta_{42}$ biogenesis. Based on these results, we focused on elucidating downstream signaling events elicited by rising intracellular C_{16}-PAF.

5.6.4 hNT neurons but not NT2/D1 precursors undergo PAFR-independent death following PAF challenge.

To establish whether C_{16}-PAF compromises neuronal viability, we compared survival of NT2/D1 (precursor cells) and hNT (neurons) cultures exposed to 0.001-1 μM C_{16}-PAF in the absence of any other toxic stimuli. Cultures were treated with exogenous C_{16}-PAF in serum-free media supplemented with 0.025% BSA as a lipid carrier. Viability of NT2/D1 precursor cells was not affected by C_{16}-PAF challenge (Fig 5.3A). hNT neurons were sensitive to C_{16}-PAF at low nM to low μM concentrations (Fig 5.3B). C_{16}-*lyso*-PAF, the immediate precursor and metabolite of C_{16}-PAF, had no effect on neuronal survival (Fig 5.3A,B).

PAF lipids signal through a G-protein coupled receptor (PAFR) as well as through

Table 5. 2: Aβ42 is toxic to hNT human neurons

Untreated	% Survival following treatment with 25 μM $A\beta_{42}$*	
	24 h (Vehicle)	24 h ($A\beta_{42}$)
100%±14	97%±4	43±2**

*Neuronal viability was determined using the TUNEL assay. Data represent the mean number of TUNEL-negative cells/field standardized to viable cell number in untreated cultures ± SEM. A total of 120 fields representing triplicate experiments/cultures were analyzed. (**$p<0.01$, ANOVA, *post-hoc* Dunnett's *t*-test).

Figure 5.3: C_{16}-PAF elicits death of hNT neurons but not NT2/D1 neural precursor cells. (A) NT2/D1 cell viability is not affected by C_{16}-PAF or its immediate metabolite C_{16}-*lyso*-PAF as measured by LIVE/DEAD cytotoxicity assay 24 h after treatment. (B) Survival of hNT neurons was compromised in concentration-dependent manner by C_{16}-PAF but not C_{16}-*lyso*-PAF (*p <0.05, * p<0.01, ANOVA, *post-hoc* Dunnett's *t*-test, n=8-11 cultures). Data represent mean ± SEM. (C) NT2/D1 and hNT cultures do not express PAFR. Random-primed cDNA from PC12 transfectants stably expressing human PAFR was used as the positive control (+). cDNA template integrity was confirmed by analysis of GAPDH as an internal control.

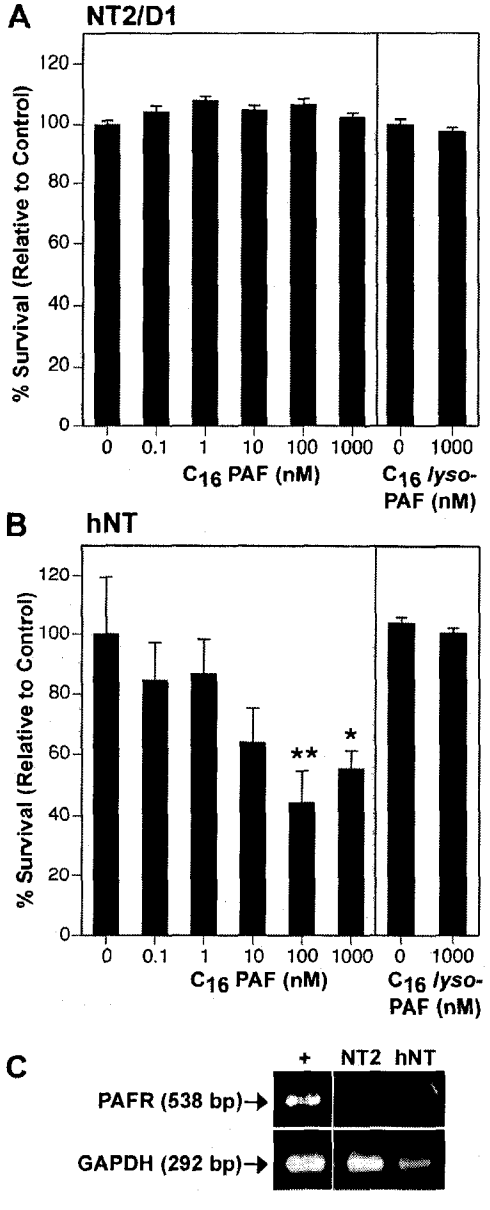

A
NT2/D1
% Survival (Relative to Control)
120
100
80
60
40
20
0
0 0.1 1 10 100 1000 0 1000
C16 PAF (nM)
C16 lyso-PAF (nM)
B
hNT
% Survival (Relative to Control)
120
100
80
60
40
20
0
**
*
0 0.1 1 10 100 1000 0 1000
C16 PAF (nM)
C16 lyso-PAF (nM)
C
+ NT2 hNT
PAFR (538 bp)
GAPDH (292 bp)

Figure 5.3

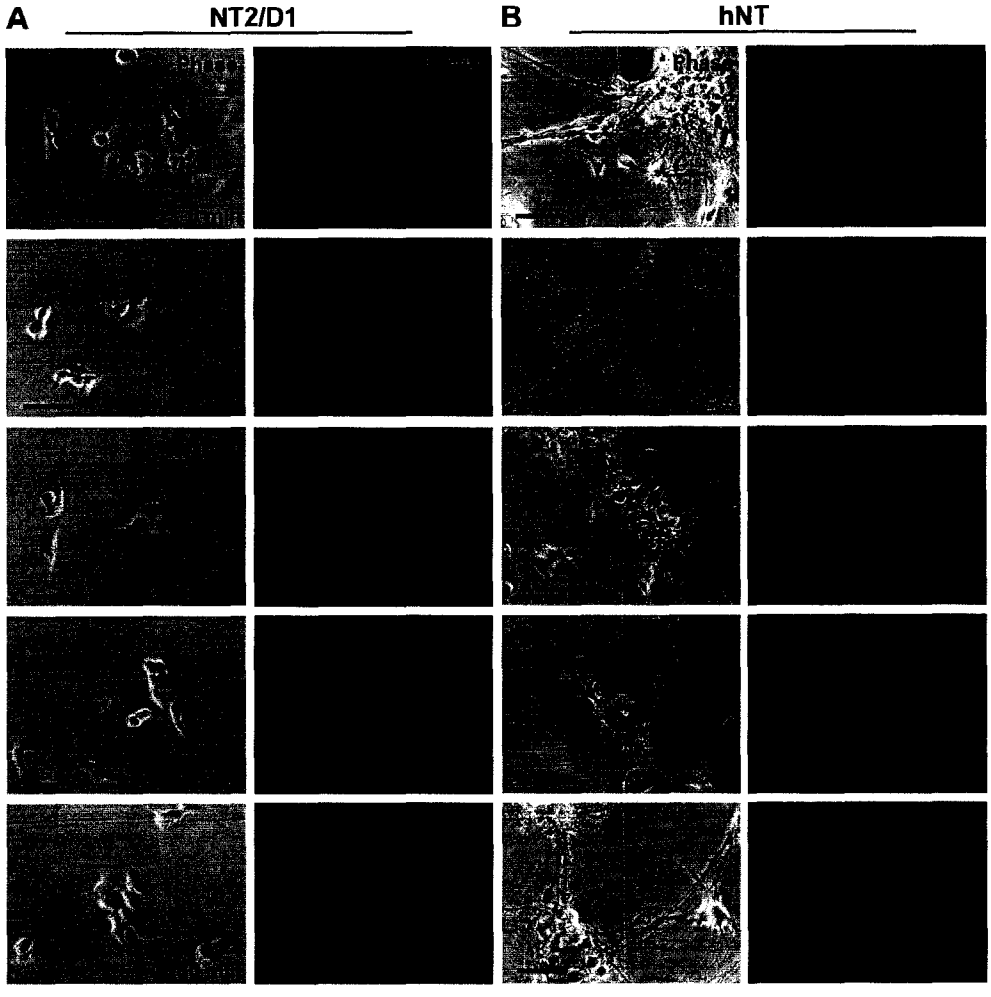
PAFR-independent pathways (Ishii and Shimizu, 2000). Receptor expression is down-regulated in injured brain tissue (Zhang et al., 2007). Moreover, PAFR activation has been shown to protect primary murine neurons from C_{16}-PAF challenge (Ryan et al., 2007). RT-PCR was performed to determine whether differences in NT2/D1 and hNT susceptibility to C_{16}-PAF are the result of differential expression of PAFR. Transcript was not detected in NT2/D1 or hNT cells (Fig 5.3C). These findings characterize a model system well-suited to the study of PAFR-independent signaling pathways that render genetically identical human cells susceptible or resistant to rising concentrations of C_{16}-PAF.

5.6.5 NT2/D1 cells and hNT neurons can be "loaded" with PAF isoforms to elicit pathological increases intracellular lipid second messengers in the absence of any other toxic stimuli

To determine whether differences in the rate of PAF internalization underlie neuronal susceptibility to ligand, cultures were treated with 1 μM of a BODIPY fluorophore-labeled PAF isoform (C_{11}-B-PAF: 1-(*O*-(11-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-*s*-indacene-3-proprionyl)amino)undecyl)-2-acetyl-*sn*-glycero-3-phosphocholine). Loosely bound lipid was removed from the extracellular leaflet of the plasma membrane and C_{11}-B-PAF internalization was visualized by live-cell imaging at 0, 5, 30, 45, and 60 minutes after PAF exposure (Bonin et al., 2004). C_{11}-B-PAF was internalized to the same extent and with the same kinetics in NT2/D1 cells as hNT neurons (Fig 5.4A-C).

To establish whether internalization of exogenous C_{16}-PAF elicits physiologically relevant increases in intracellular lipid concentrations, hNT cultures were exposed to 1

Figure 5.4: PAF is internalized by NT2/D1 cells and hNT neurons. (A) Live cell imaging of C_{11}-B-PAF internalization in NT2/D1 progenitors and (B) hNT neurons. Scale bar, 50 μm. Cultures were treated for 60 min with C_{11}-B-PAF (1 μM). The C_{11}-B-PAF-containing media was set aside at each time point and cells were washed with an acidified buffer containing 1% BSA to remove lipid loosely associated with the extracellular leaflet of the plasma membrane before imaging. (C) Quantification of cell-associated C_{11}-B-PAF 60 minutes post treatment. Values are expressed as mean fluorescence per cell and represent quantification of 25 randomly chosen cells in 5 different microscopic fields conducted in replicate experiments (n=50) ± SEM. Fold increase over background autofluorescence (0 min) is shown.


A
NT2/D1
B
hNT

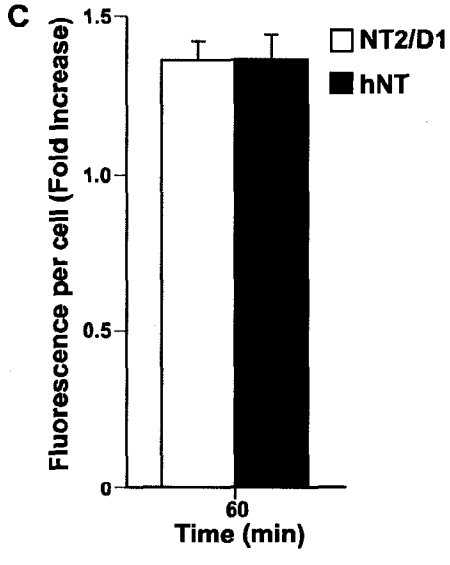

C
1.5
1.0
0.5
0
Fluorescence per cell (Fold Increase)
NT2/D1
hNT
60
Time (min)

Figure 5.4

μM C_{16}-PAF. After 0, 5 and 45 minutes of treatment, lipids loosely bound to the plasma membrane were removed. Cellular lipids were extracted and C_{16}-PAF quantified by LC-ESI-MS. Intracellular C_{16}-PAF concentrations increased 10-fold above baseline reaching 1520 pM within 5 minutes dropping to 616 pM after 45 minutes of continued treatment with exogenous C_{16}-PAF (Table 5.1). These concentrations were comparable to those elicited by $A\beta_{42}$ treatment of hNT neurons as well as to the regional concentrations detected in diseased human cortex (Table 5.1).

5.6.6 PAF hydrolysis differs between NT2/D1 cells and hNT neurons

To track the fate of internalized PAF, hydrolysis of C_{11}-B-PAF to C_{11}-B-*lyso*-PAF was assessed 0, 1, 5, 15, 30, and 60 minutes after treatment. Lipids were extracted from cells, separated by thin layer chromatography (TLC), and quantified in comparison to a standard curve of Bodipy-labeled synthetic standards. We found that the metabolism of C_{11}-B-PAF to C_{11}-B-*lyso*-PAF was, initially, faster in NT2/D1 precursors (Fig 5.5A,B, 1 min, 5 min, $p<0.05$). A significant acceleration of C_{11}-B-PAF hydrolysis was subsequently observed between 5 and 15 minutes in hNT neurons (Fig 5.5C, $p<0.05$).

5.6.7 PAF-AH I subunits are differentially expressed by NT2/D1 and hNT cells with the α_2 subunit induced in both cell systems following C_{16}-PAF challenge

These observations suggested that PAF-AH activity, elicited in response to rising intracellular concentrations of PAF, is differentially regulated in NT2/D1 precursor cells and hNT neurons. RT-PCR and Western analyses were performed to establish whether the different hydrolytic kinetics could be attributed to activity of different PAF-AH en-

Figure 5.5: The kinetics of PAF hydrolysis differs between NT2/D1 cells and hNT neurons. (A) Bligh and Dyer lipid extraction followed by TLC analysis of NT2/D1s and hNT samples treated with C_{11}-B-PAF (1 μM) detected significant differences in the rate of C_{11}-B-PAF hydrolysis to C_{11}-B-*lyso*-PAF. PAF hydrolysis was more rapid in NT2/D1 cultures at 1 and 5 minutes (*$p<0.05$, ANOVA, *post hoc* Tukey test). Acceleration in C_{11}-B-PAF hydrolysis to C_{11}-B-*lyso*-PAF was observed in hNT cultures between 5 and 15 minutes. Data represent the mean ± SEM of duplicate samples conducted over 3 independent experiments (n=6). These differences were confirmed by analysis of the mean slope of hydrolysis. (B) C_{11}-B-PAF hydrolysis was initially faster in NT2/D1 cells (*$p<0.05$, Student's *t*-test). (C) A significant acceleration was confirmed within 15 min of C_{11}-B-PAF treatment in hNT neurons (*$p<0.05$, Student's *t*-test). In (B,C), each TLC extract is depicted (white circles, NT2/D1 cells, white squares, hNT neurons). Mean ± SEM are represented by black circles (NT2/D1 cells) and black squares (hNT neurons).

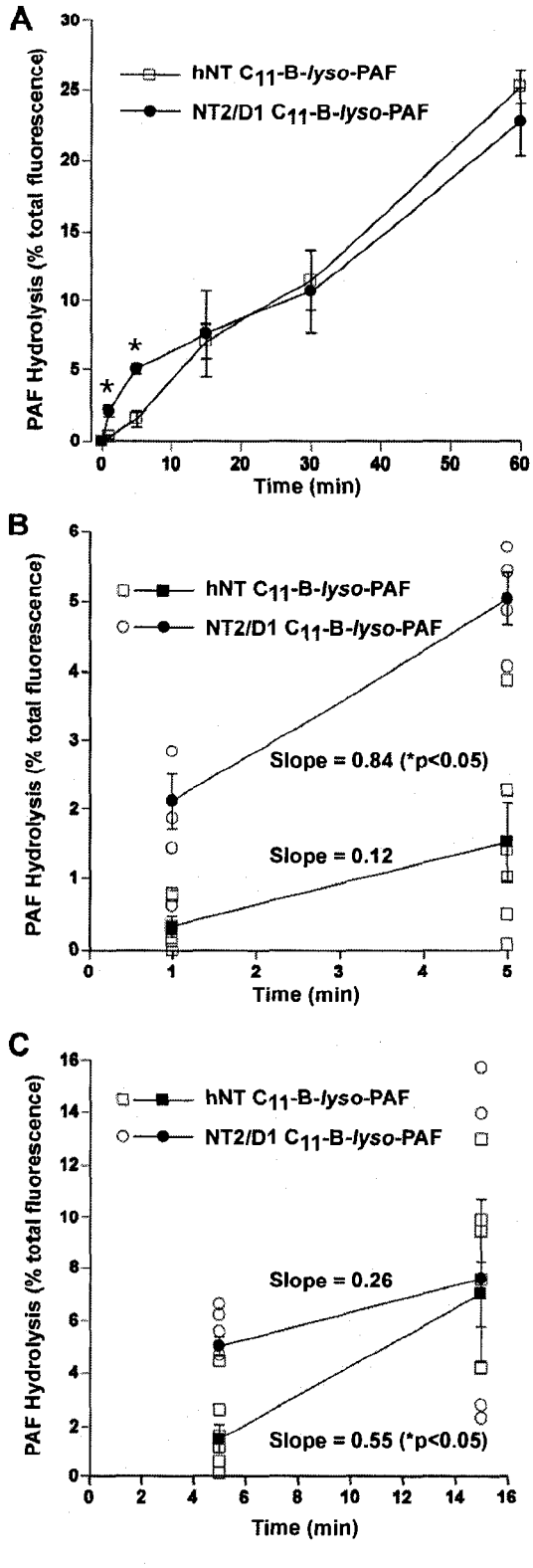

A
PAF Hydrolysis (% total fluorescence)
hNT C11-B-lyso-PAF
NT2/D1 C11-B-lyso-PAF
Time (min)
B
PAF Hydrolysis (% total fluorescence)
hNT C11-B-lyso-PAF
NT2/D1 C11-B-lyso-PAF
Slope = 0.84 (*p<0.05)
Slope = 0.12
Time (min)
C
PAF Hydrolysis (% total fluorescence)
hNT C11-B-lyso-PAF
NT2/D1 C11-B-lyso-PAF
Slope = 0.26
Slope = 0.55 (*p<0.05)
Time (min)

Figure 5.5

zymes. Transcript for the secreted PAF-AH isoform was not detected in NT2/D1 and hNT cultures (data not shown). PAF-AH II was also not observed in either cell system as determined by Western analysis and was not induced following C_{16}-PAF challenge (Fig 5.6B). All three PAF-AH I subunits were expressed by NT2/D1 cells and hNT neurons (Fig 5.6A). Individual subunits exhibited different expression patterns (Fig 5.6A). The α_1 catalytic subunit was expressed at higher levels in NT2/D1 cells (Fig 5.6A). The α_2 catalytic and LIS1 regulatory subunits were expressed at higher levels in hNT neurons (Fig 5.6A). This profile is consistent with the developmental shift in PAF-AH I catalytic subunit expression observed over the course of cerebral development (Manya et al., 1998).

To assess impact of C_{16}-PAF challenge on PAF-AH I subunit expression, Western blot analysis was performed 0, 5, 30, 45, and 60 minutes after C_{16}-PAF treatment. PAF-AH I α_2 protein levels increased 10-fold in NT2/D1 (Fig 5.7A,C) and 14-fold in hNT neurons (Fig 5.7B,D) relative to unstimulated cells. In both cell systems, PAF-AH I α_2 induction was detected within 5 minutes and peaked 45 minutes after C_{16}-PAF administration after analysis of triplicate immunoblots (Fig 5.7C,D). This increase was confirmed by immunofluorescence and immunoprecipitation (Fig 5.8A,C). A moderate decrease in PAF-AH I α_1 and no change in LIS1 expression levels was observed (Fig 5.7C,D). Note, that throughout these experiments, the exposure times for PAF-AH I subunits were optimized to provide comparable analytical signal in NT2/D1 and hNT cells given the differences in expression in unstimulated cells (Fig 5.6A). Data are expressed as fold-change in comparison to basal expression (Fig 5.7C,D).

Figure 5.6: PAF-AH I subunits are differentially expressed by NT2/D1 cells and hNT neurons. Western analysis of 30 μg of protein was performed to identify cytosolic PAF-AH isoforms expressed by NT2/D1 precursor cells and hNT neurons. (A) PAF-AH I α_2 and LIS1 expression is higher in hNT neurons. NT2/D1 cells primarily express the α_1 subunit. GAPDH was used as a loading control. (B) Equal amounts of protein (30 μg) from untreated cells (0 min) or cells treated with 1 μM PAF for 5, 30, 45, or 60 min were immunoblotted for PAF-AH II protein. NT2/D1 precursor cells and hNT neurons do not express PAF-AH II protein. PC12 cells were used as a positive control. Representative immunoblots of replicate experiments are depicted.

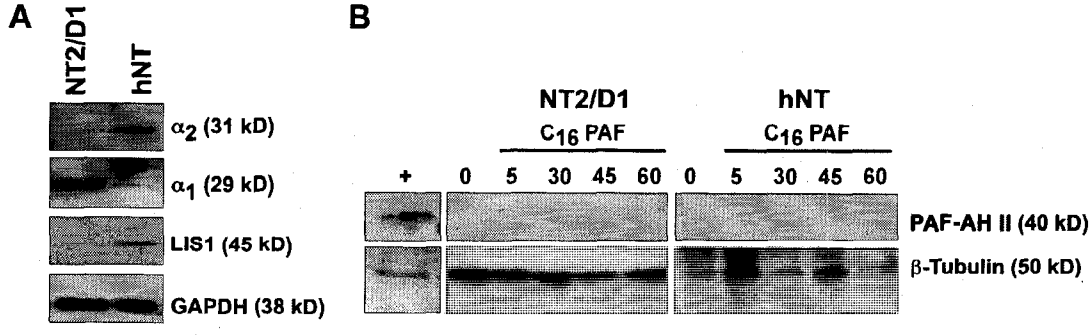

A
NT2/D1
hNT
α2 (31 kD)
α1 (29 kD)
LIS1 (45 kD)
GAPDH (38 kD)
B
NT2/D1
C16 PAF
hNT
C16 PAF
+
0
5
30
45
60
0
5
30
45
60
PAF-AH II (40 kD)
β-Tubulin (50 kD)

Figure 5.6

5.6.8 LIS1 translocates to the PAF-AH I complex in hNT neurons but not NT2/D1 precursor cells

Previous studies indicate that stress-induced expression of PAF-AH I α_2 is sufficient to enhance lipid metabolism by promoting PAF-AH I α_2/α_2 homodimer formation when cells rely primarily on the α_1 subunit for basal catalysis (Bonin et al., 2004). PAF-AH I α_2/α_2 homodimers exhibit maximal affinity for the C_{16}-PAF isoform (Manya et al., 1999). Thus, α_2/α_2 homodimer formation likely underlies the rapid rate of PAF catalysis observed in NT2/D1 cells within 5 minutes of PAF challenge as previously demonstrated in other cell systems(Bonin et al., 2004). In hNT neurons, however, the PAF-AH I expression profile already favors PAF-AH I α_2/α_2 homodimer formation. The increase in α_2 protein levels likely does not significantly impact upon homodimer formation and thus does not enhance the basal rate of catalysis.

LIS1 increases PAF-AH I α_2/α_2 catalytic activity in cell-free systems (Manya et al., 1999). However, we did not detect an increase in LIS1 expression in either NT2/D1 cells or hNT neurons in response to rising intracellular C_{16}-PAF concentrations. Moreover, LIS1 is already expressed at higher levels in hNT neurons compared to NT2/D1 precursor cells (Fig 5.6A). Given the ability of the PAF-AH I α_2/α_2 homodimer to competitively bind microtubular LIS1 (Tarricone et al., 2004; Yamaguchi et al., 2007), we next examined whether rising intracellular PAF concentration were sufficient to induce the translocation of LIS1 from microtubules to the PAF-AH I complex. hNTs were treated with C_{16}-PAF (1 μM) for 0, 15, 45 and 60 minutes. Co-localization of LIS1 with PAF-AH I α_2 was assessed by confocal microscopy (Fig 5.8A,B). An increase α_2 immunofluorescence was detected (Fig 5.8A) consistent with Western analysis (Fig 5.7B,D)

Figure 5.7: PAF-AH I α_2 expression increases in both NT2/D1s and hNTs following C_{16}-PAF challenge. (A) NT2/D1 or (B) hNT protein (30 μg) from untreated cells (0 minutes) or cells treated with 1 μM C_{16}-PAF for 5, 30, 45, or 60 minutes treatment were immunoblotted for α_1, α_2, or LIS1. β-tubulin was used as a loading control. Exposure times were optimized to provide comparable analytical signal in NT2/D1 and hNT cells given differences in basal expression in unstimulated cells (Fig 5.6A). (C, D) Densitometry was performed on n=3 immunoblots. Data are expressed fold-change compared to basal expression in each cell line.

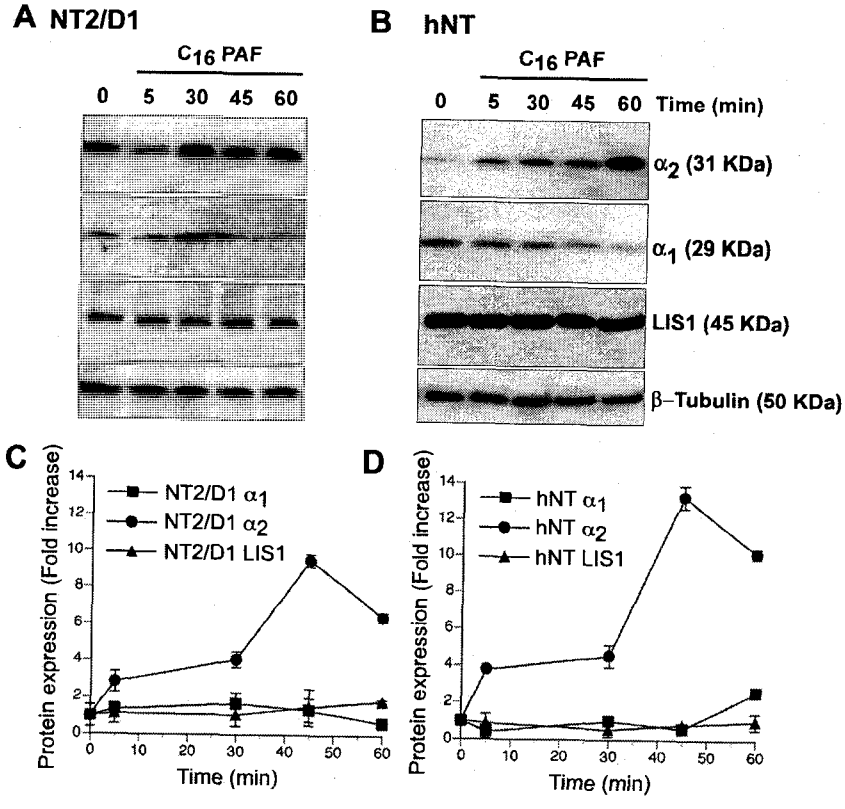

A NT2/D1
C16 PAF
0 5 30 45 60
B hNT
C16 PAF
0 5 30 45 60 Time (min)
α2 (31 KDa)
α1 (29 KDa)
LIS1 (45 KDa)
β–Tubulin (50 KDa)
C
Protein expression (Fold increase)
NT2/D1 α1
NT2/D1 α2
NT2/D1 LIS1
Time (min)
D
Protein expression (Fold increase)
hNT α1
hNT α2
hNT LIS1
Time (min)

Figure 5.7

as was a time-dependent increase in LIS1/α_2 co-localization in hNT neurons (Fig 5.8A,B). To confirm this association, we performed reciprocal immunoprecipitations and immunoblots for α_2 and LIS1 from NT2/D1 cells and hNT neurons 45 minutes after C_{16}-PAF administration. No change in LIS1/α_2 association was observed in C_{16}-PAF-resistant NT2/D1 precursor cells whereas an increase in LIS1/α_2 association was observed in C_{16}-PAF-sensitive hNT neurons (Fig 5.8C). These data suggest that the increase in co-localization observed *in situ* in hNT neurons is not simply due to the increase in α_2 expression but represents a *bona fide* interaction. To further confirm these data, we examined subcellular localization of LIS1. In addition to being part of the PAF-AH I complex, LIS1 also interacts with microtubular dynein (Smith et al., 2000). To determine whether the increase in LIS1/α_2 association resulted from C_{16}-PAF-induced recruitment of LIS1 from the microtubules, we fractionated insoluble cytoskeletal and soluble cytosolic protein (Joshi and Cleveland, 1989; Yamaguchi et al., 2007). A shift in LIS1 subcellular localization from the insoluble cytoskeletal fraction to the soluble cytosolic fraction was observed following C_{16}-PAF treatment (Fig 5.8D) coincident with cytosolic PAF-AH I α_2 induction (Fig 5.8D).

5.6.9 Preventing LIS1 translocation to the PAF-AH I complex protects neurons from C_{16}-PAF

While translocation of LIS1 to the PAF-AH I complex is likely initially elicited to enhance PAF hydrolysis, sustained sequestering of LIS1 from the microtubules in the face of sustained C_{16}-PAF challenge may also play a role in compromising neuronal integrity by destabilizing neuronal microtubules (Sapir et al., 1999; Sapir et al., 1997).

Figure 5.8: LIS1 translocates to the PAF-AH I complex in hNT neurons but not NT2/D1 precursor cells (A) Confocal microscopy of hNTs treated for 0, 15, 45, and 60 min with 1 μM C_{16}-PAF. LIS1 is not co-localized with α_2 in control cultures (merge). An increase in α_2 expression coinciding with co-localization with LIS1 is detected within 5 minutes (merge). Scale bar, 50 μm. (B) Co-localization was quantified using the Advanced Measurements Co-localization Module of OpenLab v4.5. A statistically significant time-dependent increase in co-localization was detected. (*$p<0.05$, **$p<0.01$, ANOVA, *post-hoc* Dunnett's *t*-test, n=5 fields/time point). (C) Reciprocal immunoprecipitation and immunoblotting for α_2 and LIS1 in the presence and absence of C_{16}-PAF were performed 45 min after treatment. C_{16}-PAF promotes LIS1 association with α_2 in hNT neurons not NT2/D1 cells. (D) Microtubules were fractionated based on solubility from C_{16}-PAF-treated hNT neurons 45 min after treatment. Movement of LIS1 from insoluble cytoskeletal/microtubule fraction to the soluble cytosolic fraction was observed following C_{16}-PAF treatment coinciding with the increase in cytosolic PAF-AH a_2 expression. (E) hNT neurons were transiently transfected with pcDNA3.1 containing GFP as well as empty vector (GFP) or pcDNA3.1 containing GFP and pcDNA3.1 containing PAF-AH I α_2 (PAF-AH α_2) and challenged with 1 μM C_{16}-PAF or vehicle (media control). The total number of GFP-expressing cells in each treatment condition was quantitated. Percent survival was standardized to vehicle-treated GFP-transfectants. α_2 overexpression protected hNTs from C_{16}-PAF toxicity (**$p<0.01$, ANOVA, *post hoc* Dunnett's *t*-test, n=6 fields conducted over replicate cultures). (F) hNT neurons were transiently transfected with pcDNA3.1 containing GFP and empty vector (EV) or pcDNA3.1 containing GFP and pcDNA3.1 containing PAF-AH I α_2 (α_2). Cells were challenged with C_{16}-PAF (1 μM), mc-PAF (1 μM), or vehicle (media control). PAF-AH I α_2 overexpression inhibited PAF-mediated translocation of LIS1 from the insoluble microtubule fraction to the soluble cytosolic fraction. mc-PAF does not induce LIS1 translocation. Representative immunoblots of replicate experiments are presented. Abbreviations are C, control; PAF, C_{16}-PAF-treated cultures; I, insoluble cytoskeletal/microtubule fraction; S, soluble cytosolic fraction.

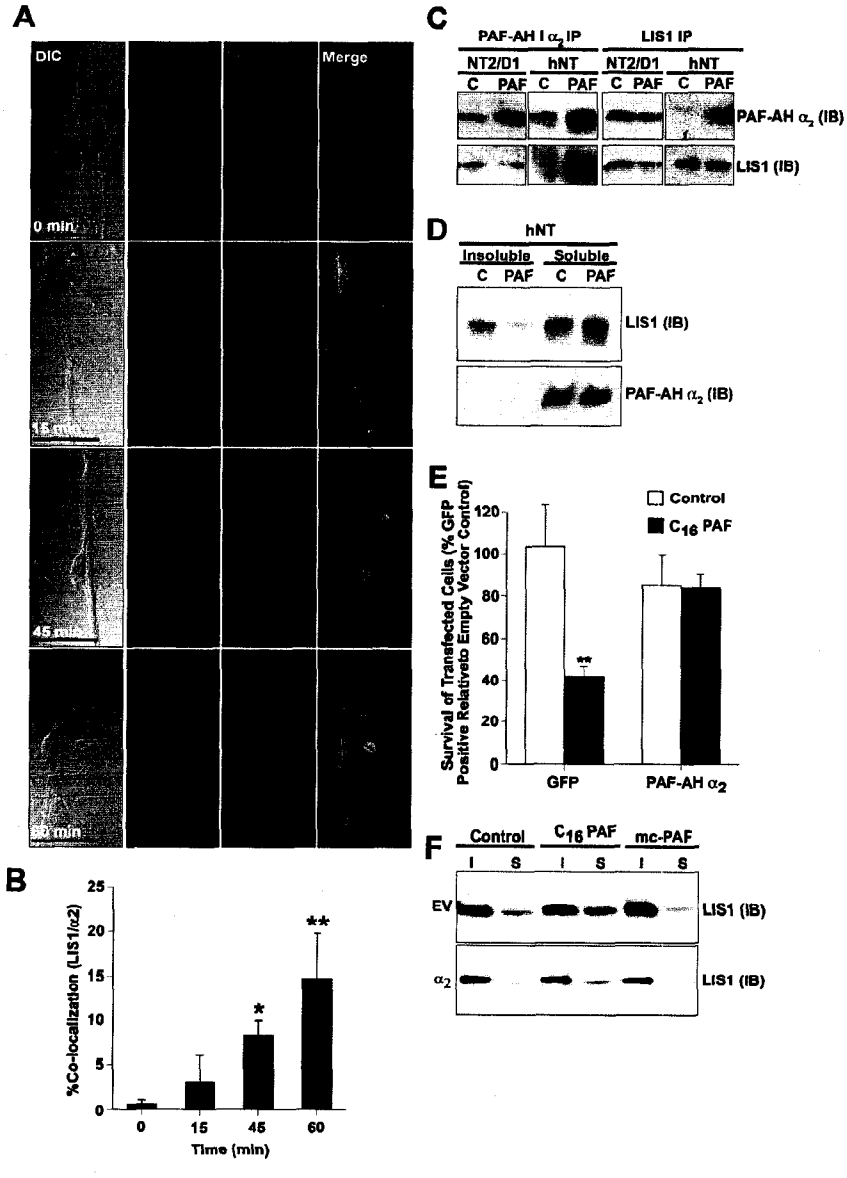

A
DIC
Merge
0 min
15 min
45 min
60 min
B
%Co-localization (LIS1/α2)
Time (min)
0
15
45
60
C
PAF-AH I α2 IP
LIS1 IP
NT2/D1
hNT
C PAF C PAF C PAF C PAF
PAF-AH α2 (IB)
LIS1 (IB)
D
hNT
Insoluble
Soluble
C PAF C PAF
LIS1 (IB)
PAF-AH α2 (IB)
E
Control
C16 PAF
Survival of Transfected Cells (% GFP Positive Relative to Empty Vector Control)
GFP
PAF-AH α2
F
Control
C16 PAF
mc-PAF
I S I S I S
EV
α2
LIS1 (IB)
LIS1 (IB)

Figure 5.8

This hypothesis predicts that promoting alternative mechanisms of enhancing PAF-AH I α_2 catalytic activity should prevent LIS1 translocation and render hNT neurons resistant to PAF-induced toxicity. Alternatively, it may be that aberrant LIS1 translocation is elicited when PAF-AH I α_2 protein levels exceed a threshold level of expression. Given the difference in basal expression between NT2/D1 cells and hNT neurons, it is possible this threshold is surpassed by the C_{16}-PAF induction of α_2 expression in neurons but not precursor cells. This latter hypothesis predicts that ectopic expression of the α_2 subunit in hNT neurons would be, in and of itself, sufficient to induce LIS1 translocation in the absence of PAF and trigger neuronal death (Yamaguchi et al., 2007).

To test both hypotheses, we transfected hNT neurons with PAF-AH I α_2. Overexpression of PAF-AH I α_2 did not induce LIS1 translocation from the microtubules (Fig 5.8F). Moreover, ectopic expression of PAF-AH I α_2 protected hNTs from C_{16}-PAF (Fig 5.8E) and prevented C_{16}-PAF-induced LIS1 translocation (Fig 5.8F). To demonstrate that the translocation of LIS1 with α_2 requires the interaction of PAF isoforms with the PAF-AH I complex, we treated hNT neurons with mc-PAF. PAF-AH I hydrolyzes the *sn*-2 acetyl group of PAF isoforms with particular affinity for the C_{16}-PAF isoform (Prescott et al., 2000). The methyl carbamyl group at the *sn*-2 position of the synthetic mc-PAF analog renders it resistant to PAF-AH I (O'Flaherty et al., 1987). mc-PAF was unable to induce LIS1 translocation in either empty vector or PAF-AH I α_2 overexpressing neurons (Fig 5.8F). Moreover, mc-PAF did not induce significant neuronal death when cultures were treated with up to 1 μM of lipid (data not shown). Collectively these data support the conclusion that LIS1 translocates for the purpose of enhancing C_{16}-PAF catabolism, that C_{16}-PAF activation of the PAF-AH I complex is required to induce this

translocation, and that rapidly removing the active glycerophosphocholine stressor through ectopic overexpression of PAF-AH I α_2 prevents LIS1 translocation and protects neurons from pathological C_{16}-PAF challenge.

5.6.10 LIS1 translocation is signaled by Cdk5 activation

These data implicate sustained LIS1 translocation in C_{16}-PAF-mediated neuronal loss when rising intracellular PAF concentrations cannot be alleviated by PAF-AH I hydrolysis. To test this hypothesis, we focused on elucidating how C_{16}-PAF signals the release of LIS1 from the microtubules and whether blocking this pathway would prevent neurotoxic signaling. Since LIS1 plays a role in regulating ER to Golgi transport, we first tested whether C_{16}-PAF alters ER function. BIP (GRP78) induction is an early marker of ER stress (Gulow et al., 2002). Expression increases following cessation of translation induced by UPR (Gulow et al., 2002; Munro and Pelham, 1986). Here, we found that BIP induction preceded LIS1 translocation to the PAF-AH I complex in hNT neurons (Fig 5.9A). BIP was not induced in NT2/D1 cells (Fig 5.9A).

Next, we assessed UPR-induced calpain activation. Spectrin is a substrate for both calpains and caspases and is cleaved and activated during apoptosis (Brown et al., 1999; Martin et al., 1995; Rotter et al., 2004; Williams et al., 2003). Proteolytic processing by calpains (m and μ) yields 149 and 136 kDa fragments and whereas caspase 2, 3, and 7-cleavage yields 148 and 137 kDa fragments (Meary et al., 2007; Rotter et al., 2004). The 148 kDa fragment can be further processed to 114 kDa and 34 kDa fragments by caspases 3 and 7 but not caspase 2 (Meary et al., 2007; Rotter et al., 2004; Williams et al., 2003). Thus, the pattern of spectrin cleavage can be used to predict apoptotic enzyme

Figure 5.9: C_{16}-PAF elicits ER stress, initiates the unfolded protein response, and activates m-calpain, and caspases 2, 3/7. (A) hNT and NT2/D1 protein (30 μg) from untreated cells (0 min) or cells treated with 1 μM C_{16}-PAF for 5, 30, 45, or 60 min treatment were immunoblotted for BIP (Grp78) and β-tubulin. Representative immunoblots show induction of BIP within 5 min of treatment in hNT but not NT2/D1 cells. (B) The same hNT protein lysates were immunoblotted for αII-spectrin and m-calpain with β-tubulin as a loading control. Cleavage of spectrin to a ~150 kDa and a ~140 kDa fragment within 15 min of C_{16}-PAF treatment and a 114 kDa fragment 60 min after C_{16}-PAF treatment was observed (upper panel). Cleavage fragments generated by calpains (149, 136 kDa) and caspases (148, 137, 114 kDa) are indicated. To distinguish between calpain and caspase cleavage of spectrin, immunoblots were performed using an antibody that detects the inactive m-calpain pro-protein but not cleaved, activated calpain. A decrease in pro-protein levels consistent with activation by cleavage was detected within 5 min of C_{16}-PAF exposure (middle panel). (C) To assess caspase activation, hNT neurons were treated with PAF for 0, 30 and 60 min. Caspase 2 activation was determined using the FLICA assay detecting cleavage of FAM-VDVAD-FMK. Caspase 3/7 activation was determined using the Caspatag assay detecting cleavage of FAM-DEVD-FMK. Data are expressed as the percentage of cells positive for activated caspases (*$p<0.05$, **$p<0.01$, ANOVA, post-hoc Dunnett's *t* test). Caspase activation was first detected 60 min after C_{16}-PAF administration. (D) Functional dependence of C_{16}-PAF-induced neuronal apoptosis on caspase activation was measured by LIVE/DEAD assay. hNTs were treated with C_{16}-PAF (1 μM) in the presence or absence of the caspase 2 inhibitor Z-VDVAD-FMK (50 μM) or the caspase 3/7 inhibitor Z-DEVD-FMK (50 μM). Data are expressed as percent survival relative to vehicle-treated cells. (**$p<0.05$, ANOVA, *post-hoc* Dunnett's *t* test).

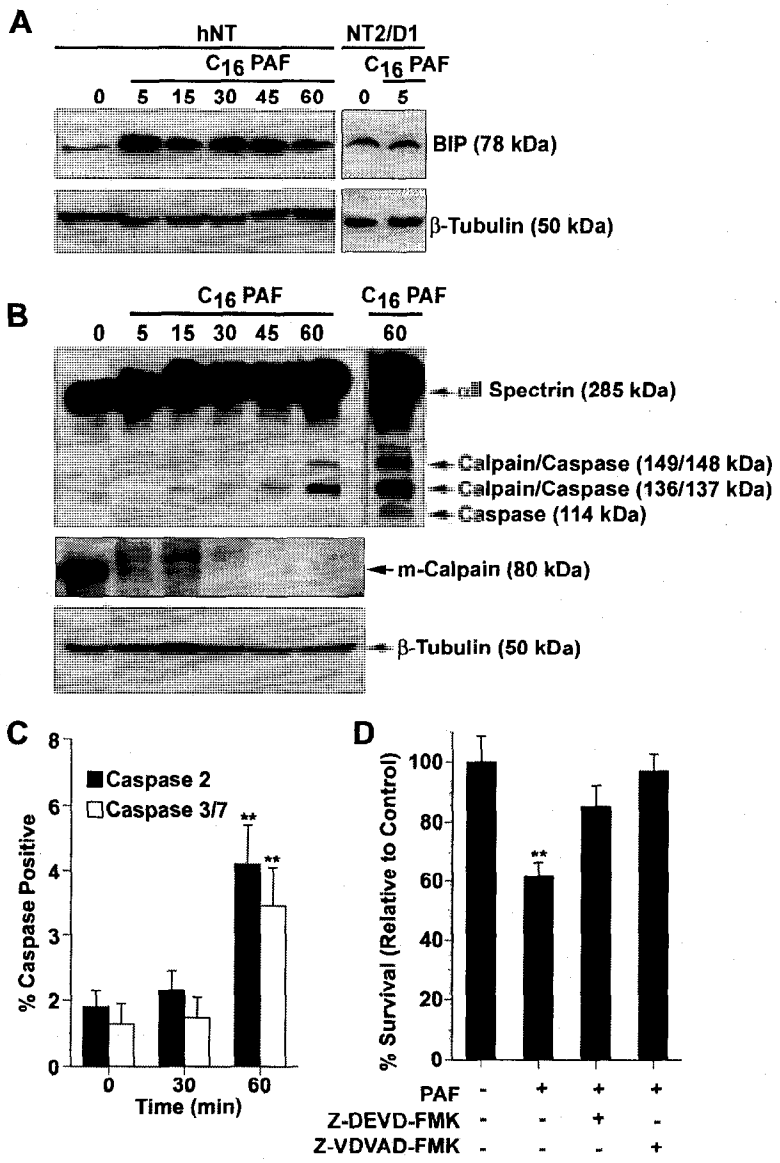

A
hNT
NT2/D1
C16 PAF
C16 PAF
0 5 15 30 45 60
0 5
BIP (78 kDa)
β-Tubulin (50 kDa)
B
C16 PAF
C16 PAF
0 5 15 30 45 60
60
Spectrin (285 kDa)
Calpain/Caspase (149/148 kDa)
Calpain/Caspase (136/137 kDa)
Caspase (114 kDa)
m-Calpain (80 kDa)
β-Tubulin (50 kDa)
C
Caspase 2
Caspase 3/7
% Caspase Positive
Time (min)
0 30 60
D
% Survival (Relative to Control)
PAF - + + +
Z-DEVD-FMK - - + -
Z-VDVAD-FMK - - - +

Figure 5.9

activation. We found that cleavage of αII-spectrin occurred coincident with the LIS1-mediated acceleration in PAF-AH I activity in hNT neurons (Fig 5.9B). An increase in spectrin cleavage, generating both ~150 kDa and ~140 kDa fragments, was observed 15 minutes after C_{16}-PAF exposure (Fig 5.9B). A marked increase in the ~140 kDa fragment was observed following 60 minutes of C_{16}-PAF treatment coupled with the appearance of the caspase-specific 114 kDa fragment (Fig 5.9B). These kinetics implicate calpains and/or caspase 2 in the initial transduction of C_{16}-PAF neurotoxicity. After 60 minutes of C_{16}-PAF treatment, activation of caspase 3/7 likely enhances the proteolyic production of the ~140 kDa fragment and further processes the ~150 kDa product to the 114 kDa spectrin fragment (Fig 5.9B)

To distinguish between calpain and caspase activation, we assessed m-calpain activation and caspase 2, 3, and 7 activity directly. Activation of m-calpain is associated with cleavage of the 80 kDa calpain pro-protein (Yoon et al., 2006). Using an antibody that detects the inactive pro-protein but not cleaved, activated calpain, we found a marked decrease in pro-m-calpain levels within 5 minutes of C_{16}-PAF exposure (Fig 5.9B). Significant caspase 2 and caspase 3/7 activation was not detected until 60 minutes after C_{16}-PAF administration (Fig 5.9C). To determine whether downstream caspase activation is ultimately responsible for C_{16}-PAF-induced neuronal death, hNT neurons were exposed to C_{16}-PAF in the presence of the caspase 2 inhibitor Z-VDVAD-FMK or the caspase 3/7 inhibitor Z-DEVD-FMK. Inhibiting either caspase 2 or caspase 3/7 attenuated C_{16}-PAF-mediated neuronal loss (Fig 5.9D). Together, these data indicate that calpain activation and LIS1 translocation to the PAF-AH I complex precede caspase activation in C_{16}-PAF-treated neurons and that downstream caspase 2 and 3/7 signal terminal neuronal failure.

Based on these data, our model (Fig 5.12) predicts that rising intracellular concentrations of C_{16}-PAF induce UPR resulting in m-calpain activation. Sustained ER stress signals the release of LIS1 from the microtubules to the PAF-AH 1 complex. Transient translocation protects neurons from acute rises in intracellular PAF concentrations. Prolonged translocation, as would be predicted in the face of the aberrant lipid metabolism associated with neurodegenerative disease, likely exacerbates C_{16}-PAF-induced stress to ER and Golgi thereby triggering a caspase 2 and 3/7-dependent apoptotic cascade.

To test this model, our final series of experiments were designed to determine whether C_{16}-PAF-induced UPR and calpain activation (Fig 5.9A,B) are responsible for the release LIS1 from the microtubules (Fig 5.8). Calpains have been shown to activate both glycogen synthase kinase 3β (GSK3β) and cyclin-dependent kinase 5 (Cdk5) leading to the hyperphosphorylation of microtubule-associated proteins including LIS1-binding partners (Goni-Oliver et al., 2007; Hallows et al., 2003; Lee et al., 2000). To test whether increases in kinase activity underlie LIS1 movement to the PAF-AH I complex, we first assessed post-translational modifications of GSK3β and its activator AKT in C_{16}-PAF challenged neurons. A decrease in GSK3β phosphorylation at Ser9, associated with GSK3β activation, was detected 45 minutes after PAF treatment (Fig 5.10A). Conversely, phosphorylation of AKT at Thr308 is associated with the inhibition of GSK3β activity. No change in AKT (Thr308) phosphorylation, was observed over the course of C_{16}-PAF exposure (Fig 5.10A). These data suggest that, while GSK3β may be activated by rising intracellular C_{16}-PAF concentrations within 45 min of PAF challenge, GSK3β kinase activity likely does not initiate LIS1 release from the microtubules within the first 15 min of C_{16}-PAF exposure.

Figure 5.10: C_{16}-PAF induces Cdk5 and GSK3β activity leading to increased phosphorylation of microtubule-associated proteins. (A) hNT protein (30 μg) from untreated cells (0 minutes) or cells treated with 1 μM C_{16}-PAF for 5, 30, 45, or 60 min were immunoblotted to assess kinase expression and post-translational modifications associated with activation. Increases in protein expression of Cdk5 and its activators p35 and p39 were detected 15 min after PAF administration. A decrease in phospho-GSK3β associated with activation was first observed 45 min after treatment. Little to no change in AKT phosphorylation at Thr308 associated with subsequent inhibition of GSK3β was detected. These kinetics are consistent with rapid activation of Cdk5 and subsequent activation GSK3β. β-tubulin was used as a loading control. (B) Cdk5 activity in hNTs was directly determined by *in vitro* assay of P^{32} labeled histone 1 of immunoprecipitated Cdk5 protein (inset). Quantification was performed by densitometry. An progressive increase in Cdk5 activity was detected following C_{16}-PAF treatment. (C,D) Comparison of Cdk5 activity in NT2/D1 precursor cells and hNT neurons 45 min after C_{16}-PAF treatment. C_{16}-PAF induces Cdk5 activity in hNT neurons but not NT2/D1 precursor cells. (E) Phosphorylation of Cdk5 and GSK3β microtubule-associated substrates. Phosphorylation of MAP2A/B (arrow) and tau protein was first detected 15 min after PAF exposure. Phosphorylation of the LIS1 binding partner Nudel was detected 45 min after PAF exposure associated with a decrease in total Nudel levels. β-tubulin was used as a loading control. Data are representative of replicate immunoblots

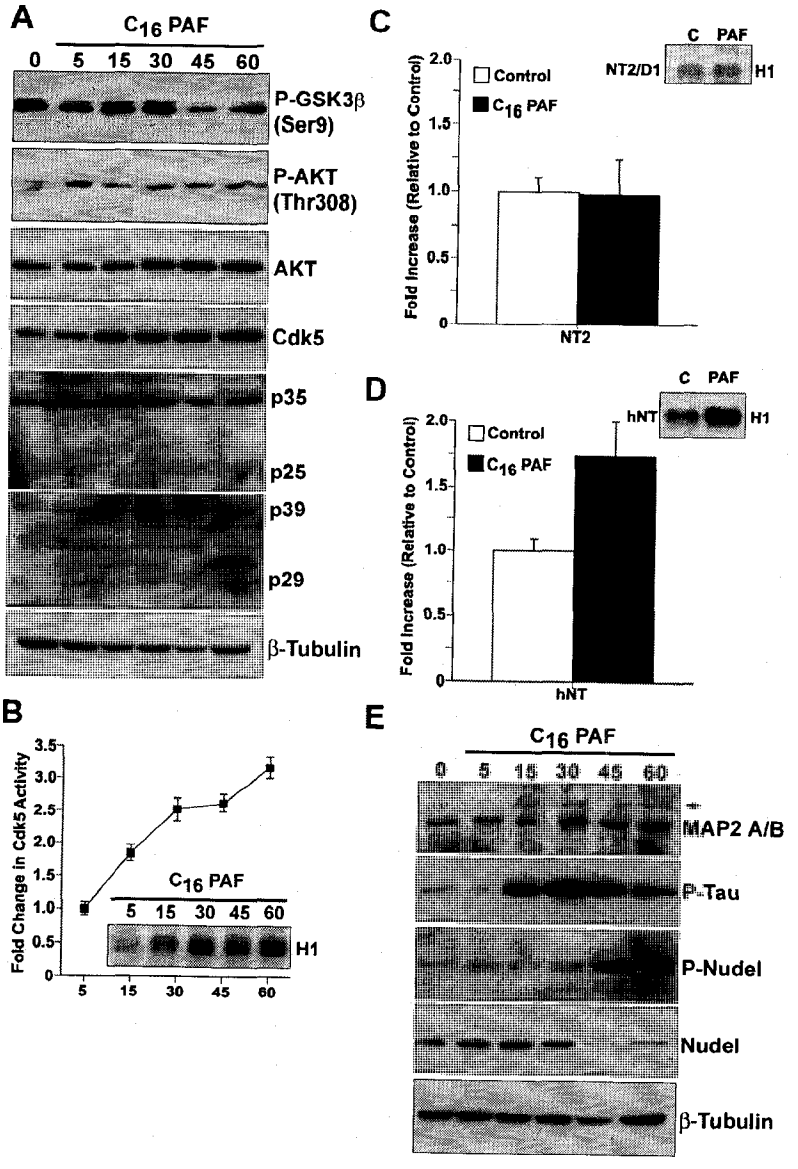

A
C16 PAF
0 5 15 30 45 60
P-GSK3β (Ser9)
P-AKT (Thr308)
AKT
Cdk5
p35
p25
p39
p29
β-Tubulin
B
Fold Change in Cdk5 Activity
C16 PAF
5 15 30 45 60
H1
C
C PAF
NT2/D1
H1
Control
C16 PAF
Fold Increase (Relative to Control)
NT2
D
C PAF
hNT
H1
Control
C16 PAF
Fold Increase (Relative to Control)
hNT
E
C16 PAF
0 5 15 30 45 60
MAP2 A/B
P-Tau
P-Nudel
Nudel
β-Tubulin

Figure 5.10

Increases in Cdk5, p35, and p39 protein levels were observed within 15 minutes of PAF administration (Fig 5.10A), coincident with the acceleration in PAF-AH I activity (Fig 5.5). These temporal kinetics implicate Cdk5 in acute C16-PAF signaling. Surprisingly, calpain-dependent cleavage of p35 to p25 and p39 to p29 was not detected over the course of C16-PAF treatment (Fig 5.10A). Previous studies have shown that Cdk5 activity can be augmented independently of p35 and p39 cleavage (Lin et al., 2007a; Lin et al., 2007b; Nguyen et al., 2002; Tang et al., 1995; Tsai et al., 1994; Tsai et al., 1993). To assess Cdk5 activity directly, Cdk5-mediated transfer of radiolabeled P32 to histone-1 was measured and quantified following C16-PAF exposure (Fig 5.10B). A time-dependent increase in Cdk5 activity was observed (Fig 5.10B). Activation was only detected in PAF-sensitive hNT neurons and was not observed in PAF-resistant NT2/D1 precursor cells (Fig 5.10C,D).

Finally, we assessed the phosphorylation of known microtubule-associated Cdk5 and GSK3β substrates. Phosphorylation of MAP2 and tau was observed within 15 minutes of C_{16}-PAF exposure (Fig 5.10E). Phosphorylation of the LIS1 binding partner Nudel responsible for interaction with microtubular dynein (Shu et al., 2004) was observed 45 minutes after challenge with C_{16}-PAF. Phosphorylation was also associated with an overall decrease in Nudel protein expression (Fig 5.10E). Nudel competes with the PAF-AH I α_2 homodimer for LIS1 binding to retain LIS1 at the microtubules (Tarricone et al., 2004). Phosphorylation and loss of Nudel is therefore predicted to decrease microtubule competition for LIS1 and thus promote association with α_2.

To establish whether Cdk5 activity is responsible for LIS1 translocation, we treated hNT cultures with the Cdk5/p35 inhibitor roscovitine (Meijer et al., 1997). Ros-

covitine protected hNT neurons from C_{16}-PAF toxicity in a concentration-dependent manner up to 5 μM (Fig 5.10A). To confirm that roscovitine inhibited C_{16}-PAF-induced Cdk5 phosphorylation of microtubule associated proteins, we immunoblotted for phospho-tau and phospho-Nudel. Roscovitine (5 μM) prevented C_{16}-PAF-induced phosphorylation of both tau and Nudel (Fig 5.11B). Finally, we tested whether Cdk5 inhibition was sufficient to prevent LIS1 association with PAF-AH I complex. hNTs were treated with PAF (1 μM) in the presence or absence of roscovitine (5 μM). Lysates were immunoprecipitated for LIS1 and immunoblotted for LIS1 and PAF-AH I α_2. Roscovitine reduced association of LIS1 with cytosolic PAF-AH I α_2 (Fig 5.11C). These data provide clear evidence that roscovitine prevents C_{16}-PAF-mediated translocation of LIS1 from the microtubules to the PAF-AH I complex and protects neurons from C_{16}-PAF neurotoxicity.

5.7 Discussion

In this study, we elucidate a new pathway of neuronal dysfunction triggered by aberrant lipid metabolism of C_{16}-PAF in Alzheimer disease. Our model is presented in Fig 5.12. Repeated demonstrations that $cPLA_2$ activity is increased in Alzheimer disease have implicated bioactive lipid second messengers in disease pathology (Chen and Bazan, 2005; Farooqui and Horrocks, 2001; Farooqui and Horrocks, 2006; Klein, 2000; Sweet et al., 2002). Little is known about the identity of pathogenic species or their impact on neuronal function. Here, we show, for the first time, that the C_{16}-PAF isoform accumulates in Alzheimer disease brain and in human neurons following $A\beta_{42}$. We demonstrate that this is an early event in TgCRND8 mice (Chishti et al., 2001). We further

Figure 5.11: Inhibition of Cdk5 prevents C_{16}-PAF-induced LIS1 translocation to the PAF-AH I complex and protects neurons from C_{16}-PAF-induced death. (A) hNT neurons, pre-treated for 15 min with increasing concentrations of the Cdk5 inhibitor roscovitine, were treated with C_{16}-PAF. Significant protection from C_{16}-PAF toxicity was observed following treatment with 1 and 5 μM of roscovitine (**$p<0.01$, *$p<0.05$, ANOVA, *post-hoc* Dunnett's *t*-test). (B) Phosphorylation of microtubule associated proteins was assessed in hNT neurons pre-treated with 5 μM roscovitine for 15 min. Roscovitine inhibited PAF-induced phosphorylation of tau and Nudel. β-tubulin immunoblots were used as loading controls. (C) Protein extracts from hNT neurons, pre-treated with 5 μM roscovitine for 15 min, were immunoprecipitated for LIS1 and immunoblotted for PAF-AH I α_2. Inhibition of Cdk5 decreased C_{16}-PAF-induced association of LIS1 with α_2.

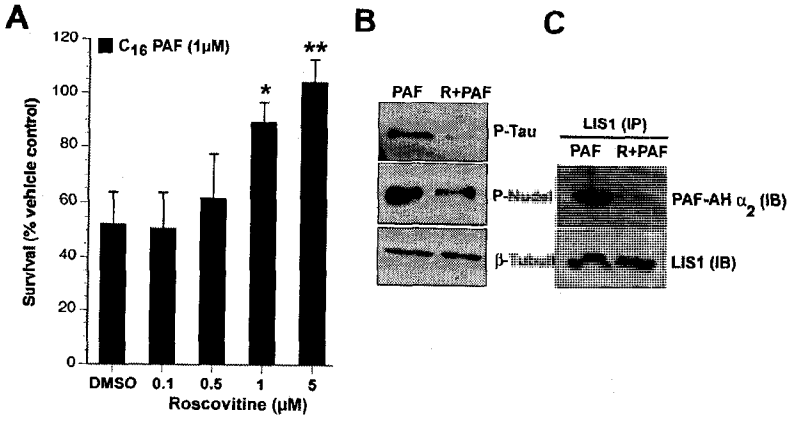

A
B
C
C16 PAF (1μM)
Survival (% vehicle control)
120
100
80
60
40
20
0
*
**
DMSO
0.1
0.5
1
5
Roscovitine (μM)
PAF
R+PAF
P-Tau
P-Nudel
β-Tubulin
LIS1 (IP)
PAF
R+PAF
PAF-AH α2 (IB)
LIS1 (IB)

Figure 5.11

show that rising intracellular concentrations of C_{16}-PAF elicit UPR triggering calpain, Cdk5, and GSK3β activation. Cdk5-mediated phosphorylation of microtubule-associated substrates facilitates the translocation of LIS1 to the PAF-AH I complex to accelerate C_{16}-PAF catalysis. When LIS1 is sequestered from the microtubules, Golgi and ER-associated caspases are activated signaling neuronal death. These findings represent the first mechanistic link between early Aβ biogenesis and the generation of specific pathogenic lipid mediators that compromise neuronal survival in Alzheimer disease.

Our data are consistent with studies indicating that LIS1 specifically increases the catalytic activity of PAF-AH I α_2 homodimers (Manya et al., 1999). Here, we show that the association of LIS1 with PAF-AH I α_2 accelerates PAF-AH I activity in human neurons. Previous studies have indicated that PAF-AH I α_2 overexpression induces centrosomal amplification, spindle deficits, and microtubule disorganization in mitotic cells through LIS1 interaction (Yamaguchi et al., 2007). While our translocation studies are consistent with these results, they also indicate that ectopic expression is not sufficient to sequester LIS1 from the microtubules in human neurons. Rather, we find that overexpression of PAF-AH I α_2 protects cells from PAF challenge likely by promoting C_{16}-PAF hydrolysis and preventing LIS1 translocation. This disconnect may reflect the differences in PAF-AH I□ catalytic subunit expression in different cell systems. As demonstrated in this study, terminally differentiated neurons have adapted to deal with high levels of PAF-AH I α_2 without affecting LIS1 localization consistent with the shift in PAF-AH I catalytic expression observed over the course of cerebral development (Manya et al., 1998).

In human neurons, activation of Cdk5 is necessary to signal LIS1 movement to

PAF-AH I α_2. Inhibiting Cdk5 activity in the presence of rising intracellular C_{16}-PAF concentrations prevents LIS1 translocation and blocks C_{16}-PAF -induced neuronal death. Subsequent GSK3β activation observed in this study may also depend upon Cdk5 given reports that Cdk5 regulates GSK3β activity and primes GSK3β phosphorylation sites (Cho and Johnson, 2003; Hallows et al., 2003). Neuronal dysfunction leading to death triggered by intracellular C_{16}-PAF accumulation is characterized by an apparent gradual impairment of microtubule function that is dependent upon the activity of these kinases (Fig 5.12). Phosphorylation of tau, MAP2, and Nudel is observed after activation of both GSK3β and Cdk5. Each target stabilizes microtubules through independent mechanisms (Harada et al., 1994; Tai et al., 2002; Teng et al., 2001) with phosphorylation compromising microtubule stability (Harada et al., 1994; Teng et al., 2001). We propose that the initial phosphorylation of immediate targets weakens the affinity of LIS1 for the dynein motor complex while the subsequent phosphorylation of Nudel stabilizes the interaction of LIS1 with PAF-AH I α_2. This conclusion is supported by evidence that phosphorylation of Nudel elicits changes in LIS1 tertiary conformation reported to be necessary for PAF-AH I α_2-LIS1 interaction (Derewenda et al., 2007; Tarricone et al., 2004). Moreover, phosphorylation of NUDEL promotes the transient interaction of LIS1with the microtubule severing protein p60 katanin required for microtubule reorganization (Toyo-Oka et al., 2005) by dissociating LIS1 from microtubule complexes (Niethammer et al., 2000; Sasaki et al., 2000; Yan et al., 2003) and, thus, likely represents a final step in the commitment of C_{16}-PAF-challenged neurons to an apoptotic program.

Finally, these data also address a new means of ER-Golgi apoptotic cross-talk signaled by choline-containing lipid second messengers. As a component of the microtubu-

Figure 5.12: Predicted model as to how rising intracellular C_{16}-PAF concentrations compromise neuronal survival. Aberrant metabolism of glycerophosphocholine lipids leads to the intracellular accumulation of C_{16}-PAF. (A) Where PAF hydrolysis by PAF-AH I in insufficient to neutralize neurotoxic lipid, (B) an ER-stress response is triggered initiating UPR and m-calpain activation. (C) Downstream Cdk5 activation mediates translocation of LIS1 from the microtubules to the PAF-AH I complex likely through (D) phosphorylation of LIS1 binding partners to accelerate (E) C_{16}-PAF to C_{16}-*lyso*-PAF hydrolysis. In the face of pathological C_{16}-PAF challenge, sustained kinase activity and sequestering of LIS1 from the microtubules is predicted to (F) destabilize neuronal microtubules activating Golgi- and ER-associated caspases as protein transport is fatally interrupted.

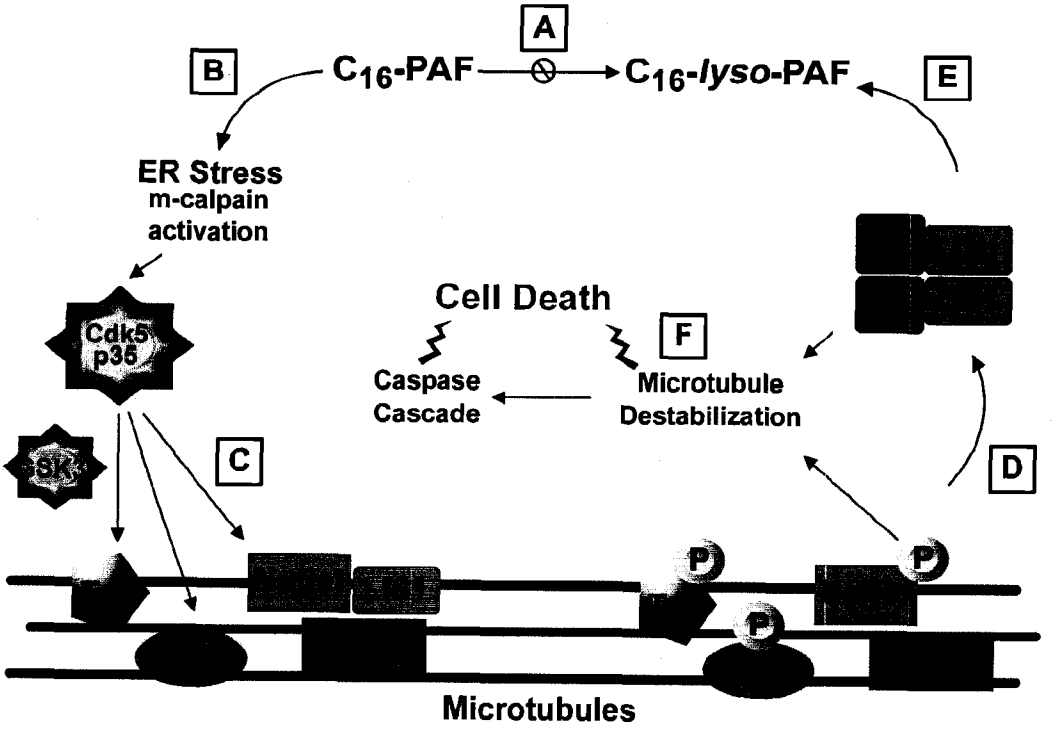

A
B
C16-PAF
C16-lyso-PAF
E
ER Stress
m-calpain
activation
Cdk5
p35
Cell Death
F
Caspase
Cascade
Microtubule
Destabilization
GSK
C
D
P
P
P
Microtubules

Figure 5.12

lar dynein complex, LIS1, regulates Golgi localization and intracellular vesicle trafficking from the ER (Smith et al., 2000). Recently, Golgi fragmentation has been implicated in the initiation of neuronal death associated with progressive neurodegenerative disorders (Nakagomi et al., 2007). Our data provide new insight into potential underlying mechanisms. LIS1-mediated disruption of vesicle trafficking has been shown to alter trafficking of pro- and anti-apoptotic proteins to the plasma membrane (Kondratova et al., 2005). Moreover, pro-apoptotic proteins such as BH3 only (Bcl-2 homology 3-only), BIM (Bcl-2 interacting mediator of cell death) and BMF (Bcl-2 modifying factor) are sequestered by the dynein complex (Day et al., 2004; Puthalakath et al., 1999; Puthalakath et al., 2001; Strasser et al., 2000). ER stress can induce BIM translocation from microtubule-associated dynein initiating an ER-dependent caspase cascade (Morishima et al., 2004). Thus, the sustained recruitment of LIS1 to the PAF-AH I complex likely exacerbates ER stress and UPR by inhibiting dynein function thereby activating Golgi- and ER-associated caspases as protein transport is fatally interrupted (Fig 5.12). We have yet to address how C_{16}-PAF elicits the UPR response leading to critical changes in calpain and Cdk5 activity. One possible mechanism involves activation the mitogen-activated protein kinase (MAPK) pathway. Extracellular signal-regulated kinase (ERK) activation has been linked to both induction of ER-stress and calpain activation (Arai et al., 2004). ERK signaling has also been shown to activate Cdk5 independently of p35 or p39 (Lee and Kim, 2004). Furthermore, ERK activity is stimulated by PAF in multiple cell types including PAFR-null mutant neurons (Hidalgo et al., 2004; Hirabayashi et al., 1998; Tsuda et al., 2007). C_{16}-PAF activation of ERK may be mediated through an interacting pro-

tein with the lipid binding pleckstrin homeodomain such as Gab1 as previously reported (Osawa et al., 2004). Clearly, this hypothesis will require further validation.

Collectively, these data elucidate a novel signal transduction pathway by which pathogenic glycerophosphocholine metabolites upregulated in Alzheimer disease, specifically C_{16}-PAF, trigger neuronal dysfunction and death. These data provide needed insight into the underlying mechanisms of neuronal degeneration associated with accumulation of bioactive glycerophospholipids in ischemia, epileptic seizure, encephalitis, meningitis, HIV dementia, and Alzheimer disease (Bate et al., 2006; Bate et al., 2004; Bazan et al., 2002; Birkle et al., 1998; Farooqui and Horrocks, 2006; Ishii and Shimizu, 2000; McLarnon et al., 2005; Perry et al., 1998; Stafforini et al., 2003) and identify, for the first time, a new neurotoxic signal transduction pathway driven by aberrant lipid metabolism.

5.8 Materials and Methods

5.8.1 Reagents: All chemicals were purchased through Sigma-Aldrich (St. Louis, MO USA) and all cell culture reagents were obtained from Invitrogen (Burlington, ON Canada) except where indicated.

5.8.2 Human tissue samples: Human postmortem samples (n=4 per group) were obtained from the Douglas Hospital Research Centre Brain Bank (Montreal, QC, Canada). Control individuals were between 56 and 94 years of age with mean age 78 $\pm$ 16 yrs who suffered sudden death for reasons other than neurological complications. Alzheimer disease patients were between 64 and 83 years of age with mean age 74$\pm$ 8 yrs at time of death. The parahippocampal gyrus from each individual was removed and flash-frozen in liquid nitrogen at autopsy. Tissue was maintained at -80°C until dissection for lipid extraction. Post-mortem delay was between 5 and 25 h and was matched between Alzheimer disease patients and controls. Tissue was weighed and placed in 1 ml of ice-cold methanol acidified with 2% acetic acid. Tissue was homogenized using a tissue tearor (Biospec) and extracted as described below.

5.8.3 Mice: TgCRND8 and NonTg littermate controls were sacrificed at 4 months of age via lethal injection of euthanol. Cerebral cortices (posterior cortex, temporal lobe) were removed on ice. Cortical tissue was weighed and placed in 1 ml of ice-cold methanol acidified with 2% acetic acid. Tissue was homogenized using a tissue tearor (Biospec) and extracted as described below.

5.8.4 Cell death assays: NT2/D1 cells, originally obtained from Stratagene (La Jolla, CA USA), were maintained in growth media consisting of Dulbecco's Modified Eagle's Medium/F12 (DMEM/F12) containing 10% FBS, 1% penicillin/streptomycin, and 2mM L-glutamine at 37°C in a 5% CO_2/95% air atmosphere. Passage 57 NT2/D1 precursors were terminally differentiated into hNT neurons as described (Boucher and Bennett, 2003). Cells were treated in serum-free growth media containing 0.025% BSA for with 24 h with PAF (0.01-1 μM, Biomol Research Laboratories Inc.), C_{16}-*lyso*-PAF (1 μM, Biomol Research Laboratories Inc), mc-PAF (0.01-5 μM, Biomol Research Laboratories Inc.) or $A\beta_{42}$ (25 μM) for 24 h. $A\beta_{42}$ was prepared as described (Klein, 2002). Briefly, 0.1 mg of Aβ (Chemicon) was dissolved in 100 μl of isopropanol, separated into 10 μg aliquots and drying under steady stream nitrogen. To prevent aggregation, appropriate volumes of treatment media were added at 4°C to give a final concentration of 25 μM immediately prior to treatment. Cell survival was assessed by the LIVE/DEAD Viability/Cytotoxicity (Molecular Probes) as we have described (Ryan et al., 2007). For lipid analysis, media was removed and cells washed with PBS followed by 2.72% Na acetate, 0.146% NaCl, and 1% BSA (w/v) for 1 minute at 4°C to remove loosely bound lipids from the cell surface (Ammitt and O'Neill, 1997; Bonin et al., 2004).

5.8.5 Lipid extraction and LC-ESI-MS: Stock chemicals were purchased from J.T. Baker (Phillipsburg, NJ). Glycerophospholipids were extracted according to a modified Bligh/Dyer procedure (Bligh and Dyer, 1959) described in detail in (Whitehead et al., 2007). Briefly, lipids were extracted using a volumetric ratio of 0.95 of chloroform and

0.8 of 0.1 M Na acetate (aq) per volume of MeOH in acid-washed borosilicate glass tubes (Fisher, Ottawa, ON). Phospholipids were collected from the organic phase after layer separation by centrifugation. The aqueous phase was back-extracted three times in the organic phase of a wash solution prepared by combining DMEM/F12+ 0.025% BSA, methanol, chloroform, and sodium acetate in the volumetric ratio of 1:2.5:3.75:1. The organic fractions were combined, evaporated under a stream of nitrogen gas, and dissolved in 300 µl EtOH. LC-ESI-MS was performed as we have described previously (Whitehead et al., 2007). For each LC-MS run, 40 µL of lipid preparation were loaded for analysis, consisting of 8 µl of lipid extracts or diluents, 5 µL of exogenous standards, and 27 µl H_2O of with 0.1% formic acid. To prevent column from being overloaded, brain extracts were diluted 1:4, 1:8, or 1:16 in EtOH before 8 µL of diluent were taken for analysis. For cell extracts, 8 µl were taken directly from lipid extracts without dilution. Five µl of a standard solution containing known amounts of C_{16}-PAF were then spiked into the sample, and the final volume was brought to 40 µL by adding 27 µl of H_2O with 0.1% formic acid. Five standard solutions containing 200, 400, 600, 800, and 1000 pg each of C_{16}-PAF were used in the experiment. At each standard addition concentration, samples were analyzed in triplicate. All other methodological details are as described in (Whitehead et al., 2007).

5.8.6 RT-PCR: Total RNA was isolated with Trizol (Invitrogen) and treated with 1 U/µg RQ1-DNAse1 (Promega) to eliminate residual genomic DNA. Two µg of RNA were reverse-transcribed using pdN6 random primers with Superscript II H RT according to manufacturer's protocol. Negative controls included reaction processed identically with-

out addition of RT. Two μl of cDNA was PCR amplified in a total volume of 50 μl using the BD Advantage 2 Polymerase (Clontech) in a Whatman Biometra T-Gradient (Montreal-Biotech Inc.) using dNTPs (200 μM each, Promega), 10 pmol per primer for GAPDH, and 25 pmol per primer for PAFR. Cycling conditions were: 94°C for 5 minutes, 35 cycles of 94°C for 25 s, 59°C for 50 s, and 72°C for 1 mi, followed by a final extension of 7 minutes at 72°C. Primer sequences were: sense 5'-GCATCCTACTTCCTCATCCT-3' and antisense 5'-ACTTCAGTGACCGTATCCGT-3' defining a 538 bp amplicon of human PAFR and sense 5'-TGGTGCTGAGTATGTCGTGGAGT-3' and antisense 5'-AGTCTTCTGAGTGGCAGTGATGG-3' defining a 292 bp amplicon of GAPDH.

5.8.7 Live cell imaging: NT2/D1 and hNT cells were cultured in growth media overnight on poly-D-lysine (100 μg/ml)/laminin (20 μg/ml). Cells were incubated with 1 μM C_{11}-B-PAF (custom synthesized by Molecular Probes) in serum free growth media supplemented with 0.025% BSA for 0, 5, 30, 45, and 60 minutes. Following incubation media was removed, cells were washed with an acidified buffer consisting of 2.72% Na acetate, 0.146% NaCl, and 1% BSA (w/v) for 1 minute at 4°C to remove loosely bound lipids from the cell surface (Ammitt and O'Neill, 1997; Bonin et al., 2004). Live cell imaging under phase and fluorescence of identical cell fields was performed to track C_{11}-B-PAF internalization was performed as described (Bonin et al., 2004) using a DMR inverted microscope (Leica) equipped with a QICAM digital camera (Quorum Technologies) and captured using OpenLab software v4.5 (Improvision). Quantitation of fluorescence intensity of individual cells was performed using the Advanced Measurement Module of

OpenLab v 4.5 software.

5.8.8 PAF-AH I α_2 overexpression: EGFP plasmid and PAF-AH I α_2 plasmid (Dr H. Arai, University of Tokyo, Tokyo, Japan) in pcDNA3.1 (Invitrogen) backbones were co-transfected into hNT cells by calcium phosphate transfection. Maximal EGFP expression was observed 72 h after transfection. Control transfections were EGFP co-transfected with empty vector pcDNA3.1. Efficiency of transfection was comparable between PAF-AH I α_2 and empty vector as established by counting $EGFP^+$ cells immediately before treatment. Cell survival in serum-free media following PAF (1 μM) treatment was calculated as $EGFP^+$ cell number following treatment / mean $EGFP^+$ cell number in the vehicle control x 100.

5.8.9 TLC: Cultures were incubated at 37°C with 1 μM C_{11}-B-PAF in serum-free growth media containing 0.025% BSA, for 0, 1, 5, 15, 30, and 60 minutes. Lipids were extracted and developed on TLC plates as described (Bonin et al., 2004). C_{11}-B-PAF and C_{11}-B-*lyso*-PAF were used as authentic markers. Fluorescent lipids were visualized under UV light using AlphaImager-1220 software (Alpha Innotech Corporation). Fluorescence intensity corresponding to lipid yield was determined by densitometry using the Advanced Measurement Module of OpenLab v4.5.

5.8.10 Western analysis: Proteins were isolated in RIPA buffer (10 mM PBS, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 30 μl/ml aprotinin, 10 mM Na orthovanadate,

100 µl/ml PMSF). Protein samples (30 µg) were separated by SDS-PAGE under reducing conditions. Western analyses were performed using monoclonal anti-α_1, monoclonal anti-α_2, monoclonal anti-PAF-AH II (1:1000, Dr H. Arai, University of Tokyo, Tokyo, Japan), monoclonal anti-GAPDH (1:2000, IMGENEX), monoclonal anti-LIS1 clone 210 (1:5000, Dr. O. Reiner), monoclonal anti-BiP/GRP78 (1:1000, Cell Signalling), monoclonal anti-αII-spectrin (Fodrin, 1:1000, Sigma), monoclonal anti-m-calpain (1:500, Sigma), polyclonal anti-Cdk5 (C-8, 1:1000, Santa Cruz), polyclonal anti-p35 (C-19, 1:500, Santa Cruz), polyclonal anti-p39 (C-20, 1:500, Santa Cruz), polyclonal anti-phospho-GSK3β(Ser9) (1:1000, Cell Signalling), polyclonal anti-AKT (1:1000, Cell Signalling), polyclonal anti-phospho-AKT(Thr308), monoclonal anti-tau (AT8, 1:1000, Sigma), polyclonal anti-Nudel (1:1000, ProSci) monoclonal anti-phospho-Nudel (N219T, 1:1000, MBL International), monoclonal anti-MAP2 (1:1000, Sigma), and monoclonal anti-β-tubulin (1:10000, Sigma) as primary antibodies. Secondary antibodies were horseradish peroxidase-conjugated anti-mouse IgG (1:2000, Jackson Immunolabs), horseradish peroxidase-conjugated anti-rabbit IgG (1:5000, Jackson Immunolabs) and horseradish peroxidase-conjugated anti-goat IgG (1:800, Jackson Immunolabs). Immunoreactive bands were visualized using SuperSignal West Pico (MJS BioLynx Inc). Quantification of protein expression levels was performed by densitometric analysis of individual bands using ImageJ analysis software (NIH).

5.8.11 Immunoprecipitations: Protein was isolated from hNTs treated for 45 minutes with PAF or vehicle (0.025% BSA) and immunoprecipitated for LIS1 or PAF-AH I α_2 using Microlink Protein Coupling Kit (Pierce) or Immunoprecipitation Kit (Protein G) (Roche).

Antibodies used in this procedure were polyclonal anti-LIS1 (Chemicon) and monoclonal anti-α_2 (Dr H. Arai). Following protein isolation samples were separated by SDS-PAGE and analyzed by Western blot.

5.8.12 Separation of soluble cytosolic and insoluble cytoskeletal protein fractions: Soluble cytosolic and insoluble cytoskeletal protein fractions were separated according to (Joshi and Cleveland, 1989). Briefly, cells were washed twice with a microtubule stabilizing buffer (0.1 mol/L N-morpholinoethanesulffonic acid, pH 6.75, 1 mmol/L $MgSO_4$, 2 mmol/L EGTA, 0.1 mmol/L EDTA, 4 mol/L glycerol) followed by a 5 minute incubation at 37°C in 500 µl of microtubule stabilizing buffer plus 0.1% Triton X-100 (Polysciences Inc, Warrington, PA USA). The soluble cytosolic fraction was then removed and centrifuged at 300g for 2 minutes to pellet any insoluble cytoskeletal debris. The pellet was recombined with the insoluble cytoskeletal fraction remaining in the culture dish and proteins of both insoluble cytoskeletal and soluble cytosolic fractions were isolated in RIPA buffer as described above.

5.8.13 Immunofluorescence: Cells were washed with 10mM PBS prior to incubation. Primary antibodies were polyclonal rabbit anti-α-LIS1 and monoclonal mouse anti-α_2. Secondary antibodies were Cy3-conjugated anti-rabbit IgG (1:400; Jackson ImmunoResearch) and FITC-conjugated anti-mouse IgG (1:100; Jackson ImmunoResearch). Antibodies were diluted in antibody buffer (10 mM PBS, 0.3% Triton X-100, and 3% bovine serum albumin). Photomicrographs were obtained using an Olympus FV1000 confocal microscope (Olympus Canada). An argon laser was used to excite the 495 nm secondary

antibody FITC and the Red HeNe laser to excite the 512 nm Cy3 secondary. Co-localization was evaluated using the Advanced Measurements Co-localization Module of OpenLab Software, version 4.5 (Improvision, Lexington, MA).

5.8.14 Caspase Activation: Caspase activity in live cells was measured using CaspaTag (caspase 3/7, Chemicon) or FLICA (caspase 2, Intergen) assays in 96-well plates according to manufacturer's instructions. Phase contrast and fluorescent photos were taken of 6 random fields per well for 5 – 10 replicate wells in duplicate or triplicate experiments. The mean number of cells with active caspases in vehicle-treated cultures was subtracted from the number of positive cells in experimental cultures and data standardized against untreated controls to determine fold-change in caspase activity attributable to treatment. Functional dependence of PAF-induced neuronal apoptosis on caspase 2 activation was assessed by LIVE/DEAD cytotoxicity assay. hNTs were treated with PAF (1 μM) or vehicle in the presence and absence of the caspase 2 inhibitor Z-VDVAD-FMK (50 μM). Survival was expressed as % relative to control treated cells.

5.8.15 Statistical analysis: Data were analyzed using Student's *t*-test or factorial ANOVA as applicable using Instat v3.0. Following detection of a statistically significant difference in a given series of treatments by ANOVA, *post hoc* Dunnett's *t*-tests or Tukey tests were performed where appropriate. Where analysis of slope following linear regression difference is indicated, the rate of PAF hydrolysis between cultures was calculated as the difference between the two slopes divided by the standard error of the difference between the slopes subsequently analyzed Student's *t*-test. P values under 0.05 were considered

statistically significant (shown as *); P values under 0.01 were considered highly statistically significant (shown as **).

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Author contributions: SDR and SALB conceived and designed the experiments and analyzed the data. SDR performed the majority of the experimental work and wrote the manuscript with SALB. TCM contributed data to Fig 2. JR and DQ performed the Cdk5 kinase assays with guidance in design and data analysis from DSP. PF contributed new reagents/analytical tools. SDR, SNW, WH, LS, ME, AJGB performed and analyzed the LC-ESI-MS experiments with assistance from SALB and DF.

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Chapter 6: General Conclusion: Bioactive glycerophospholipid mediators in neurodegenerative disease

6.1 Summary: A novel PAF-induced death cascade independent of PAFR

This thesis sought to identify whether PAF signalling is implicated in neurodegenerative disease pathology driven by the hypothesis that aberrant PAF glycerophospholipid metabolism induces neuronal dysfunction and death. In testing this hypothesis, I determined that PAF neurotoxicity is isoform-specific with signalling dependent upon the repertoire of PAF effector proteins expressed by target neurons. First, PAF species triggered neuronal apoptosis in neurons devoid of PAFR (Chapters 2, 3, 4 and 5). In PC12 cells, primary murine neurons, and terminally differentiated human neurons, toxicity was specific to PAF and was not induced by its immediate metabolite *lyso*-PAF (Chapters 2, 3 and 5). Next, PAFR expression protected neurons from C_{16}-PAF but not C_{18}-PAF demonstrating that carbon chain length at the *sn*-1 position has significant impact on biological activity (Chapters 3 and 4). To intervene in PAF toxicity, I identified a novel PAF inhibitor (orsellinic acid) capable of protecting from the deleterious effects of C_{16}-PAF without impacting on PAFR-mediated neuroprotection (Chapter 3). To establish whether PAF isoforms signal neuronal death through distinct pathways, I compared C_{16}-PAF and C_{18}-PAF signalling. C_{18}-PAF was found to induce caspase-dependent neuronal apoptosis through a PAFR-dependent pathway and non-apoptotic (caspase-independent) neurodegeneration through a PAFR-independent pathway whereas the C_{16}-PAF species triggered neuronal apoptosis only in the absence of PAFR (Chapter 4). Finally, I delineated a novel signal transduction pathway whereby rising concentrations of C_{16}-PAF target the

ER to elicit apoptosis in human neurons (Chapter 5). Underlying mechanisms involve the translocation of microtubule-associated LIS1 to cytosolic PAF-AH I α_2 (Chapter 5). In collaboration with other members of the Bennett laboratory, I found that C_{16}-PAF and C_{16}-*lyso*-PAF concentrations are elevated in post-mortem AD temporal cortex (Chapter 5). Using TgCRND mice overexpressing the human APP protein under control of the prion protein promoter, again in collaboration, I demonstrated that this increase is an early event in AD etiology (Chapter 5). Finally, I show that Aβ triggers C_{16}-PAF accumulation in human neurons thereby providing direct evidence that aberrant lipid metabolism contributes to neuronal dysfunction and death by targeting the ER (Chapter 5).

6.1.1 New roles for PAFR and PAF-AH I α_2 in regulating neuronal fate

This thesis identifies new anti-apoptotic actions of both PAFR and the α_2 subunit of the PAF-AH I enzymatic complex. PAF-like lipids have been implicated as key apoptotic second messengers induced by a variety of pathological stressors (Bate et al., 2006; Bate et al., 2004; Bazan et al., 2002a; Bazan et al., 2002b; Birkle et al., 1998; Farooqui and Horrocks, 2006; Hershkowitz and Adunsky, 1996; Ishii and Shimizu, 2000; McLarnon et al., 2005; Perry et al., 1998; Stafforini et al., 2003). While previous studies have implicated PAFR-mediated excitotoxicity and activation of the intrinsic caspase cascade in PAF-toxicity (Bazan and Allan, 1996; Bennett et al., 1998; Chen et al., 2007; Ma and Bazan, 2001), the cerebral expression pattern of PAFR leaves little doubt that PAFR independent death mechanisms must also be at play. The importance of this alternative death mechanism is emphasized by the fact that neurons express low levels of PAFR in discrete brain regions (Bennett et al., 1998; Mori et al., 1996), and yet PAF is a principal

mediator of widespread neuronal loss in epileptic seizure, encephalitis, ischemia reperfusion injury, HIV-1 associated dementia and Alzheimer's like dementia (Bate et al., 2006; Bate et al., 2004; Bazan et al., 2002a; Bazan et al., 2002b; Birkle et al., 1998; Farooqui and Horrocks, 2006; Hershkowitz and Adunsky, 1996; Ishii and Shimizu, 2000; McLarnon et al., 2005; Perry et al., 1998; Stafforini et al., 2003). When this thesis was initiated, it was not known how PAF confers apoptotic signalling in the absence of PAFR.

Here, pharmacological inhibition of PAF hydrolysis and mRNA silencing studies demonstrated that the duration of PAF apoptogenic signalling was regulated primarily by the PAF-AH I α_2 subunit. In support of this finding, protection of cells from PAF toxicity by the non-competitive PAF-antagonists BN 52021 and FR 49175 was achieved, in part, by indirect promotion of PAF-AH I α_2 homodimer activity in PC12 cells (Chapter 2). These results were subsequently confirmed in a primary murine neuronal system (Chapter 3). This subunit-specific role for PAF-AH I in neuroprotection was unexpected. In mitotic cells, constitutive overexpression of either PAF-AH I α_1 or PAF-AH I α_2 is toxic, with conditional overexpression of PAF-AH I α_2 causing centromere amplification, nuclear collapse, and microtubule disorganization (Yamaguchi et al., 2007). This toxicity may explain why endogenous induction of PAF-AH I α_2 is insufficient to protect PC12 cells completely from PAF challenge as demonstrated in this thesis. PC12 cells induce PAF-AH I α_2 to hydrolyse rising intracellular concentrations of PAF, however, as excess PAF-AH I α_2 is equally undesired, expression plateaus prior to complete PAF catalysis (Chapter 2). In contrast to previously published data using mitotic cell systems (Yamaguchi et al., 2007), this thesis demonstrates that PAF-AH I α_2 overexpression protects

neurons from PAF challenge, suggesting that the toxic impact of PAF-AH I on the nucleus is restricted to actively dividing cells (Chapter 5).

PAFR-independent apoptosis does not involve NO or NMDAR signalling pathways previously implicated in PAFR-dependent excitotoxicity (Xu and Tao, 2004). Neither the NMDAR antagonists MK 801 nor the NOS inhibitor L-NAME protected $PAFR^{-/-}$ neurons from cytotoxic PAF challenge (Chapter 3). These results are consistent with previous reports that NO production requires PAFR activation (Han et al., 2006; Santiago et al., 2006) and that PAF-mediated excitotoxicity is a PAFR-dependent event (Bazan and Allan, 1996; Bennett et al., 1998; Clark et al., 1992, 1994; Marcheselli and Bazan, 1993; Xu and Tao, 2004; Zhu et al., 2004). Here, PAFR activation was found to inhibit C_{16}-PAF but not C_{18}-PAF -mediated cell death of both mitotic PC12 cells and post-mitotic neurons. PAFR-expressing WT neurons were resistant to challenge by pathophysiological concentrations of C_{16}-PAF whereas $PAFR^{-/-}$ neurons were sensitive to PAF-toxicity (Chapter 3 and 4). Ectopic expression of PAFR rendered $PAFR^{-/-}$ neurons resistant to C_{16}-PAF challenge (Chapter 3). Collectively these data provide novel evidence that PAFR-independent death mechanisms are activated in neurodegenerative disease models and suggest a potential mechanism underlying differential neuronal susceptibility to Aβ and other stimuli enhancing PAF synthesis.

To intervene in PAFR-independent apoptosis, I screened a series of natural benzoic acid derivatives. Natural products have received considerable attention as potential neuroprotectants. Many phytochemicals of natural origin have demonstrated protective activities in a multitude of neurodegenerative disease models (Gelinas and Martinoli, 2002; Heo and Lee, 2004; Ono et al., 2003). In this thesis, a benzoic acid derivative,

orsellinic acid and the non-competitive PAFR antagonists BN 52021 and FR 49175 reduced PAF neurotoxicity in $PAFR^{-/-}$ neurons without impacting upon PAF-mediated neuroprotection (Chapter 3). In PC12 cells, BN 52021 alleviates PAF toxicity by promoting rapid intracellular degradation to its non-toxic form, *lyso*-PAF (Bonin et al., 2004). Whether BN 52021 and orsellinic acid act though similar mechanisms in primary neurons remains to be determined. The two compounds are structurally dissimilar and are unlikely to share common direct cellular targets although downstream events may ultimately be similar. The biological activity of orsellinic acid is largely unknown and, beyond physiological PAF antagonism, the same is true for FR 49175. Both are, however, phenolic fungal derivatives raising the possibility of common cellular targets. By contrast, BN 52021 has been extensively studied and ascribed a variety of biological activities, from PAF antagonism to antioxidant properties (Maclennan et al., 2002). Further investigation is required to determine whether orsellinic acid is also multifunctional. In summary, these data demonstrate that PAFR-independent neurotoxicity may be selectively targeted to prevent neuronal loss identifying a novel PAF inhibitor, orsellinic acid, as a potential therapeutic candidate for further investigation.

6.1.2 Pathogenicity is regulated by heterogeneity in the PAF sn-1 chain

PAF lipids display considerable heterogeneity in carbon chain length and degree of unsaturation at the *sn*-1 position of glycerol backbone. C_{16}-PAF and C_{18}-PAF are the most predominant PAF family members produced (Callea et al., 1999; Oda et al., 1985; Ramesha and Pickett, 1987). The relative distribution of these two PAF species differs, with C_{18}-PAF being more abundant in the cerebrospinal fluid (Nishida and Markey,

1996). When this thesis began, it was not known whether PAF isoforms differentially impacted on neurotoxicity. Previous studies in the periphery had shown that the potency of PAF signalling was tightly tied to the chain length of fatty acid present (Carolan and Casale, 1990; Snyder, 1987). Differences in activity were the result of PAF binding to PAFR with the C_{16}-PAF isoform having an order of magnitude greater affinity for PAFR than the C_{18}-PAF isoform (Prescott et al., 2000; Snyder, 1987). My early interpretation was that both PAF species signalled through PAFR sharing similar toxicities with the only difference being their relative potency. To test this hypothesis, my subsequent investigations focused on whether subspecies of PAF triggered PAFR-independent signalling. Surprisingly, these studies revealed that the length of the *sn*-1 carbon chain of PAF glycerophospholipids has significant impact upon downstream apoptotic signalling and neuronal fate. I found that both C_{16}-PAF and C_{18}-PAF were toxic to neurons that lack PAFR but initiated different cell death pathways (Chapter 4). When PAFR-deficient neurons were exposed to PAF, C_{16}-PAF triggered caspase-dependent apoptosis whereas C_{18}-PAF-induces caspase-independent neurodegeneration. Isoform-specific neurotoxicity was further modulated by PAFR expression. Ectopic or endogenous PAFR expression protected neurons from C_{16}-PAF whereas PAFR expression shifted C_{18}-PAF-mediated neurotoxicity from a caspase-independent to a caspase-dependent signalling pathway. This finding is significant when placed in context with studies using apolipoprotein E knockout mice indicating that loss of apolipoprotein E, a risk factor associated with AD, alters the traffic of polyunsaturated lipids from astrocytes to neurons, skewing the composition of long-chain fatty acids in phosphatidylcholines of synaptosomes towards hexadecyl (16:0) species (Igbavboa et al., 2002). Thus, the redistribution of octadecyl to

hexadecyl phospholipids is likely to impact upon the type of neurodegeneration associated with aberrant lipid metabolism. These data provide insight into the underlying cell death pathways initiated following PAF challenge that, in part, reconcile reports of both pro- and anti-apoptotic PAF signalling (Brewer et al., 2002; Li et al., 2003; Southall et al., 2001).

6.1.3 A novel cell death pathway: Role of LIS1 in linking aberrant PAF metabolism to microtubule dysfunction

Having identified the PAF species responsible for PAFR-independent apoptogenic signalling (C_{16}-PAF), I proceeded to identify underlying signalling mechanisms in terminally differentiated human neurons. These studies were designed to elucidate the means by which aberrant lipid metabolism triggers neuronal apoptosis and to place my research in the larger context of impact in neurodegenerative disease. I found that extrinsic exposure to pathological PAF concentrations in the absence of PAFR led to rapid lipid internalization and increased protein expression of PAF-AH I α_2 (Chapter 5). Rising intracellular concentrations of C_{16}-PAF resulted in LIS1 translocation from human neuronal microtubules to the cytosolic PAF-AH I complex where it acts to accelerate PAF hydrolysis. Translocation was initiated following PAF-induced UPR that resulted in m-calpain activation and led to induction of Cdk5 and GSK3β kinase activity. PAF-induced kinase activity elicited phosphorylation of microtubule associated proteins MAP2A/B, Tau and the LIS1 binding partner Nudel, presumably contributing to the release of LIS1 from the microtubules. This interpretation is consistent with evidence that Cdk5-mediated phosphorylation of Nudel is predicted to unravel the coiled coil domain of

Nudel purported to facilitate a conformational change in LIS1 allowing for interaction of LIS1 with the PAF-AH I α_2 catalytic subunit as suggested by structural examination of Nudel/LIS1 interactions (Derewenda et al., 2007). In our model (Fig 5.12), sustained sequestration of LIS1 from the microtubules triggered an ER-Golgi-dependent caspase cascade involving caspases 2, 3, and 7 that ultimately resulted in neuronal death. Promoting alternate means of PAF hydrolysis through PAF-AH I α_2 overexpression rapidly inactivated the death ligand and protected neurons from PAF challenge. Furthermore, inhibiting Cdk5 activity in the presence of rising intracellular PAF concentrations prevented LIS1 translocation and blocked PAF-induced neuronal death. Finally, preventing PAF-mediated caspase activation also protected human neurons from PAF toxicity. These data elucidate a novel signal transduction pathway whereby cytotoxic PAF signalling is transduced to the cytoskeleton in the absence of PAFR activation.

Aberrations in many of the signalling events described above are associated with the pathology of AD. In this thesis, we present evidence suggesting that altered bioactive lipid synthesis and degradation unifies these signalling pathways and collectively underscore neurodegenerative disease progression. Altered PAF signalling occurs in patients with Alzheimer's associated senile dementia (McLarnon et al., 2005). In this thesis, I have demonstrated how PAF signalling may participate in associated neurodegeneration. Mutations in ER protein presenilin-1 found in familial AD (1995) results in aberrant Aβ processing (Lemere et al., 1996) and increased neuronal susceptibility to ER stress (Katayama et al., 1999). ER stress further promotes Aβ generation (Sai et al., 2002). Moreover ER stress-mediated activation of Cdk5 results in hyper-phosphorylation of Tau protein (Ahlijanian et al., 2000; Patrick et al., 1999; Saito et al., 2007), a clinical sign of AD.

Here, I describe how PAF impacts on neuronal loss through an ER stress mechanism resulting in enhanced Cdk5 activity and aberrant Tau phosphorylation. Furthermore, LIS1 is mobilized to protect from acute PAF-induced ER stress in neurons. Sustained loss of LIS1 from the dynein complex may disrupt dynein function (Sapir et al., 1999; Sapir et al., 1997; Smith et al., 2000). Pro-apoptotic proteins such as BH3 only, BIM and BMF are sequestered by the dynein complex (Day et al., 2004; Puthalakath et al., 1999; Puthalakath et al., 2001; Strasser et al., 2000). ER stress can induce BIM translocation from microtuble-associated dynein to the ER and initiate an ER-dependent caspase cascade (Morishima et al., 2004). Taken, together, these data provide needed insight into how accumulation of bioactive glycerophospholipids evident in neurodegenerative disease may participate in signalling neuronal dysfunction and death (Chen and Bazan, 2005; Farooqui and Horrocks, 2001; Farooqui and Horrocks, 2006; Klein, 2000; Sweet et al., 2002)

6.1.4 LIS1: A pleiotropic regulator of neuronal function

Studies performed in this thesis have identified phosphorylation of LIS1 binding partners in the regulation of LIS1 localization. While I have implicated PAF-mediated changes in Cdk5 activity as a modulator of LIS1 subcellular localization, the absolute determinants of LIS1 function have yet to be identified. Cdk5 signalling serves numerous purposes, as does LIS1 protein (Nguyen et al., 2002; Vallee et al., 2001). The two converge on a role in cerebral development. The diverse functionality of these signalling pathways may outline a means by which LIS1 moves throughout the cell, from the microtubules to cytosol and back again. Investigations into the role of LIS1 in neuronal posi-

tioning have found that LIS1 is involved in reelin signalling during neuronal migration (Gressens, 2006). Reelin signalling mediated by very low density lipoprotein receptor (VLDLR) recruits the PAF-AH I complex to VLDLR following activation (Zhang et al., 2007). Receptor activation induces tyrosine phosphorylation of disabled protein 1 (Dab-1) by Src-family-of-tyrosine-kinase (SFK) members (Assadi et al., 2003; Zhang et al., 2007). Phosphorylated Dab-1 binds LIS1 directly (Assadi et al., 2003) and competes for LIS1 binding to PAF-AH I α_2 (Fig 6.1) (Zhang et al., 2007). The Interaction of LIS1 with phosphorylated Dab-1 mediates the association of LIS1 and Dab-1 with the dynein complex; this underlies reelin influence over neuronal positioning (Pramatarova et al., 2006). Complementary reports have shown that Dab-1 is a Cdk5 substrate and Cdk5 can phosphorylate Dab-1 independent of reelin signalling (Keshvara et al., 2002). While Cdk5 phosphorylates Dab-1 at Ser 491 and reelin signalling results in tyrosine phosphorylation of Dab-1, Cdk5 phosphorylation has been shown to augment tyrosine phosphorylation of Dab-1 by the SFK member Fyn tyrosine kinase (Ohshima et al., 2007). I believe this may be explain LIS1 translocation from the PAF-AH I complex back to the dynein complex following neutralization of the PAF threat (Fig 6.1, modified from Zhang et al., 2007).

Cdk5 activation following PAF challenge would, in theory, phosphorylate both Nudel, releasing LIS1 from the microtubules, and Dab-1, returning LIS1 to the microtubules creating redundant loop. The regulator would thus be PAF-AH I α_2. When PAF-AH I α_2 levels are induced following PAF challenge, they become sufficient to compete for LIS1 binding with phosphorylated Nudel. Once PAF cytotoxic signalling is terminated and PAF-AH I α_2 levels are restored to basal levels, phosphorylated Dab-1

Figure 6.1: Predicted model of LIS1 translocation throughout the cell. Insufficient PAF hydrolysis leads to increased steady state levels of PAF. In the presence of elevated PAF (red) PAF-AH I α_2 is induced and Cdk5 activated leading to phosphorylation of microtubule associated proteins including the LIS1 binding partner Nudel. Microtubule phosphorylation facilitates LIS1 association with PAF-AH I α_2 increasing PAF hydrolysis. Once steady state PAF levels return to basal (blue) enhanced PAF hydrolysis is no longer necessary and LIS1 is shuttled back to the microtubules by phosphorylated Dab-1. Modified from Zhang et al., 2007.

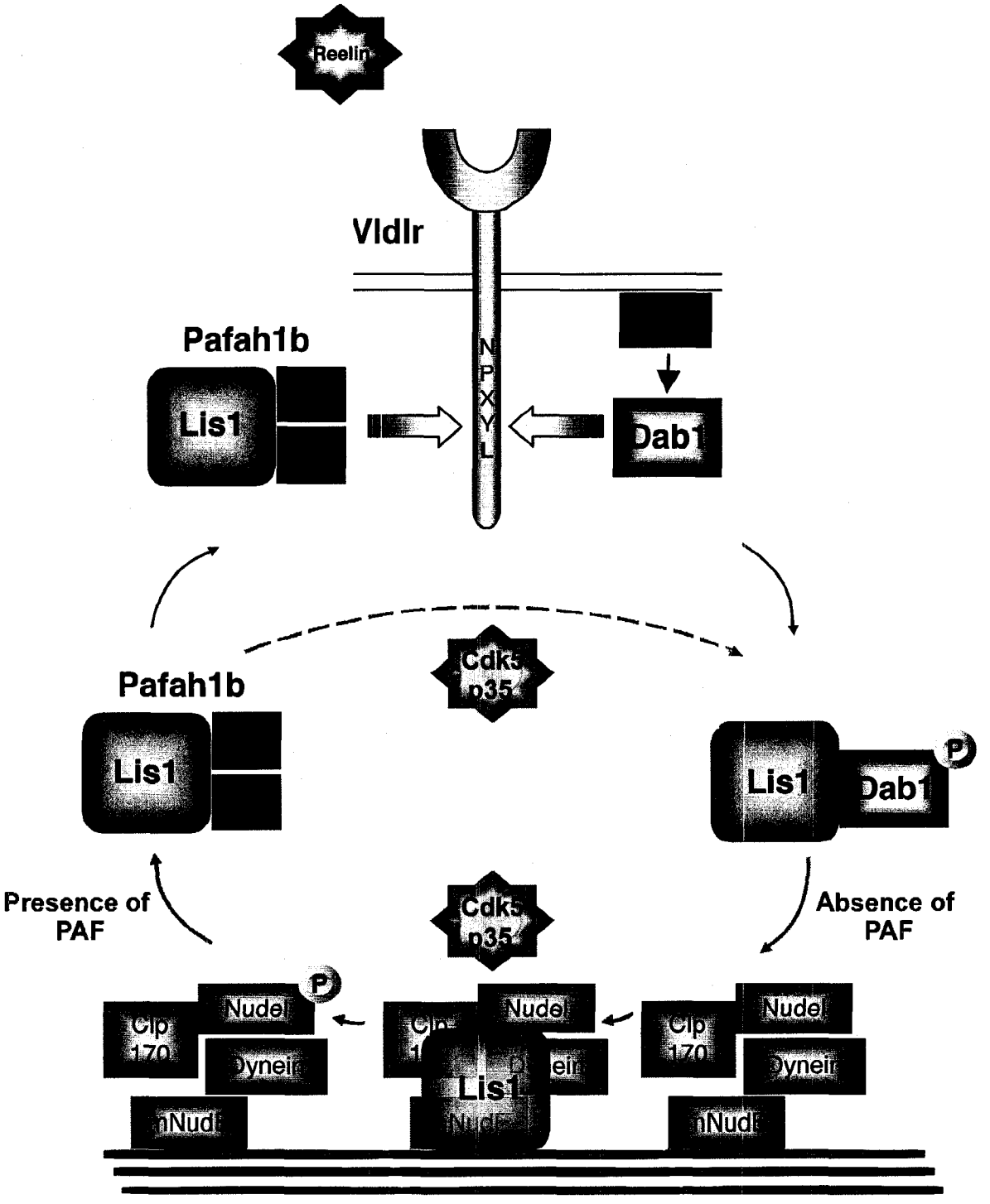
Reelin
VldIr
Pafah1b
Lis1
NPXYL
Dab1
Cdk5
p35
Pafah1b
Lis1
Lis1
Dab1
P
Presence of
PAF
Cdk5
p35
Absence of
PAF
Nudel
P
Clip
170
Dynein
Lis1
Nudel
Clip
170
Dynein
nNudl
Nudel
Clip
170
Dynein
nNudl

Figure 6.1

binds LIS1 returning it to the dynein complex. Further understanding of the regulation of LIS1 translocation is essential if the PAFR-independent death pathway is to lead to a new means of disease intervention. This theory, of course, requires empirical validation and future studies should investigate the kinetics of DAB phosphorylation following PAF challenge.

Functional mutation of the LIS1 gene results in a cortical layering disorder known as Miller Dieker Lisencephaly Syndrome (Reiner et al., 1993). This disorder is characterized by microcephaly and a thickened cortex with 4 rather than 6 layers (Dobyns et al., 1991; Miller, 1963). While point mutations in the LIS1 gene have been identified that underlie the defects in cortical layering (Reiner et al., 1993) other symptoms of the syndrome are believed to be the result of deletions distal to the LIS1 gene on the same chromosome (17p) (Dobyns et al., 1983; Toyo-oka et al., 2003). The "Reeler" animal model of this disorder results from a functional mutation in the reelin gene product localized to chromosome 5p (Landrieu and Goffinet, 1981). No previous link to clinical Miller Dieker Lisencephaly in humans has been reported as no homologous genes have been identified between chromosome 5p in mice and chromosome 17p in humans (Ledbetter et al., 1989). The model described herein may denote the link between reelin mutations and lisencephaly. Functional reelin mutation may reduce the ability of LIS1 to be shuttled back to the dynein complex following removal by PAF-AH I α_2 association. As such, a novel interpretation of the mechanism underlying Miller Dieker Syndrome may result from these studies.

6.2 The bigger picture: Implications for neurodegenerative disorders

This novel PAFR-independent apoptogenic pathway may underlie cerebral dysfunction in multiple neurodegenerative diseases. Oligomeric Aβ-mediated increases in PLA_2 activity (Kriem et al., 2005) resulting in plasmalogen turnover to ethanolamine PAF (Farooqui and Horrocks, 2001) coupled with pharmacological data on the effects of PAF antagonism in AD (Bate et al., 2004; Farooqui and Horrocks, 2001; Kanfer et al., 1998; Sweet et al., 2002) have implicated PAF as a downstream effector of Aβ neurotoxicity but direct evidence had yet to be provided. In this thesis, in collaboration with members of the Bennett laboratory, I provide direct evidence that the pathogenic C_{16}-PAF isoform accumulates in cortical tissue early in AD using a transgenic mouse model of accelerated Aβ biogenesis and that exposure of human neurons to Aβ elicits a pathogenic rise in the C_{16}-PAF isoform. These findings are recapitulated in our analysis of post-mortem AD tissue. Moreover, I have demonstrated that apoptogenic PAF signalling induces Tau protein hyperphosphorylation in human neurons. This occurs via an ER-stress pathway-mediated induction of Cdk5 and GSK3β kinase activity described above. Aberrant activation of these kinase pathways is believed to underscore the development of Alzheimer's neurofibrillary tangles of which hyperphosphorylated tau protein is a major constituent (Cruz et al., 2003; Noble et al., 2003; Takashima, 2006). Several studies have elucidated that soluble Aβ deposition likely precedes neurofibrillary tangle formation in animal models of AD (Oddo et al., 2007; Oddo et al., 2006a; Oddo et al., 2006b). Thus soluble Aβ-mediated increases in neuronal PAF synthesis may be one of the root causes of subsequent pathology both in terms of Aβ fibrillar plaque formation (Mandal et al., 2004) and neurofibrillary tangle formation. Over the course of these investigations

we have demonstrated that PAF-mediated neuronal apoptosis and PAF-mediated Tau protein hyperphosphorylation can be inhibited though manipulation of PAF signal transduction. Together, these findings provide a novel link between the amyloid hypothesis and tau deposition characteristic of AD amenable to intervention.

In AIDS dementia the efficacy of PAF-antagonists at impeding viral replication coupled with the efficacy of anti-retroviral treatment at antagonizing PAF-mediated apoptosis has also suggested a causal link between PAF signalling and retroviral replication (Serradji et al., 2000; Serradji et al., 2004; Weaver et al., 2005). The ability of LIS1 to translocate from the microtubules to the PAF-AH I complex may also underlie these effects. The HIV-1 viral transcription factor TAT has been shown to interact with LIS1 (Epie et al., 2005). *In vitro* this interaction has been shown to promote viral transcription (Epie et al., 2006), postulated to be due to LIS1-mediated inhibition of protein phosphatase 2A (PP2A) (Epie et al., 2006). In a system where rising intracellular PAF levels are cytotoxic and PAF-AH I activity thus essential, the TAT protein may sequester LIS1 mobilized in response to PAF, thus promoting viral transcription while preventing the cell from neutralizing the impending threat. I speculate that LIS1-mediated PP2A inhibition may function to maintain phosphorylation of microtubule associated proteins such as Nudel and thus allow more LIS1 to be recruited by TAT. Cytoskeletal proteins have previously been reported to be hijacked by viral proteins following HIV-1 infection to allow the virus to navigate the cell without impediment by cytoplasm, organelles or other cellular constituents (Matarrese and Malorni, 2005; Naghavi and Goff, 2007). As such LIS1 interaction with TAT may allow HIV-1 genes to replicate and help translated proteins navigate for viral assembly and exit from the cell. While HIV-1 has not been reported to

infect neurons, perivascular macrophages, microglia, and astrocytes are susceptible (Power, 2001). Infected macrophages and microglia secrete significant amounts of PAF and PAF metabolites throughout the brain (Gelbard et al., 1994; Genis et al., 1992). Furthermore, astrocytes function to regulate and remove cerebral toxins, such as PAF (Kentroti et al., 1991; Ransom et al., 2003). In this function, astrocytes maintain homeostatic balance and have been shown to be protective against neuronal loss (Heales et al., 2004). Infection of astrocytes by HIV-1 (and hijacking of the LIS1 machinery) would prevent astrocytes from metabolizing PAF, thus further promoting rather than preventing disease pathology.

6.3 Future Directions: Lipidomics of Neurodegenerative Disease

When evaluating the impact of lipid signalling on neuronal viability in neurodegenerative disease one must be aware of the changes that may occur in all potential bioactive lipid species. Analysis from this perspective, however, is exceedingly difficult. Nonetheless, the potential gains from a broad scale lipidomic approach to neurodegeneration are vast. Analyses such as those conducted in this thesis manuscript can only be fully interpreted when the impact of rising intracellular PAF levels on inter-related signalling lipids and their targets are known. Thus we may conclude that PAF is apoptogenic in neurons devoid of PAFR and that PAF initiates the caspase cascade, however, we cannot exclude other bioactive lipid mediators as effectors of PAF toxicity. Analysis of the impact of extracellular PAF and its metabolite *lyso*-PAF on steady state levels of bioactive lipids in neurons, with particular focus on other families of lipid second messengers such as phosphatidic acid, DAG, LPA and inositol phospholipids, would be an

important next step if we hope to manipulate lipid metabolism therapeutically. Using a transgenic mouse model of AD and post-mortem AD tissue, I provide proof of principle that a lipidomic approach can be used to quantify glycerophospholipid aberrations, showing conclusively that elevated PAF levels are associated with neurodegenerative disease. Thus, a wider analysis compiling the impact of discrete metabolic changes on overall lipid metabolism may lead to new therapeutic interventions. With continuous advances in lipidomic technology, challenges associated with identifying, quantifying, and tracking individual bioactive lipid species within complex backgrounds of several thousand lipids will no doubt soon be addressed.

From a hypothesis-driven perspective, a crucial first step in this approach will be determining whether the *in vivo* neuroprotective effects of PAFR from C_{16}-PAF toxicity outweigh the PAFR-mediated toxic effect of C_{18}-PAF in disorders associated with elevated PAF levels. Transgenic animal models can be used to address this issue. Histological analysis coupled with lipidomic profiling could determine whether changing C_{16}-PAF and C_{18}-PAF levels in experimental models of AD progression such as the TgCRND8 mice used in this study can be correlated with disease pathology. A subsequent *in* vivo approach could be used to directly test the significance of findings described in this thesis. I hypothesize that crossing Alzheimer's transgenic mice with PAFR transgenic mice will protect from neuronal loss, if C_{16}-PAF is the predominant pathological stressor following Aβ deposition, while PAFR overexpression would exacerbate neuronal loss if steady state levels of C_{18}-PAF are increased. Conversely, TgCRND8 x $PAFR^{-/-}$ mice will likely display enhanced neuronal pathology as they would be susceptible to all PAF species. Finally, TgCRND8 x PAF-AH I α_2 transgenic

mice should neutralize steady state changes in all PAF species thus making them resistant to Alzheimer's pathology. This double transgenic approach would enable us to determine 1) whether PAF is an effector of Aβ *in vivo* through assessment of neuronal loss 2) whether PAF enhances Aβ aggregation *in vivo* through comparison of non-aggregated verses fibrillar Aβ 3) whether PAF enhances neurofibrillary tangle formation *in vivo*, and 4) whether preventing either PAFR-dependent or PAFR-independent PAF signalling by enhancing PAF hydrolysis improves overall Alzheimer's pathology. On a molecular level, further investigation is required to determine how aberrant metabolism of glycerophospholipids and generation of specific pathogenic isoforms results in accumulation of unfolded proteins and initiation the of ER-stress response. PAF has been reported to halt amino acid uptake and protein translation in skeletal muscle (Buripakdi and Karlstad, 1994). While the precise mechanism of PAF mediated inhibition of protein translation is unknown, PAF mediated oxidation of amino acids has been implicated (Karlstad et al., 2000). Reduced amino acid availability has been shown to induce UPR (Novoa et al., 2003), however, this theory requires validation with respect to how PAF activates UPR. Nonetheless, this is the first demonstration that rising intracellular concentrations of this bioactive lipid mediator specifically impacts on ER function.

6.4 Summary

In summary, this thesis has delineated a novel PAF-mediated pathway of PAFR-independent apoptosis in human neurons, relevant to the progression of neurodegenerative disease. Furthermore, we demonstrate that neurotoxic PAF-signalling can be inhibited by altering lipid metabolism and, as a result, that bioactive lipid signalling can be

targeted for neurodegenerative disease intervention. Further investigation should delineate whether PAFR-independent signalling can be used to reduce disease pathology and improve behavioural outcome *in vivo* in experimental models of neurodegeneration.

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Yamaguchi, N., Koizumi, H., Aoki, J., Natori, Y., Nishikawa, K., Natori, Y., Takanezawa, Y., and Arai, H. (2007). Type I platelet-activating factor acetylhydrolase catalytic subunits over-expression induces pleiomorphic nuclei and centrosome amplification. Genes Cells *12*, 1153-1161.

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Appendix 1: Curriculem Vitae

RYAN, Scott D

DEGREES

Ph D (Biochemistry),
University of Ottawa, ON, Canada, (Thesis Submitted April 2008)

Thesis: Molecular mechanisms underlying platelet activating factor-mediated neuronal death
Supervisor: Dr. Steffany Bennett

B. Sc. Honours (Biochemistry/Nutrition),
Memorial University of Newfoundland, St. John's, NL, Canada, 2003

Thesis: Effects Of Various Fatty Acids on Gene Expression
Supervisor: Dr. Sukhinder Kaur

TEACHING EXPERIENCE

2007 Invited Lecturer- Biochemistry of Disease (BCH 4009)
Department of Biochemistry, Carelton University, Ottawa, ON
Course Coordinator: Dr. Shawn Whitehead

2007 Teaching Assistant- Introduction to Biochemitry (BCH 2333)
Department of Biochemistry, Microbiology and Immunology, University of Ottawa, Ottawa, ON
Course Coordinator: Dr. Vasek Mezl

2004 Teaching Assistant- Cellular Regulation (BCH 4125)
Department of Biochemistry, Microbiology and Immunology, University of Ottawa, Ottawa, ON
Course Coordinator: Dr. Doug J. Franks

2002 Student Research Assistant
Department of Biochemistry, Memorial University of Newfoundland, St. John's, Newfoundland And Labrador
Supervisor: Dr. Sukhinder Kaur

2001 Instructor

Future Set (Scientists Engineers And Technologists), Association Of Professional Engineers And Geoscientists Of Newfoundland, St. John's, Newfoundland And Labrador
Supervisor: Adam Brown

RESEARCH HONOURS

2006-2008	Ontario Mental Health Foundation, Studentship Awardee
2006-2008	University of Ottawa, Excellence Scholarship Awardee
2005	Dalhousie University, Neuroscience Competition Awardee
2005	University of Calgary, National Neuroscience Competition Awardee
2003-2005	Neuroregeneration Laboratory (NRL), Research Funding Award
2003	Ontario Mental Heath Foundation, Fundable Proposal
2003	Biochemistry, Microbiology and Immunology, Graduate Student Award of Excellence (Masters)

SCHOLARLY AND PROFESSIONAL ACTIVITIES

Professional Invitations

2006	Invited Reviewer, Journal of Life Science
2005	Invited Speaker, Biochemistry Undergraduate Recruitment, "Honours in Biochemistry" University of Ottawa (Oct 2005)
	Invited Evaluator, Summer Studentship Medical Research Forum, Faculty of Medicine, University of Ottawa (Sept 2005)
2004	Invited Speaker, Biochemistry Undergraduate Recruitment, "Honours in Biochemistry" University of Ottawa, Honours Day (Jan 2004)

Seminar and Poster Presentations

2007	Platelet activating factor-induced neuronal apoptosis is initiated independently of its G-protein coupled PAF receptor and is inhibited by the benzoate orsellinic acid. The 3rd International Conference On Phospholipases A2 And Lipid Mediators. Sorrento, Italy
2006	LIS1 binding, a life or death situation. Institute for Systems Biology Mini-symposium. Ottawa, Canada
2005	Neuroprotective properties of platelet activating factor acetylhydrolase I over the course of in vitro neuronal differentiation: rodent verses human

comparison. Society for Neuroscience 35rd Annual Meeting. Washington D.C., USA

2005 PAF-AH and Neuronal Death, University of Ottawa Seminar Series, Ottawa Canada

2004 Cytoprotective properties of platelet activating factor acetylhydrolase 1B in the absence of platelet activating factor receptor. Joint Conference on PAF and PLA2. Berlin, Germany

2003 Cytosolic platelet activating factor acetylhydrolases I and II protect PC12 cells from apoptotic loss. Society for Neuroscience 33rd Annual Meeting. New Orleans, USA

Cytosolic platelet activating factor acetylhydrolases I and II protect PC12 cells from apoptotic loss. Neuroscience Day, NRC-Institute for biological Sciences. Ottawa, Canada

Cytosolic platelet activating factor acetylhydrolases I and II protect PC12 cells from apoptotic loss. University of Ottawa, BMI Poster Day. Ottawa, Canada

Medical Research Advocacy

2005-Present Participating Neuroscientist, Neuroscientists/Teachers Partners Program, Society for Neuroscience

2006 Organizational Assistant, Progress in Systems Biology 2006, Ottawa Institute for Systems Biology in collaboration with the National Research Council of Canada, Ottawa ON (November 2006)

2004 Profiled as a member of the University of Ottawa Research Community at the Canadian Celebration of excellence in Health Research presented by the Canadian Institute of Health Research, Health Charities Council of Canada and the Council for Health research in Canada (March 4, 2004)

Organization Assistant, Canadian Celebration of Excellence in Health Research presented by the Canadian Institute of Health Research, Health Charities Council of Canada and the Council for Health research in Canada (March 4, 2004)

2003 Research laboratory profiled in "Berry Important Questions; Blueberries as protector of Brain Cells?" by Holly Lake, The Ottawa Sun (Oct 4, 2003)

Membership on Professional Societies

Society for Neuroscience
International Brain Research Organization
Canadian Society of Neuroscience

Professional Certifications

2004	WHIMIS
2003	Biohazard Safety
2003	Laboratory Safety
2002	Radioisotope Safety

Other Academic Activities

2007-2008	Work in Progress Seminar Series (Biochemistry), Member
2003-2007	Graduate Student Seminar Series (Biochemistry), Member
2003-2007	Biochemistry Undergraduate Mentor Program, Mentor to Fan Mo (B.Sc Honours) and Serena Wong (B. Sc. Honours), André Bourgouis (B. Sc. Honours)
2003-2004	Coordination Assistant, Departmental Seminar Series (Biochemistry)

PUBLICATIONS

Published Manuscripts:

Ryan SD, Harris CS, Carswell CL, Baenziger JE, Bennett SA. (2008) Heterogeneity in the *sn*-1 carbon chain of platelet activating factor glycerophospholipids determines pro-or anti-apoptotic signaling in primary neurons. *J Lipid Res.* doi:10.1194/jlr.M800263-JLR200

Scott D. Ryan, Cory S. Harris, Fan Mo, Haemi Lee, Sheng T. Hou, Nicolas G. Bazan, Pierre S. Haddad, John T. Arnason, Steffany A.L. Bennett. (2007) Platelet activating factor-induced neuronal apoptosis is initiated independently of its G-protein coupled PAF receptor and is inhibited by the benzoate orsellinic acid. J Neuro Chem. Oct; 103(1):88-97

Bonin F, Ryan SD, Migahed L, Mo F, Lallier J, Franks DJ, Arai H, Bennett SAL (2004) Anti-apoptotic actions of the platelet activating factor acetylhydrolase I α_2 catalytic subunit. J Biol Chem. 10;279(50):52425-36

Submitted Manuscripts:

Ryan SD, Moffat TC, Bourgeois AJG, Rashidian J, Park DS, Bennett SAL. Aberrant lipid metabolism compromises neuronal viability in Alzheimer disease. Submitted to PLoS Biology

Abstracts:

Scott D. Ryan, Cory S. Harris, Fan Mo, Haemi Lee, Sheng T. Hou, Nicolas G. Bazan, Pierre S. Haddad, John T. Arnason, Steffany A.L. Bennett. (2007) Platelet activating factor-induced neuronal apoptosis is initiated independently of its G-protein coupled PAF receptor and is inhibited by the benzoate orsellinic acid. The 3rd International Conference On Phospholipases A2 And Lipid Mediators. Sorrento, Italy

S. D. Ryan, T. C. Moffat and S. A. L. (2005) Neuroprotective properties of platelet activating factor acetylhydrolase I over the course of in vitro neuronal differentiation: rodent verses human comparison. Society for Neuroscience 35rd Annual Meeting. Washington D.C., USA

Ryan SD, Mo F, Migahed L, Bennett SAL (2004) Cytoprotective prperties of platelet activating factor acetylhydrolase 1B in the absence of platelet activating factor receptor. Joint Conference on PAF and PLA2. Berlin, Germany.

Ryan SD, Mo F, Migahed L, Bennett SAL (2004) Cytoprotective prperties of platelet activating factor acetylhydrolase 1B in the absence of platelet activating factor receptor. Society for Neuroscience 34th Annual Meeting. (San Diego, USA, poster presentation by SD Ryan)

Moffat TC, Ryan SD, Hill J, Bennett SAL (2004) Platelet activating factor acts downstream of β-amyloid and can be targeted to reduce neuronal loss-implication for Alzheimer disease. Joint Conference on PAF and PLA2. (Berlin, Germany, oral presentation by SAL Bennett)

Ryan SD, Bonin F, Moffat TC, Bennett SAL (2003) Cytosolic platelet activating factor acetylhydrolases I and II protect PC12 cells from apoptotic loss. Society for Neuroscience 33rd Annual Meeting. (New Orleans, USA, poster presentation by SD Ryan)

Bennett SA, Melanso-Drapeau L, Davé S, Ryan SD, Hebb ALO (2003) Cyclic Fluctuations in Progenitor Proliferation Across the Estrous Cycle are Mediated by Connexin 32. Society for Neuroscience 33rd Annual Meeting. (New Orleans, USA, poster presentation by SAL Bennett)