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CYTOPROTECTIVE EFFECTS OF INTRACELLULAR PLATELET ACTIVATING FACTOR ACETYLHYDROLASES

Fanny Bonin

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THESIS ABSTRACT

Platelet activating factor (PAF) is a biologically active phospholipid implicated in the developmental brain disorder Miller-Dieker Syndrome (MDS) and purported to be a primary mediator of cell death in HIV-dementia, ischemia, and epilepsy. As part of my honour's thesis, I demonstrated that PAF can elicit cell death independently of its G-protein coupled receptor (PAFR) in PC12 cells. In my M.Sc. research, I have sought to identify how PAF-mediated cell death is regulated in PC12 cells. PAF is inactivated in brain by two intracellular PAF-acetylhydrolases (PAF-AHs): PAF-AH I and PAF-AH II. PAF-AH I is a trimeric complex composed of two catalytic subunits (α1 and α2) and one regulatory subunit (β). Mutations in the *Lis1* gene, coding for the β subunit of PAF-AH I, are the genetic determinant of MDS. However, it is not clear whether these mutations impact on PAF-AH I enzymatic activity in MDS. Furthermore, it is not known whether cytosolic PAF-AH activity regulates the kinetics of neuronal loss following pathophysiological challenge. To begin to address these questions, I sought to identify an *in vitro* model system suitable for study of PAF-AH activity. In my first series of experiments, I have shown that the kinetics of PAF-induced cell death is regulated by functional PAF-AHs, identified for the first time in PC12 cells. Using RT-PCR, it was demonstrated that PC12 cells and rat primary cultures express PAF-AH I (α1, α2 and *Lis1*) and PAF-AH II transcripts. Because the rat PAF-AH II gene had not been cloned, I amplified and sequenced full-length PAF-AH II cDNA using primer pairs homologous to the mouse and human genes (Sequence accession number: AY225592). By enzymatic assay

and thin layer chromatography (TLC), I have demonstrated that PAF is internalized in PC12 cells in the absence of the known PAFR and that intracellular PAF-AHs rapidly inactivate PAF to *lyso*-PAF in PC12 cells. The inactive metabolite *lyso*-PAF is subsequently released into the extracellular space and is apparently not reinternalized by the cells. The serine proteases inhibitor diisopropyl fluorophosphate (DFP) has been used at 0.1 mM and 1 mM to inhibit PAF-AH I and PAF-AH II respectively. Results obtained from these experiments suggested that both isoforms are functional in PC12 cells. Moreover, I show, for the first time, that the gliotoxin derivative FR 49175, accelerates cytosolic PAH-AH activity and protects PC12 cells from PAF-mediated cell death. To address the question of whether LIS1 mutation affects PAF-AH I enzymatic activity, I engineered two adenoviral constructs to overexpress functional LIS1 or mutant LIS1. These constructs were packaged by the Adenoviral Core Facility (Lab of Dr. Ruth Slack, University of Ottawa). Virion will be used in the future to determine the effect of LIS1 and mutant LIS1 overexpression on death susceptibility to PAF in our *in vitro* neuronal and PC12 cell models.

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Je veux finalement remercier ma famille pour avoir cru en moi depuis le tout début, et pour m'avoir donné tous les outils afin de réaliser mes rêves.

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LIST OF ABBREVIATIONS

ACF	artificial cerebrospinal fluid
AD	Alzheimer's disease
ANOVA	analysis of variance
AraC	Cytosine b-D-arabinofuranoside
B-*lyso*-PAF	Bodipy-*lyso*-platelet activating factor
B-PAF	Bodipy platelet activating factor
bp	base pair
BSA	bovine serum albumin
C-terminus	carboxy-terminus
Ca^{2+}	calcium
cDNA	complementary deoxyribonucleic acid
CMV	cytomegalovirus
CNS	central nervous system
ConA	concanavalin A
DFP	diisopropyl fluorophosphate
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
DTNB	5,5'-dithiobis (2-nitrobenzoic acid)
E19	embryonic day 19
EDTA	ethylenediaminetetra-acetic acid
EtOH	ethanol
FCS	fetal calf serum
FudR	5-fluoro-2'-deoxyuridine
GAPDH	Glyceraldehyde-3'-phosphate dehydrogenase
HIV	human immunodeficiency virus
HS	horse serum
LTP	long-term potentiation
MAPK	mitogen-activated protein kinase
mc-PAF	methyl-carbamyl platelet activating factor
MDS	Miller-Dieker Syndrome
mRNA	messenger ribonucleic acid
N-terminus	amino-terminus
NCS	newborn calf serum
NF-H_2O	nuclease free double-distilled water
NGF	nerve-growth factor
P6-10	postnatal day 6-10
PAF	platelet activating factor
PAF-AH	platelet activating factor-acetylhydrolase
PAFR	platelet activating factor receptor
PBS	phosphate buffered saline
PKC	protein kinase C
PLA_2	phospholipase A_2
PMNL	polymorphonuclear leukocytes
PMSF	phenylmethylsulfonylfluoride

ROS	reactive oxygen species
rPAF-AH	recombinant platelet activating factor-acetylhydrolase
RT-PCR	reverse transcriptase polymerase chain reaction
$t_{1/2}$	Half-life
TLC	thin layer chromatography
UV	ultraviolet
WT	wild-type

CHAPTER 1: GENERAL INTRODUCTION

1.1 Platelet Activating Factor: Overview

In the early seventies, Benveniste *et al.* isolated a soluble substance that they named platelet activating factor (PAF; 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine), reflecting the capacity of this lipid autacoid to elicit aggregation of rabbit platelets when released from basophils (Benveniste et al., 1972). Since this discovery, it has been established that PAF is not a single entity but is composed of a family of *sn*-2 acetylated, choline-containing phosphoglycerides that are structurally related, but differ markedly in biological action and potency (Pinckard et al., 1994). Based upon the chemical linkage at the *sn*-1 position, the PAF family is separated into three subclasses: alkyl-, alkenyl- and acyl-PAF. The majority of PAF research has focused primarily on the most potent (and predominant) molecular species of PAF (16:0- and 18:0-alkyl-PAF)[1] (Maclennan et al., 1996).

PAF phospholipids possess a wide spectrum of potent proinflammatory actions and other biologically relevant actions (for a review, see (Prescott et al., 2000)). As a result, the designation PAF is something of a misnomer given that this bioactive phospholipid can be synthesized by, and acts upon, diverse cell types, including polymorphonuclear leukocytes (PMNLs), mast cells, monocytes/macrophages, vascular endothelial cells, lymphocytes, microglia, and neurons (McManus and Pinckard, 2000). The majority of PAF-mediated effects

[1] In this thesis, I will refer to $C_{16}H_{33}$ and $C_{18}H_{35}$ alkyl PAF using the family name PAF unless specifically stated.

are recognized to be receptor-mediated although PAF can also be metabolized to other bioactive phospholipid mediators (for a review see Ishii and Shimizu, 2000). In fact, PAF was the first lipid molecule for which a protein receptor was identified (Honda et al., 1991). The PAF receptor (PAFR) is highly conserved between species and it is a member of the seven transmembrane G-protein coupled receptor family (Honda et al., 1991; Bito et al., 1994). Binding of PAF to PAFR mediates turnover of phosphatidylinositols, elevations in intracellular calcium (Ca^{2+}) concentration, and activation of many kinases, e.g., protein kinase C (PKC), and mitogen-activated protein kinase (MAPK) (for review see (Ishii et al., 2002)). In addition to intercellular signaling, it has been proposed that PAF has intracrine effects, acting as a second messenger mobilized in response to glutamate, and acting on one or more intracellular receptors (for a review see (Prescott et al., 2000) (Bazan, 1998). Intracellular binding sites for PAF have been pharmacologically characterized in rat brain cortical lysates (Marcheselli et al., 1990) and human neutrophils (Svetlov and Nigam, 1993) but have yet to be identified at the molecular level and have yet to be rigorously characterized.

Several groups have examined the transfer of PAF into cells, a transmembrane process referred to as internalization. However, the conclusions regarding the role of the PAF receptor have been somewhat conflicting. PAF internalization has been shown to be accelerated by expression of PAFR, demonstrating that the PAF/PAFR ligand complex is rapidly internalized (Ohshima et al., 2002). However, many other reports support the concept that the cellular processing of PAF, at least under some conditions, can also occur

independently of a receptor-ligand pathway. Thus, it has been suggested that PAF signaling can be initiated following the internalization of PAF by transbilayer movement (flipping) across the plasma membrane, through passive incorporation of PAF into the lipid bilayer, or through active transport of PAF across the plasma membrane by a PAF-specific transglutaminase (Bratton et al., 1992; Ishii and Shimizu, 2000). Moreover, intracellular PAF levels can be elevated by active synthesis in response to stimulation (Francescangeli et al., 2002). The concentrations of PAF required to elicit biological responses are significantly less (1 pM to 1 μM) than its critical micelle concentration (2.5 to 3 μM) (Blank et al., 1981) and thus, PAF would exist in the aqueous cell cytosol unless it is compartmentalized or located in the lipid bilayer of a membrane. As indicated above, PAF can act at picomolar concentrations and is now believed to have a multitude of physiological and pathological functions (for reviews, see (Braquet et al., 1987; Venable et al., 1993)). Pathological concentrations of 100 nM to 1 μM have been shown to elicit apoptotic death (Brewer et al. 2002, Bonin et al., submitted). Exposure to higher concentration of PAF (3.5 to 10 μM) is cytotoxic (Kornecki and Ehrlich, 1988) and elicits necrotic cell lysis by disrupting the plasma membrane (Brewer et al., 2002).

1.2 PAF and its role in the central nervous system

At the first international PAF conference in Paris (1984), it was asked whether there was any possible neurologic involvement for PAF: there was no answer (Bussolino et al., 1995). In 2003, numerous studies have focused on

elucidating the role of PAF in the central nervous system (CNS) as a potential therapeutic target for numerous neurodegenerative conditions. The involvement of PAF in cellular signaling in the nervous tissue is further supported by the presence of receptors located in plasma and intracellular membranes (Marcheselli et al., 1990) and by the generation of second messengers induced by PAF in nerve cells (Feurstein et al., 1990; Kornecki and Ehrlich, 1991). PAF production has been demonstrated in cultured rat cerebellar granule cells (Yue et al., 1990), human fetal neurons and glial cells in culture (Sogos et al., 1990; Jaranowska et al., 1995). Cells of the nervous system are both a target for and a source of PAF, and this lipid mediator serves as a communication signal within the CNS (for review see (Prescott et al., 2000)). In brain, PAF serves as a critical mediator in diverse biological processes. It induces Ca^{2+} mobilization (Kornecki and Ehrlich, 1991). It modulates the levels of a variety of neuropeptides (Rougeot et al., 1990) and controls cerebral blood flow (Kochanek et al., 1988). The involvement of PAF in long-term potentiation (LTP), a long-lasting increase in synaptic efficacy following brief high-frequency stimulation of a monosynaptic pathway, suggests that PAF also acts as a retrograde neurotransmitter in memory formation (Kato et al., 1994; Kornecki et al., 1996; Chen et al., 2001). However, in some pathological conditions, elevated levels of PAF accumulate in the brain resulting in neuronal injury (Pulliam et al., 1998). It has been repeatedly demonstrated that diverse neuropathologies are associated with sustained PAF exposure in the brain (for a review see (Maclennan et al., 1996)).

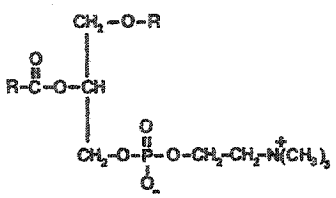
In the adult brain, elevated levels of PAF are associated with neuronal death following epileptiform seizure and ischemia (Kumar et al., 1988; Braquet et al., 1989; Bazan et al., 1991; Domingo et al., 1994; Shmueli et al., 1999) and with the pathogenesis of neurodegenerative diseases such as human immunodeficiency virus (HIV)-associated dementia (Gelbard et al., 1994; Perry et al., 1998). It is also postulated that PAF may play a role in Alzheimer's disease (AD) (Hershkowitz and Adunsky, 1996), and unpublished data from our laboratory suggests that PAF may act as a second messenger transducing β-amyloid-induced neurotoxic signals (Tia Moffat, Ph.D thesis, in progress). In the developing brain, nerve cell development encompasses proliferation, differentiation and maturation, including outgrowth of cellular processes and formation of cellular contacts (Ved et al., 1991; Francescangeli et al., 1997; Dichmann et al., 2000). Sustained PAF exposure *in vitro* has been shown to elicit neuronal growth cone collapse (Clark et al., 1995) and inhibit neuronal motility (Adachi et al., 1997; Bix and Clark, 1998).

1.3 PAF metabolism

There are several mechanisms that individually or together can control the biological activity of PAF under physiological and pathophysiological conditions. These include tightly controlled synthetic pathways and efficient PAF degradative enzymes that are constitutively present in most PAF-responsive cells (see Fig.1.1).

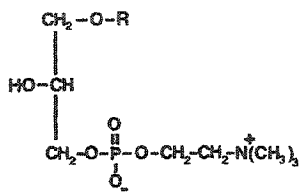
Figure 1.1: Pathways of PAF biosynthesis (remodeling and *de novo*) and degradation (for comments see text).

Remodeling



1-*O*-alkyl-2-acyl-*sn*-glycero-3-phosphocholine

phospholipase A2 ↓

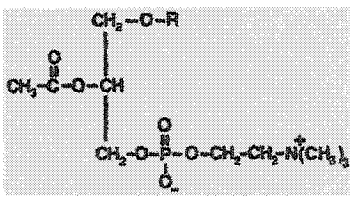


1-*O*-alkyl-2-lyso-*sn*-glycero-3-phosphocholine
(lyso-PAF)

Catabolism

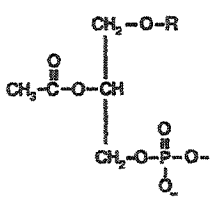
PAF acetylhydrolase (PAF-AH)

lyso-PAF acetyltransferase ↓



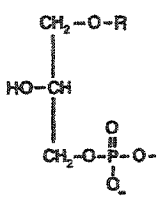
1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine
(PAF)

phosphocholinetransferase
phosphohydrolase ↑



1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphate

acetyltransferase ↑



1-*O*-alkyl-2-lyso-*sn*-glycero-3-phosphate

De novo

1.3.1 Anabolism

PAF can be synthesized via two distinct pathways: the *de novo* pathway and the remodeling pathway, both of which have been reviewed extensively (Snyder, 1994, 1995). The *de novo* pathway is a three-step process that starts with 1-*O*-alkyl-2-lyso-*sn*-glycero-3-phosphate, which is acetylated by an acetyltransferase to form 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphate. Following the removal of the phosphate by a phosphohydrolase, choline-P is added by a phosphocholinesterase to form PAF (Francescangeli et al., 1997). Alternatively, the synthesis of PAF via the remodeling pathway requires the conversion of 1-*O*-alkyl-2-acyl-*sn*-glycero-3-phosphocholine by phospholipase A_2 (PLA_2), into 1-*O*-alkyl-2-lyso-sn-glycero-3-phosphocholine (lyso-PAF), the immediate precursor of PAF. The final step is catalyzed by *lyso*-PAF acetyltransferase, and involves the addition of an acetate to the *lyso*-PAF molecule to form PAF (Baker, 1995; Maclennan et al., 1996). It has been proposed that the remodeling route plays a crucial role in generating PAF following pathophysiological challenge, whereas *de novo* synthesis appears to be of more physiological importance in that it is thought to maintain the resting state levels of PAF in various tissues and blood (Francescangeli et al., 1996).

1.3.2 Catabolism

As mentioned above, the final molecular composition of PAF in the tissues and its biological activity also depend on the activation of catabolic pathways. It has been proposed that the extracellular and intracellular levels of PAF are

mainly regulated by its inactivation, catalyzed by PAF-acetylhydrolases (PAF-AHs), which remove the acetyl group from the *sn*-2 position of the glycerol backbone of PAF (Stafforini et al., 1997). This hydrolytic activity from PAF-AHs produces *lyso*-PAF, a lipid that lacks the biological activities associated with PAF (Baker, 2000). As categorized by enzymatic activity, PAF-AHs are isozymes with a Ca^{2+}-independent PLA_2 activity (Kudo and Murakami, 2002). PAF-AHs only act on a unique type of phospholipids with an ether linkage at the *sn*-1 position, that are characterized as either biologically active or toxic. Consistent with the ubiquitous distribution of PAF and PAF-like lipids, PAF-AHs enzymes are present in plasma (Farr et al., 1980) and in most tissues (Blank et al., 1981), including brain (Hattori et al., 1993). Three isoforms have been cloned: plasma PAF-AH, cytosolic PAF-AH II, and cytosolic PAF-AH I (for a review see (Derewenda and Ho, 1999)). Within the family of PAF-AHs, there is a range of substrate specificities. Plasma PAF-AH and intracellular PAF-AH II can hydrolyze PAF analogues with *sn*-2 propionyl and butyroyl moieties (Stremler et al., 1991; Hattori et al., 1995a), while PAF-AH I minimally hydrolyzes these phospholipids (Hattori et al., 1993). Intracellular PAF-AH I is the only member that has an absolute requirement for acetate at the *sn*-2 position and, therefore, is specific for PAF (Hattori et al., 1993). Intracellular PAF-AHs have been shown to be inhibited by 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB), phenylmethylsulfonylfluoride (PMSF), and diisopropyl fluorophosphates (DFP) (Hattori et al., 1995a; Karpouza and Vakirtzi-Lemonias, 1997). In contrast,

purified PAF-AH from human plasma is not affected by any of these treatments (Stafforini et al., 1987).

1.3.2.1 Plasma PAF-AH

The initial studies on PAF-AHs were performed on the secreted form of PAF-AH found in mammalian plasma (e.g. plasma PAF-AH). The enzyme was purified from human plasma in 1987 (Stafforini et al., 1987) and its cDNA was cloned in 1995 (Stafforini et al., 1996a). The 45 kD monomer is the only secreted PAF-AH characterized to date (Tjoelker et al., 1995a). Synthesized by hematopoietic lineage cells (Asano et al., 1999), the isoform circulates in blood as a complex with lipoproteins (Stafforini et al., 1987; Blencowe et al., 1995). Deficiency of extracellular (plasma) PAF-AH is associated with increased severity of asthma (Stafforini et al., 1999), and stroke (Hiramoto et al., 1997). Consistent with this observation, recombinant plasma PAF-AH (rPAF-AH) may have therapeutic benefits to patients with allergic airway inflammation (Henderson et al., 2000). In fact, plasma PAF-AH prevents not only inflammatory conditions induced by administration of exogenous PAF but also diseases in which the production of PAF and/or oxidized phospholipids is suspected to occur (for a review see (Stafforini et al., 1997)). It is known that the extracellular PAF-AH transcript is expressed in thymus, tonsil and placenta, but not in heart, kidney or cerebral cortex, despite the fact that expression is detected in other areas of the brain presumably by endothelial cells or hematopoetic cells circulating in brain capillary (Tjoelker et al., 1995b). However, it is not known whether the plasma form of PAF-AH is responsible for regulating the amount of PAF in the brain

since messenger RNA (mRNA) for intracellular PAF-AHs is present in specific brain regions but has yet to be localized to CNS-specific cell types (Hattori et al., 1994a; Hattori et al., 1995b).

1.3.2.2 PAF-AH II

It has been found that a bovine brain soluble fraction contains at least two types of PAF-AHs, namely PAF-AH I and PAF-AH II (Hattori et al., 1993). PAF-AH II is a monomeric polypeptide that was initially purified from the bovine liver cytosol and that shares 42% identity with the extracellular plasma PAF-AH (Hattori et al., 1995a). Isoform II is the predominant intracellular isoform expressed by most tissues including brain (Hattori et al., 1996). PAF-AH II is the only isoform that possess transacetylase activity, meaning that it can transfer an acetyl group from PAF to acceptor lipids such as lysophospholipids and sphingosine (Bae et al., 2000). This enzyme translocates to the membrane during oxidative stress, and inhibits oxidative stress-induced apoptosis (Matsuzaya et al., 1997). As a result, PAF-AH II may scavenge oxidized phospholipids, which are thought to be involved in diverse pathological processes, including disorganization of membrane structure and PAF-like proinflammatory actions (Matsuzaya et al., 1997). Overexpression of PAF-AH II protects against oxidative cytotoxicity (Marques et al., 2002).

1.3.2.3 PAF-AH I

Intracellular type I PAF-AH is an oligomeric phospholipase complex composed of two homologous catalytic subunits, α1 (29 kD) and α2 (30 kD), and a non-catalytic subunit, referred to as β or LIS1 (45 kD). Recent evidence demonstrates that the β subunit likely modulates PAF-AH I enzymatic activity (Shmueli et al., 1999), and that this regulation is dependent on catalytic dimer composition (Manya et al., 1998). The PAF-AH I isoform is commonly referred as PAF-AH Ib or as the brain PAF-AH I isoform in the literature (Ho et al., 1997; Manya et al., 1999). The PAF-AH Ia isoform is composed of the two catalytic α subunits without the β/*Lis1* subunit characterized *in vitro*, but this configuration has not been identified *in vivo*. The expression of the α1 subunit in the newborn brain correlates with period of active neuronal migration (Ishii et al., 1997). α1 expression decreases once neuronal migration is complete. The β subunit is the regulatory subunit of the PAF-AH I complex, and it is suspected to inhibit enzymatic activity of dimers containing the α1 subunit (Manya et al., 1998), which could result in PAF accumulation, coupled with neuronal migration. This suggests that PAF-AH I may play a role in brain development and homeostasis. Conversely, it has been demonstrated that the β subunit stimulates the enzymatic activity of α2/α2 homodimers, which are expressed in the adult brain, where inappropriate PAF accumulation could result in seizures in the absence of adequate PAF hydrolysis (Manya et al., 1998). The importance of the PAF-AH I β subunit has been highlighted by the discovery that it was identical to the product of *Lis1*, the proposed disease-associated gene of a neuronal migration disorder (Reiner et al., 1993; Hattori et al., 1994a).

1.4 LIS1

Known to encode the non-catalytic subunit of the intracellular PAF-AH I isoform, *Lis1* was the first human neuronal migration gene to be cloned (Reiner et al., 1993; Hattori et al., 1994a). It is located on human chromosome 17p13.3 and consists of 11 exons with a coding region of 1233 bp. Point mutations or deletions in one allele of the human *Lis1* gene are the genetic determinant of a severe developmental brain malformation characterized by a classical or type 1 lissencephaly ("smooth brain") (Dobyns et al., 1993). Since LIS1 protein is expressed predominantly in fetal and adult brain (Mizuguchi et al., 1995), cerebral development is severely impaired in lissencephalic children. The specific distribution of neurons within the cerebral cortex is disrupted, a condition that arises from inadequate migration of neuronal progenitors to their cortical destinations (Reiner et al., 1995). Consequently, the cortex is inappropriately layered and underpopulated with neurons. The surface of the brain is smooth rather than folded as it is in normal individuals (Reiner et al., 1995). The one in 30 000 children that are born with this devastating condition do not reach measurable development stages, suffer from recurrent seizures, and often face a reduced life expectancy (Cardoso et al., 2002). Type I lissencephaly occurs either as an isolated abnormality or in association with dysmorphic facial appearance in patients with Miller-Dieker Syndrome (MDS) (Dieker, 1967; Miller, 1973).

In addition to its participation in the PAF-AH I enzymatic complex, the LIS1 protein has been implicated in regulation of microtubule dynamics by virtue of its

ability to bind tubulin and reduce microtubule catastrophe (Sapir et al., 1997; Sapir et al., 1999). It has been assumed that only a proportion of LIS1 molecules in a cell are associated with microtubules while other LIS1 molecules act as the regulatory subunit of PAF-AH I, and these two interactions do not occur simultaneously (Sapir et al., 1999). As reported above, reduced levels of *Lis1* activity in both humans and mice have been shown to result in a defect in neuronal migration (Dobyns et al., 1993; Hirotsune et al., 1998). Using mouse embryos, it has been demonstrated that a half dosage of *Lis1* affects neuronal migration only slightly in the developing cortex, whereas further gene dosage reduction severely obstructs cortical and hippocampal organization (Hirotsune et al., 1998). The complete loss-of-function phenotype of *Lis1* mutations in neurons has not been described, as mice that are homozygous-null for *Lis1* die in early embyrogenesis before formation of the nervous system (Hirotsune et al., 1998).

Interestingly, Clark and colleagues have demonstrated in a yeast two-hybrid system that point mutations found in patients with lissencephaly interfere with the association of LIS1 for the α1 and α2 subunits of PAF-AH I (Sweeney et al., 2000), suggesting that the heterotrimeric PAF-AH I complex may also be involved in regulating migration. Biochemically, the mutant LIS1 protein is not capable of dimerization, and enzymatic activity has been shown to be elevated in heterozygous embryos, demonstrating the *in vivo* role of LIS1 as a subunit of PAF-AH I (Cahana et al., 2001). Further evidence indicates that PAF interferes with binding of LIS1 to the catalytic subunits of PAF-AH I (Ross and Walsh, 2001). The precise role of PAF in neuronal migration is uncertain, although it has

been proposed that PAF affects migrating neurons by influencing cell morphology or adhesion properties (Albrecht et al., 1996). Studies demonstrate that addition of PAF or inhibition of PAF-AH decreases migration of cerebellar granule cells in vitro (Adachi et al., 1997; Bix and Clark, 1998), and addition of PAF to cultured hippocampal neurites produces growth cone collapse, neurite retraction and neurite varicosity formation (Clark et al., 1995). *Lis1* loss of function may also prevent the PAF-AH I complex from recognizing its substrate, thereby leading to abnormal PAF homeostasis during cerebral development, and subsequent impairment of neuronal migration (Clark et al., 1995; Stafforini et al., 1996b). Thus, the role of PAF may be to mobilize the LIS1 protein from PAF-AH I to exert a downstream effect on neuronal migration. Furthermore, identification of PAF-AH I as a non-traditional G-protein like trimer and LIS1 as a microtubule-associated protein family member support the hypothesis that *Lis1* participates in signaling downstream of PAF hydrolysis (Ho et al., 1997; Sapir et al., 1999).

1.5 Objectives

Intervention into PAF signaling pathways may represent a possible adjuvant treatment of neurodegenerative diseases, specifically AD, and neurodevelopmental disorders, specifically lissencephaly. The overall objective is to characterize the enzymatic control of apoptotic signaling initiated by PAF. The specific goals of the present study are to establish whether intracellular PAF-AH I and II activity protect cells from PAF-mediated apoptosis, and to develop tools in order to test the hypothesis that mutant *Lis1* disrupts PAF catabolism,

thereby leading to abnormal levels of active ligand during neurogenesis and migration.

This thesis is divided into the following three experimental chapters designed to address these specific goals:

- Characterization of an *in vitro* model to study PAF-AH activity.
- Characterization of PAF-AH activity in PC12 cells.
- Elaboration of tools to determine the effect of functional *Lis1* and mutant *Lis1* overexpression on PAF-AH activity and apoptotic susceptibility to PAF.

CHAPTER 2: CHARACTERIZATION OF AN *IN VITRO* MODEL TO STUDY PAF-AH ACTIVITY

2.1 Introduction

As reviewed in Chapter 1, PAF is a potent phospholipid serving as a critical mediator in diverse physiological and pathophysiological processes. In concert with the ubiquitous distribution of PAF and PAF-like lipids, PAF-AHs enzymes, responsible for inactivation of PAF to *lyso*-PAF, are found both in blood plasma and cell cytosol. Three isoforms have been cloned: plasma PAF-AH, cytosolic PAF-AH II, and cytosolic PAF-AH I (for a review see (Derewenda and Ho, 1999)). Plasma PAF-AH is expressed and secreted by vascular endothelial and hematopoietic cells (Stafforini et al., 1990). PAF-AH II is the predominant intracellular isoform expressed by most tissues including brain (Hattori et al., 1996). Cytosolic PAF-AH I is a G-protein-like trimeric enzymatic complex composed of homodimer or heterodimer catalytic subunits (α1 and α2), and a regulatory β-subunit (for a review see (Derewenda and Ho, 1999)). Acetylhydrolase activity has been characterized primarily at the biochemical level by *in vitro* assay (Hattori et al., 1994a, b; Hattori et al., 1995b; Tjoelker et al., 1995b; Watanabe et al., 1998), but the role of these enzymes in regulating PAF-mediated biological functions have yet to be elucidated (Stafforini et al., 1997).

As reviewed in Chapter 1, chronic exposure to elevated concentrations of PAF is cytotoxic. While exogenous administration of rPAF-AH or adenoviral-induced overexpression of plasma PAF-AH in primary neurons or vascular

endothelial cells reduces cell loss associated with excitotoxicity and hypercholesterolemia (Ogden et al., 1998; Hirashima et al., 2000; Chen et al., 2003), the roles of cytosolic PAF-AH I and II in regulating the kinetics of PAF-induced cytotoxicity are not known.

2.2 Objectives

The purpose of the present study was to test the hypothesis that cytosolic PAF-AHs represent potential therapeutic targets that limit PAF-mediated cytotoxicity. To achieve this objective, it was essential to identify an *in vitro* model sensitive to PAF that expressed intracellular PAF-AH isoforms, but not plasma PAF-AH. Our laboratory has previously demonstrated that PAF elicits apoptotic loss of PC12 cells (Brewer et al., 2002). As such, the PC12 cell model was chosen as a starting point to test the hypothesis that endogenous PAF-AH activity is cytoprotective. The pattern of PAF-AH isoform gene expression was analyzed by reverse transcriptase polymerase chain reaction (RT-PCR) and Western blot analyses[2]. To determine whether PAF-AH activity functionally limits the kinetics of PAF-mediated cell death, I evaluated cell loss following transient PAF exposure, repeated PAF exposure, or treatment with a PAF-AH resistant PAF analog (mc-PAF: methyl-carbamyl PAF).

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[2] Please note that results from the protein analyses are discussed but not presented in this thesis, since these experiments were performed by Scott Ryan, an M.Sc. candidate in the Bennett's laboratory

2.3 Materials and Methods

Cell culture

PC12-AC cells, a clonal derivate of the PC12 pheochromocytoma cell line (American Tissue Culture Collection) derived in our laboratory, were cultured in complete media composed of RPMI 1640 containing 10% horse serum (HS) and 5% newborn calf serum (NCS) at 37°C in a 5% CO_2/95% air atmosphere. Culture reagents were obtained from Invitrogen. Stock cultures were fed with complete media every 2-3 days and passaged when sub-confluent.

Cell death assays

PC12-AC cells (8800 cells/cm^2) were plated overnight in complete media in 6 cm diameter tissue culture plates (VWR). Cells were treated in serum-free media containing 0.025% bovine serum albumin (BSA) for 24-72 h with ethanol (EtOH) (0.1%), PAF (10 nM-1 μM, Biomol Research Laboratories Inc.), mc-PAF (100 nM-1 μM, Biomol Research Laboratories Inc), or *lyso*-PAF (10 nM-1 μM, Biomol Research Laboratories Inc). Cell survival was assessed by hemocytometer cell counts of Trypan Blue-excluding cells. Cell viability was determined by a colorimetric assay based on the ability of mitochondrial dehydrogenases from viable cells to cleave tetrazolium salt, WST-1, into formazan salt (measured at A_{450}-A_{690} nm) (Roche). In this assay, absorbance is directly proportional to the number of metabolically active cells.

Reverse transcriptase polymerase chain reaction (RT-PCR) analysis

Total RNA was isolated from PC12-AC cells 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. As a positive control, rat brain RNA was prepared from Wistar rats approximately 3 months of age or C57Bl/6 mice approximately 2 months of age (Charles Rivers). Rodents were deeply anesthetized with sodium pentobarbital and euthanized by decapitation. Two µl of the cDNA RT reaction mix were PCR amplified in a total volume of 50 µl containing 1X PCR buffer (20 mM Tris-HCl (pH 8.4), 50 mM KCl), deoxynucleotide triphosphates (dNTPs; 200 µM each, Promega), 5 U of *Taq* DNA polymerase, 1 mM $MgCl_2$, and 10 pmol per primer for GAPDH, 20 pmol per primer for PAF-AH II pairs # 2, 3 and 4, 25 pmol per primer for PAF-AH I α1, PAF-AH I α2, PAF-AH I LIS1 and PAF-AH II pair #1. The PCR reaction was brought up to a final volume of 50 µl with nuclease free double-distilled water (NF-H_2O) (Promega) and amplified in a GeneAmp PCR System 2400 (Applied Biosystems): 94°C for 5 minutes, 30-35 cycles of 94°C for 30 seconds, 55°C for 60 seconds, and 72°C for 2 minutes, followed by a final incubation at 72°C for 7 minutes. Primer pairs are indicated in Table 2.1. Primers were synthesized at BRI, University of Ottawa. Additional control reactions included PCR performed in the absence of template to detect reagent contamination and in the absence of primers to detect artifactual false priming.

Cloning of the rat PAF-AH II

Four PCR primer pairs were designed using Clone Manager software (Scientific & Educational Software) to obtain the full-length rat PAF-AH II cDNA: primers recognizing homologous sequences in human, mouse, and bovine PAF-AH II (primer pair #1), primers recognizing 5' murine PAF-AH II sequence (primer pair #2), primers recognizing 3' murine PAF-AH II sequences (primer pair #3), and primers recognizing the complete coding sequence of the rat PAF-AH II (primer pair #4). The PCR product obtained from the first amplification (primer pair #1) and a PCR marker (Promega) was size-fractionated by electrophoresis on a 1.2% (w/v) low melting point agarose gel (Life Technologies). The PAF-AH II cDNA was extracted and purified using the QIAquick gel extraction kit (Qiagen) and sequenced on both strands at BRI, University of Ottawa. Overlapping amplicons were generated using primer pair #2, sequenced on both strands, and aligned with the sequence generated using primer pair #1. This procedure was repeated with primer pair #3, and primers were designed based on this aligned sequence to amplify complete rat PAF-AH II coding sequence from random-primed Wistar rat brain cDNA. The full-length PAF-AH II was ligated into the pGEM-T vector using T4 DNA Ligase supplied with the PGEM-T vector system (Promega). Transformation was performed using JM109 High Efficiency Competent Cells (Promega). Standard plasmid miniprep procedures were used to screen and isolate recombinant plasmid DNA (Promega). Based on these screening results, large-scale recombinant plasmid DNA preparation was performed according to the protocol provided by the manufacturer (Qiagen). The

resulting cDNA was sequenced on both strands with the T7 forward primer and the SP6 reverse primer (BRI, University of Ottawa).

Statistical analysis

Data were analyzed using one-way factorial analysis of variance (ANOVA) or unpaired Student's-t-tests, as applicable. Following detection of a statistically significant difference by analysis of variance (ANOVA), *post hoc* Dunnett's t-tests or Tukey tests were performed as appropriate. P values less than 0.05 were considered statistically significant (shown as *); P values less than 0.01 were considered highly statistically significant (shown as **).

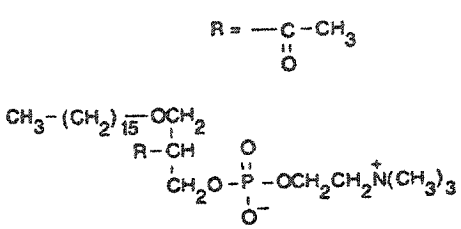
2.4 Results

2.4.1 PAF-mediated cell death is dependent upon sustained exposure to active ligand.

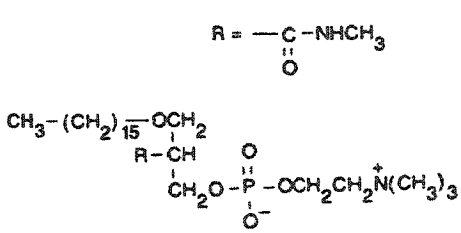
To determine whether the kinetics of PAF-mediated cell death depend upon chronic exposure to active ligand, PC12-AC cells were treated with PAF (Fig 2.1A), the synthetic PAF analog, mc-PAF (1-*O*-hexadecyl-2-*O* -methylcarbamyl-*sn*-glycero-3-phosphocholine) (Fig 2.1B), or the immediate PAF metabolite, *lyso*-PAF (Fig 2.1C) in serum-free media containing 0.025% BSA. The methyl-carbamyl group at the *sn*-2 position of mc-PAF is resistant to degradation by PAF-AHs (O'Flaherty et al., 1987). Both mc-PAF and PAF elicited comparable concentration-dependent cell death after a 24 h treatment. *Lyso*-PAF had no significant effect on cell viability (Fig 2.2A). Treatment with mc-

Figure 2.1: Chemical structures of PAF, mc-PAF, and *lyso*-PAF.
(**A**) PAF (1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine), (**B**) the non-metabolizable PAF analogue mc-PAF (1-*O*-hexadecyl-2-*O*-methylcarbamyl-*sn*-glycero-3-phosphocholine, (**C**) the immediate PAF metabolite *lyso*-PAF (1-*O*-alkyl-2-hydroxyl-*sn*-glycero-3-phosphocholine).

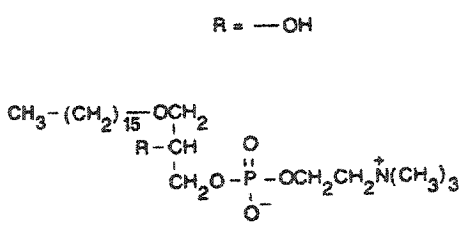
A



B



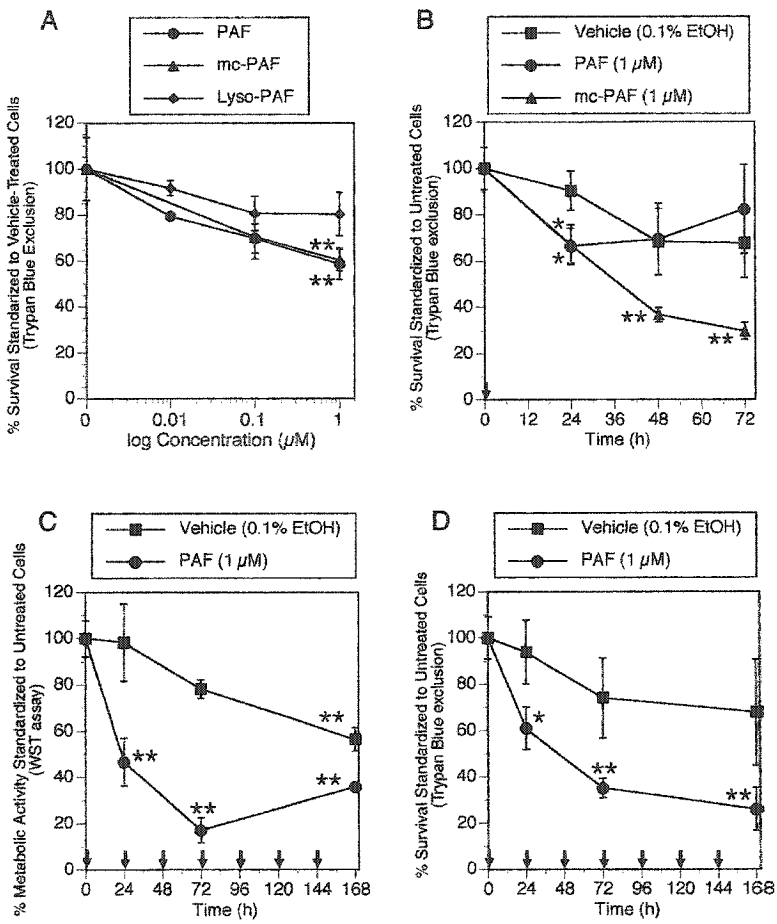
C



PAF effectively extended the kinetics of cell loss relative to PAF (Fig 2.2B). PAF (1 μM) resulted in a 33% cell loss relative to untreated cells after 24 h of treatment (Fig 2.2B, PAF). No additional reductions in cell number were observed at 48 and 72 h (Fig 2.2B, PAF). mc-PAF (1 μM) triggered progressive cell loss (Fig 2.2B, mc-PAF). A 33%, 63%, and 70% reduction in cell number relative to untreated cells was observed 24 h, 48 h, and 72 h after administration (Fig 2.2B, mc-PAF). PC12-AC viability was mildly compromised within the first 48 h of sustained EtOH exposure (0.1%); no further reductions were observed after 72 h of treatment (Fig 2.2B, EtOH).

To establish the effect of chronic PAF exposure on PC12-AC viability, cells were repeatedly treated with PAF (Fig 2.2C,D). Medium was replaced and PAF (1 μM) or vehicle (0.1% EtOH) was added every 24 h. Metabolic activity and percent survival of PC12-AC cells were assessed. Metabolic activity was defined by the ability of mitochondrial dehydrogenases in viable cells to reduce the formazan salt, WST, resulting in a detectable shift in substrate absorbance (Fig 2.2C). Progressive impairment in the ability of PC12-AC cells to reduce WST (Fig 2.2C) and incremental loss in cell number (Fig 2.2D) were observed after repeated PAF treatment. Taken together, these data demonstrated that transient exposure to PAF elicits limited cell loss but that chronic exposure or treatment with a hydrolase-resistant analog (mc-PAF) effectively extends these kinetics and triggers incremental cell loss over protracted periods.

Figure 2.2: PAF-mediated cell death is dependent upon sustained exposure to active ligand.
(**A**) PC12-AC cells were treated for 24 h with vehicle (0.1% ETOH, 0 lane) or mc-PAF (0.1-10 μM) in low serum media. A dose-dependent decrease in cell number was observed after 24 h of treatment. (*$p<0.05$, **$p<0.01$, ANOVA, post-*hoc* Dunnett's *t* test). (**B**) PAF (1 μM) treatment resulted in quantifiable cell loss 24 h after treatment with no additional cell loss at 48 and 72 h. Treatment with mc-PAF (1 μM) extended the kinetics of cell loss over a 72 h period. (*$p<0.05$, **$p<0.01$, ANOVA, post-*hoc* Dunnett's *t* test). (**C**) Repeated PAF (1 μM) administration decreased the metabolic activity of cells for up to 48 h. Media was replaced and PAF (1 μM) or vehicle (0.1% EtOH) was added every 24 h (arrows). **$p<0.01$, ANOVA, post-*hoc* Dunnett's *t* test). (**D**) Progressive cell loss was observed 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). Note that, in (**A**), data are expressed as percent survival of vehicle-treated cultures and, in (**B,C,D**) data are expressed as percent survival or percent metabolic viability of untreated cells cultured in low serum media to demonstrate the effect of vehicle treatment on cell survival. Results are reported as Mean$\pm$ SEM of n=5-26 cultures per data point. Details are as described in Materials and Method.


A
PAF
mc-PAF
Lyso-PAF
% Survival Standarized to Vehicle-Treated Cells
(Trypan Blue Exclusion)
log Concentration (μM)
B
Vehicle (0.1% EtOH)
PAF (1 μM)
mc-PAF (1 μM)
% Survival Standardized to Untreated Cells
(Trypan Blue exclusion)
Time (h)
C
Vehicle (0.1% EtOH)
PAF (1 μM)
% Metabolic Activity Standardized to Untreated Cells
(WST assay)
Time (h)
D
Vehicle (0.1% EtOH)
PAF (1 μM)
% Survival Standardized to Untreated Cells
(Trypan Blue exclusion)
Time (h)

2.4.2 Design and optimization of rodent-specific PAF-AH PCR primer pairs

The first step in establishing the pattern of PAF-AH expression in the PC12-AC cell line was to reveal PAF-AH mRNA expression. To this end, it was essential to design and optimize rodent-specific PAF-AH PCR primer pairs to be used in RT-PCR analysis. A primer designer program (Primer Designer 4, v4.2, Scientific & Educational Software) was used as a tool to generate optimized primers for the following rodent PAF-AH genes: LIS1, PAF-AH I α1, PAF-AH I α2, PAF-AH II and plasma PAF-AH. Optimal primer design implicated the consideration of several criteria, including length, GC content, melting temperature, 3' dimers, any dimers, stability, runs of bases, dinucleotide repeats, hairpins and worst-case false priming. It was also essential to design primer pairs specific to their target and non-homologous to other genomic sequences (as determined by BLAST sequence). The rat PAF-AH primer pair sequences and amplicon sizes are provided in Table 2.1.

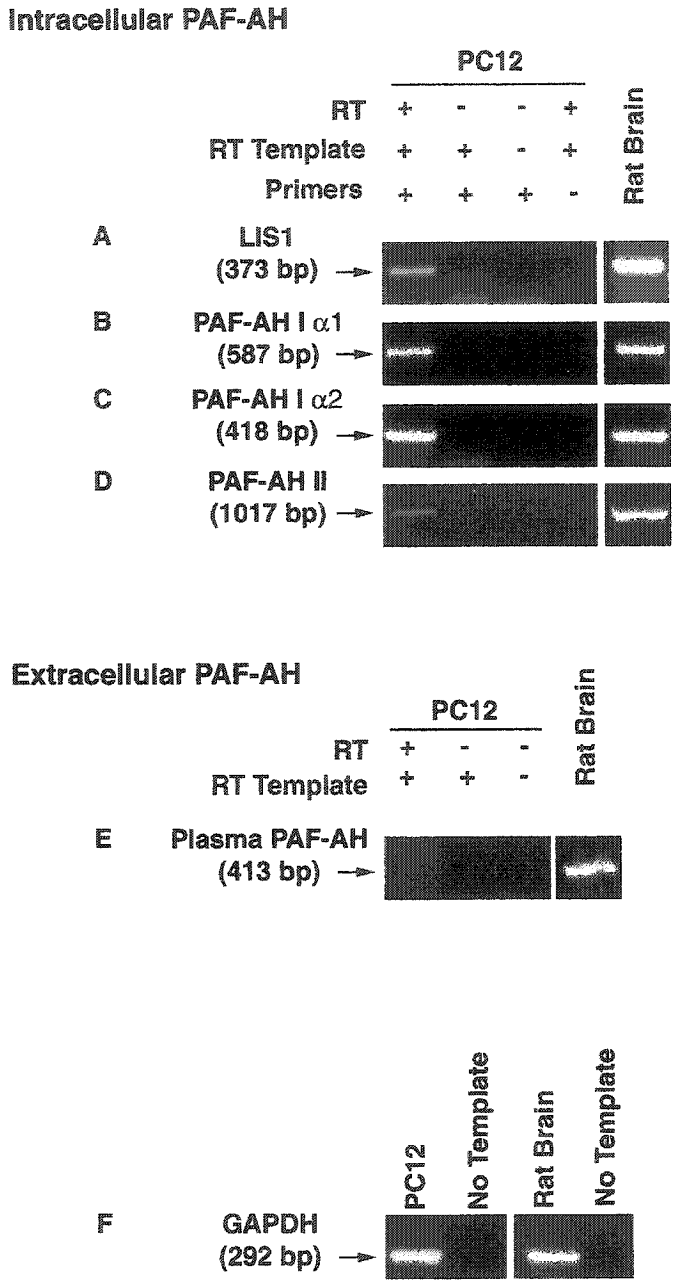
2.4.3 PC12-AC cells express endogenous LIS1, PAF-AH I α1, PAF-AH I α2, and PAF-AH II mRNA transcripts, but not plasma PAF-AH

Expression of extracellular and intracellular PAF-AH mRNA was investigated by RT-PCR. PC12-AC cells expressed *Lis1* (Fig 2.3A), PAF-AH I α1 (Fig 2.3B), PAF-AH I α2 (Fig 2.3C) and PAF-AH II (Fig 2.3D), as defined by

Table 2.1: Rat-specific PAF-AH PCR primer pair sequences and amplicon sizes.

Gene	Strand	Sequence (5'-3')	Amplicon size (bp)
GAPDH	Sense	TGGTGCTGAGTATGTCGTGGAGT	292
	Antisense	AGTCTTCTGAGTGGCAGTGATGG	
PAF-AH I α1	Sense	GACGGACGCTGGATGTCTCT	587
	Antisense	AGACGAAGCAGCAAGGAGTG	
PAF-AH I α2	Sense	TGCAGCAGTACGAGATATGG	418
	Antisense	AACATGTCGTGGCAGGAGAT	
PAF-AH I LIS1	Sense	CTGCTTCAGAGGATGCTACA	373
	Antisense	ATCAGAGTGCCGTCCTGATT	
PAF-AH II Pair #1	Sense	GGATGTGATGGAGGGTC	1017
	Antisense	TGCTTCTGCAGGAAGGCCAA	
PAF-AH II Pair #2	Sense	CAGCTGGTGATGAGATGG	224
	Antisense	GCGCTTGTTATACTGCAGGT	
PAF-AH II Pair #3	Sense	TGAGCCGAGTGGCTGTGATG	472
	Antisense	CCCTTGGATGAGCGATGGTC	
PAF-AH II Pair #4	Sense	CAGCTGGTGATGAGATGG	1250
	Antisense	CAACCTAGAGGCTGGACAGA	
Plasma PAF-AH	Sense	GGGGCATTCTTTTGGAGGAG	413
	Antisense	GACAGTCCCACTGATCAAAGTC	

Figure 2.3: PC12-AC cells express all three PAF-AH I subunits (LIS1, α1, α2) and PAF-AH II mRNA but not extracellular PAF-AH mRNA.
RT-PCR was performed for (**A**) LIS1, (**B**) PAF-AH I α1, (**C**) PAF-AH I α2 and (**D**) PAF-AH II, defining amplicons of 373, 587, 418 and 1017 bp respectively (PC12-AC lanes). The absence of genomic DNA contamination and reagent contamination was demonstrated by performing the following control reactions in parallel: 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 PAF-AH (Rat Brain lane). (**E**) Template integrity of t random-primed PC12-AC RT product was verified using GAPDH primers, defining a 292 bp amplicon.


Intracellular PAF-AH
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)
Extracellular PAF-AH
PC12
RT
RT Template
Rat Brain
E
Plasma PAF-AH
(413 bp)
PC12
No Template
Rat Brain
No Template
F
GAPDH
(292 bp)

amplicons of 373, 587, 418 and 1017 base pairs (bp) respectively. PC12-AC cells do not express plasma PAF-AH (Fig 2.3E). Several control reactions were carried out in parallel to exclude false positive and false negative results for each transcript analyzed (i.e., RT reaction carried out in the absence of enzyme to detect genomic DNA contamination in the RNA template, no template to detect reagent contamination, and no primers to detect false priming artifacts). Glyceraldehyde-3'-phosphate dehydrogenase (GAPDH), a "housekeeping" gene, was also amplified as an internal standard (Fig 2.3F).

2.4.4 Cloning of the rat PAF-AH II

Because the rat PAF-AH II sequence had not been deposited with GenBank prior to this study, 4 primer pairs were designed against highly conserved sequences in mouse, bovine, and human genes spanning the complete open reading frames (Table 2.1). A 1017 bp amplicon was identified by RT-PCR in PC12-AC cells (Fig 2.3D), sequenced on both forward and reverse strands, and found to be 88% homologous to murine PAF-AH II. The 1173 bp open reading frame was subsequently amplified from adult Wistar rat brain and placed into the pGEMT-cloning vector. Direct submission to GenBank was accepted under accession number AY225592. The full-length rat PAF-AH II cDNA is 83% and 92% homologous to the human and mouse nucleotide sequences respectively (Fig 2.4). The rat cDNA encodes a 390 amino acid protein with a predicted molecular weight of 43.5 kD and a 80% and 92% homology to the human and mouse protein sequences (Fig 2.5).

Figure 2.4: Nucleotide sequence of rat PAF-AH II and homology with murine and human cDNAs.
Nucleotide residues are numbered from 5' to 3'. The start codon (ATG) encoding the initiating methionine is surrounded by a box. Nucleic acid sequences with complete homology between all three species are indicated by (*). The full-length rat PAF-AH II cDNA, containing 1173 bp, is 92% and 83% homologous to the mouse and human nucleotide sequences respectively.

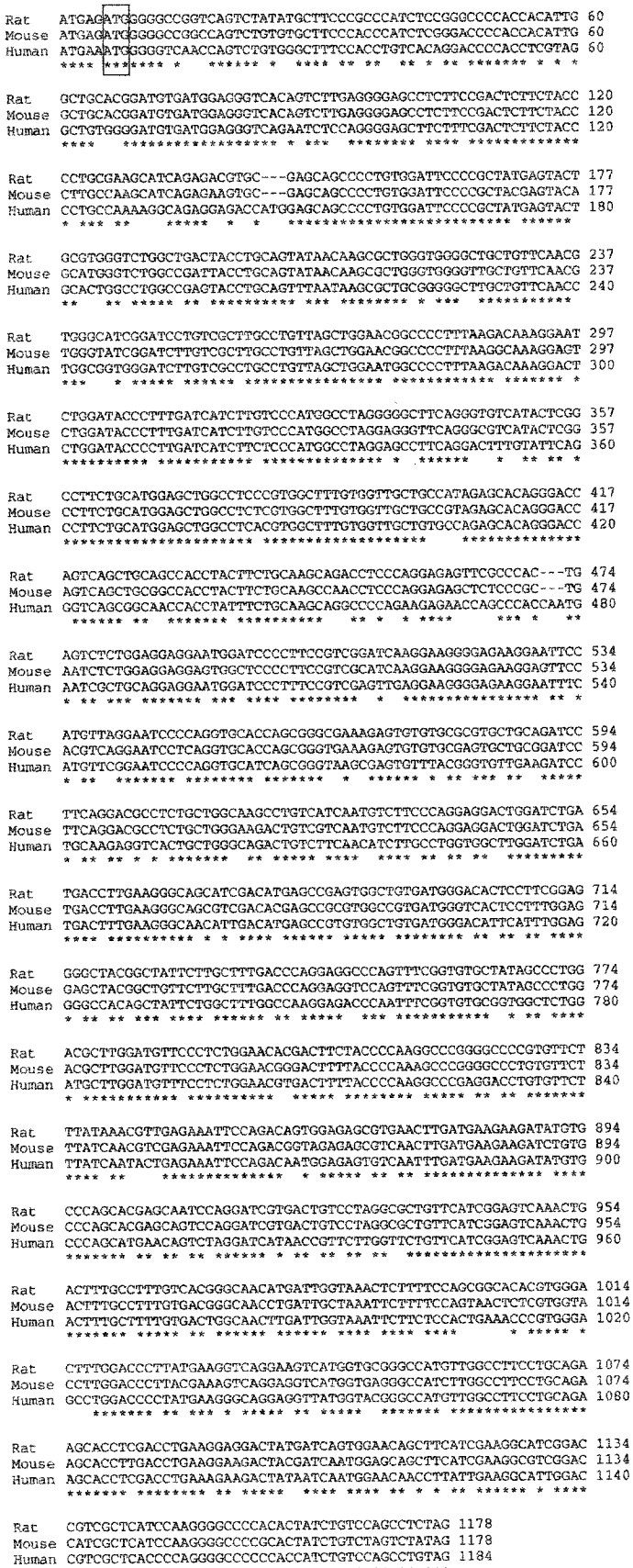


Figure 2.5: Predicted amino acid sequence of rat brain PAF-AH II cDNA and homology with murine and human protein.
The predicted amino acid sequence of rat, mouse, and human protein with complete homology between all three species are indicated by (*). The rat cDNA encodes a 390 amino acid protein with a predicted molecular weight of 43.5 kD and a 92% and 80% homology to the mouse and human protein sequence.

```
Rat    MGAGQSICFPPISGPHHIGCTDVMEGHSLEGSLFRLFYPCE-ASETCEQPLWIPRYEYCV 59
Mouse  MGAGQSVCFPPISGPHHIGCTDVMEGHSLEGSLFRLFYPCQ-ASEKCEQPLWIPRYEYSM 59
Human  MGVNQSVGFPPVTGPHLVGCGDVMEGQNLQGSFFRLFYPCQKAEETMEQPLWIPRYEYCT 60
       **..**: ***::*** :** *****:.*:**:*******: *.*. ***********.

Rat    GLADYLQYNKRWVGLLFNVGIGSCRLPVSWNGPFKTKESGYPLIILSHGLGGFRVSYSAF 119
Mouse  GLADYLQYNKRWVGLLFNVGIGSCRLPVSWNGPFKAKESGYPLIILSHGLGGFRASYSAF 119
Human  GLAEYLQFNKRCGGLLFNLAVGSCRLPVSWNGPFKTKDSGYPLIIFSHGLGAFRTLYSAF 120
       ***:***:***  *****:.:**************:*:*******:*****.**. ****

Rat    CMELASRGFVVAAIEHRDQSAAATYFCKQTSQESSPT-ESLEEEWIPFRRIKEGEKEFHV 178
Mouse  CMELASRGFVVAAVEHRDQSAAATYFCKPTSQESSPA-ESLEEEWLPFRRIKEGEKEFHV 178
Human  CMELASRGFVVAVPEHRDRSAATTYFCKQAPEENQPTNESLQEEWIPFRRVEEGEKEFHV 180
       ************. ****:***:***** :.:*..*: ***:***:****::********

Rat    RNPQVHQRAKECVRVLQILQDASAGKPVINVFPGGLDLMTLKGSIDMSRVAVMGHSFGGA 238
Mouse  RNPQVHQRVKECVRVLRILQDASAGKTVVNVFPGGLDLMTLKGSVDTSRVAVMGHSFGGA 238
Human  RNPQVHQRVSECLRVLKILQEVTAGQTVFNILPGGLDLMTLKGNIDMSRVAVMGHSFGGA 240
       ********..**:***:***:.:**:.*.*::***********.:* *************

Rat    TAILALTQEAQFRCAIALDAWMFPLEHDFYPKARGPVFFINVEKFQTVESVNLMKKICAQ 298
Mouse  TAVLALTQEVQFRCAIALDAWMFPLERDFYPKARGPVFFINVEKFQTVESVNLMKKICAQ 298
Human  TAILALAKETQFRCAVALDAWMFPLERDFYPKARGPVFFINTEKFQTMESVNLMKKICAQ 300
       **:***::*.*****:**********:**************.*****:************

Rat    HEQSRIVTVLGAVHRSQTDFAFVTGNMIGKLFSSGTRGTLDPYEGQEVMVRAMLAFLQKH 358
Mouse  HEQSRIVTVLGAVHRSQTDFAFVTGNLIAKFFSSNSRGTLDPYESQEVMVRAILAFLQKH 358
Human  HEQSRIITVLGSVHRSQTDFAFVTGNLIGKFFSTETRGSLDPYEGQEVMVRAMLAFLQKH 360
       ******:****:**************:*.*:**: :**:*****.*******:*******

Rat    LDLKEDYDQWNSFIEGIGPSLIQGAPHYLSSL 390
Mouse  LDLKEDYDQWSSFIEGVGPSLIQGAPHYLSSL 390
Human  LDLKEDYNQWNNLIEGIGPSLTPGAPHHLSSL 392
       *******:**..:***:****  ****:****
```

2.5 Discussion

Accumulating evidence suggests that protracted exposure to pathophysiological concentrations of PAF is a principal mediator of neuronal death in ischemia, encephalitis, epileptic seizure, meningitis, and HIV-1 dementia, and a primary mediator of lethality associated with systemic anaphylaxis (Bazan et al., 1995; Bazan, 1998; Birkle et al., 1998; Perry et al., 1998; Langley et al., 1999; Schifitto et al., 1999; Fukuda et al., 2000). We and others have demonstrated that PAF elicits apoptosis in a variety of cell types at concentrations comparable to that observed under certain conditions of trauma and injury, and necrosis when concentrations exceed the critical micelle concentration (Pulliam et al., 1998; Brewer et al., 2002; Murohisa et al., 2002). The mechanisms underlying PAF-mediated apoptotic signaling are not clear. While PAF can be metabolized to other biologically active lipids, the majority of PAF effects are understood to be receptor-mediated (for a review see Prescott et al 2000). We have previously demonstrated that PAF elicits cell death in PC12 cells in the absence of the G-protein coupled PAFR, and that transfection and overexpression of PAFR is cytoprotective (Brewer et al., 2002). These data and other observations from our laboratory (unpublished) suggest that PAF may act through a novel PAF binding protein to elicit apoptosis – an important distinction in the development of new therapy to reduce cell death associated with lipid mediators. Whether specific intracellular receptors for PAF exist is still a debated issue, although strong experimental evidence in favor of their existence has been published (Marcheselli et al., 1990; Ihida et al., 1999). The role of the known G-

protein coupled PAFR in the regulation of PAF-mediated apoptotic loss is also controversial. Other groups have shown that PAFR expression both protects cells from apoptotic loss and exacerbates apoptosis depending on cellular response to NF-kB activation (Southall et al., 2001; Li et al., 2003). These controversies underline the importance of establishing how PAF degradation is regulated at the intracellular level in context of PAF inactivation following pathophysiological challenge.

Degradation of PAF has been extensively studied in various cell types and tissues. However, no evidence has yet been provided for the exact catabolic kinetics of cytosolic PAF-AH in cells sensitive to PAF-mediated loss. The present thesis focused on furthering our understanding of how intracellular PAF-AHs regulate the kinetics of PAF-mediated cell death and, furthermore, on establishing whether these enzymes can be targeted to promote cell survival following injury or insult. The first steps in this investigation were to identify a PAF-sensitive system (PC12 cells) that expresses intracellular but not extracellular PAF-AH isoforms, and to provide experimental evidence that these enzymes limit the extent of PAF-mediated cell death. To this end, we investigated the effects of different concentrations of PAF treatment on PC12 cell viability. The critical micelle concentration of PAF is estimated to be 2.5 to 3 μM (Blank et al., 1981). Presence of higher concentration of PAF (3.5 to 10 μM) has been shown to produce cytotoxic effect (Kornecki and Ehrlich, 1988) and elicit necrotic cell death by disrupting the plasma membrane (Brewer et al., 2002). Although PC12 cells showed responsiveness to nanomolar concentrations of

PAF (100 nM), significant cell death was apparent at micromolar concentrations (1 μM). We have previously demonstrated that this cell death occurs through an apoptotic mechanism (Brewer et al., 2002) and have recently confirmed that PAF-mediated apoptosis is caspase-3-dependent (Bonin et al, submitted). These data were not included in this thesis as they were performed as part of my Honours research project and in collaboration with two undergraduate students I mentored over the summer of 2003. In this thesis, we extend these observations to demonstrate that the extent of PAF-mediated apoptotic loss[3] depends upon sustained exposure to active ligand. A single exposure to PAF in serum-free media containing 0.025% BSA resulted in a significant reduction in PC12 cell survival 24 h after treatment, with no subsequent decreases in cell viability. Chronic PAF treatment or single exposure to a non-hydrolyzable analogue, mc-PAF, triggered incremental apoptotic cell death over 72 h, resulting in a loss of approximately 70% of cultured cells. Salient to this finding, mc-PAF, a PAF analog known to have a lower affinity for PAF binding proteins than PAF itself (O'Flaherty et al., 1987) produced the same magnitude of cell loss in PAFR-negative PC12 cells. This effect is in keeping with the sustained actions of the ligand despite reduced receptor affinity, a phenomenon we have previously observed in other cell types (Bennett et al., 1993). Other groups have also demonstrated that exposure to both PAF, and the non-metabolizable form of it (mc-PAF) produced comparable effects upon cultured hippocampal neurites,

[3] I refer to PAF-mediated cell death as apoptotic, based on the data presented in Brewer et al 2002 and as a result of the caspase analysis performed by Fan Mo and Lamiaa Migahed, undergraduate students in the Bennett laboratory. These data are not presented in this thesis.

including growth cone collapse, neurite retraction, and neurite varicosity formation despite the reduced affinity of mc-PAF for PAF binding proteins relative to PAF (McNeil et al., 1999). Given the fact that an acute PAF administration caused a decrease in cell survival following 24 h, but, unlike mc-PAF, did not result in incremental cell loss, it is likely that PAF-AHs limit the extent of PAF-mediated apoptotic signaling in PC12 cells. Intracellular PAF-AH activities have been reported to regulate the intracellular PAF concentrations in other cell types (Matsuzaya et al., 1997; Marques et al., 2002).

Intracellular PAF-AH enzymatic activity has been shown to be controlled by two distinct isoforms designated PAF-AH I and II (Manya et al., 1998). The PAF-AH genes have since been cloned (for a review see (Arai, 2002)) with isoform I proven to have more than one catalytic subunit (α1 and α2) and a regulatory subunit (β or LIS1) (Manya et al., 1998). The expression of PAF-AH genes was investigated for mRNA and protein expression in the PC12 cell line. RT-PCR and protein analysis revealed that PC12 cells express PAF-AH II and all three subunits of PAF-AH I. Semi-quantitative RT-PCR analysis suggested that the α2 catalytic subunit expression was more predominant than the α1 catalytic subunit expression[4]. This differential expression is predicted to impact upon enzyme kinetics (Manya et al., 1998), a hypothesis tested in the next Chapter. The activity of dimeric enzymatic complexes is modulated by the β (LIS1) subunit in a catalytic subunit composition-dependent manner. The rate of PAF hydrolysis by the α2/α2 homodimer is accelerated approximately 4-fold by the addition of

[4] This observation was confirmed at the protein level by Western analysis by Scott Ryan, a M.Sc. student in the Bennett laboratory.

the β subunit, whereas the rate of hydrolysis by the α1/α1 is suppressed by it. α1/α2 activity is not affected by LIS1 (Manya et al., 1999). Therefore, according to the observation that the α2 subunit expression predominates in PC12 cells, it is likely that PAF-AH I is mostly composed by α2/α2 and α1/α2 dimers. This hypothesis was tested in the next Chapter.

In addition to investigating expression of PAF-AHs in PC12 cells, we have cloned rat PAF-AH II from Wistar rat brain and confirmed expression in PC12-AC cells. Prior to this research, mouse and human but not rat PAF-AH II sequences had been deposited with GenBank. The cDNA encoding the intracellular cytosolic rat PAF-AH II was isolated from Wistar rat brain, based on predicted sequence homology with mouse and human PAF-AH II, and registered under accession number AY225592. RT-PCR analysis using primers based on this novel sequence detected PAF-AH II in PC12 cell cultures, which marks the first report of biological expression of the PAF-AH II gene in a rat derivate cell system. The rat cDNA encodes a 390 amino acid protein with a predicted molecular weight of 43.5 kD and a 80% and 92% homology to the human and mouse protein sequence (Clustal analysis). The amino acid sequence of isoform II has no homology with any subunits of PAF-AH I but is 41% identity with that of the plasma PAF-AH, consistent with previously published analyses of mouse and human homologies (Hattori et al., 1995a; Hattori et al., 1996). The intracellular PAF-AH II has been shown to be an antioxidant phospholipase in a number of systems (Matsuzaya et al., 1997). It has been demonstrated that the intracellular PAF-AH II translocates between cytosol and membrane in response to a redox

state of the cells (Matsuzaya et al., 1997). Furthermore, cells overexpressing PAF-AH II protein have shown to be resistant to reactive oxygen species (ROS)-induced apoptosis, suggesting that hydrolysis of oxidized PAF-like phospholipids protects against ROS-induced apoptosis (Matsuzaya et al., 1997), consistent with our hypothesis that PAF-AH activity limits the extent of apoptotic loss in PC12 cells. Isoform II has been demonstrated to transfer an acetyl group from exogenously added PAF to endogenous acceptor lipid(s) in CHO-K1 cells (Bae et al., 2000). Whether this mechanism is occurring in an identical manner in PC12 cells remains to be tested.

In summary, this series of experiments reports the cloning of the intracellular PAF-AH II isoform from rat. This study also marks the first report of endogenous PAF-AH I (LIS1, α1, α2) and PAF-AH II expression in the PC12-AC cell line. The kinetics of PAF-mediated cell death and mc-PAF-mediated cell death suggest that these intracellular enzymes are playing a protective role limiting PAF cytotoxicity in PC12 cells.

CHAPTER 3: CHARACTERIZATION OF PAF-AH ACTIVITY IN PC12 CELLS

3.1 Introduction

As reviewed in Chapter 1, PAF-AHs are structurally diverse isoenzymes that catalyze the hydrolysis of the primary substrate PAF by removing the acetyl moiety at the *sn*-2 position of the glycerol backbone, generating the immediate PAF metabolite, *lyso*-PAF (for a review see (Stafforini et al., 1996a)). Mammalian PAF-AHs can be divided into two types: intracellular PAF-AHs found in the cytosol (PAF-AH I and PAF-AH II), and extracellular PAF-AH secreted to blood plasma (plasma PAF-AH) (for a review see (Arai, 2002)). PAF-AH I is a G-protein-like trimer composed of two homo or heterodimer catalytic subunits (α1, 29 kD; α2, 30 kD), and a non-catalytic 45 kD regulatory β subunit called LIS1 (Hattori et al., 1993; Ho et al., 1997). Intracellular PAF-AH II is a 40 kD enzyme expressed by most tissues including brain (Hattori et al., 1996). Isoform II differs from isoform I with respect to its polypeptide composition, substrate specificity, and tissue distribution, suggesting that it may serve a different physiologic function from isoform I in tissues (Hattori et al., 1995a). In support of this hypothesis, PAF-AH II has been shown to recognize both PAF and PAF acyl analogs with moderate length *sn*-2 chains (for a review see (Prescott et al., 2000)). PAF-AH I has been shown to have a strict requirement for the *sn*-2 acetyl group in PAF and PAF-like lipids (Min et al., 2001). The 45 kD monomer plasma PAF-AH is the most permissive PAF-AH enzyme capable of inactivating not only PAF but also structurally related analogs with relatively long *sn*-2 acyl

chains (Tjoelker et al., 1995a; Prescott et al., 2000). It has been proposed that PAF-AH activity regulates PAF levels, which can be toxic at high concentrations during brain development (Tjoelker and Stafforini, 2000).

In vitro studies of the rodent PC12 cell have shown that PAF initiates apoptotic cell death when administered at concentrations observed following pathological tissue insult (Brewer et al., 2002). The goals of the present study were to characterize the kinetics of cytosolic PAF-AH I and II and to test the hypothesis that PAF-AH enzymatic activity protects cells against PAF-mediated cytotoxicity.

3.2 Objectives

To elucidate the role of intracellular PAF-AHs in PAF degradation and in protection against PAF-mediated cell death, we developed a novel and reliable assay using a fluorescent PAF substrate capable of quantifying intracellular PAF-AH activity (I and II) and thereby determining the half-life of PAF. The advantage of this assay is that both PAF and PAF metabolites can be followed over time in living cells and following fractionation. Previous assays have used substrates shown to inhibit PAF-AH II activity and thereby cannot be used for kinetic analyses, or radioactive PAF substrates that are not amenable to live cell imaging.

In this series of experiments, we investigated the presence of functional PAF-AH activity in PC12 cells. To establish the kinetics of PAF internalization and metabolism in PC12 cells, cultures were analyzed at single-cell level by

quantitative time-lapse fluorescence microscopy, and at the population level by lipid extraction and thin layer chromatography (TLC) using Bodipy FL C_{11}-PAF (B-PAF) (Fig 3.1B). Cell survival and kinetics studies were also performed in the presence and absence of DFP, a potent inhibitor of serine proteases and acetylcholinesterase, known to inhibit PAF-AH activity in concentration-dependent fashion (Hattori et al., 1995a; Hattori et al., 1996; Manya et al., 1999), and three different PAF antagonists (CV 3988, CV 6209, and FR 49175).

3.3 Materials and Methods

Cell culture

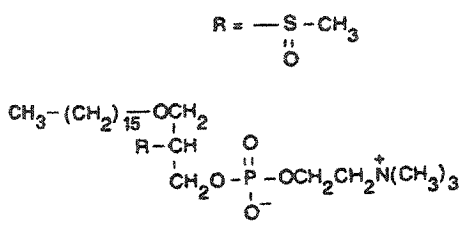
PC12-AC cells were cultured as described in Chapter 2. Stock cultures were fed with complete media every 2-3 days and passaged when sub-confluent.

PAF-AH activity assay

PAF-AH activities in complete media, serum-free RPMI, PC12-AC cells, PC12-AC conditioned media, and mouse brain (positive control) were determined using a commercial PAF-AH assay kit (Cayman Chemicals) (Fig 3.2). Cells and tissue were homogenized in 250 mM sucrose, 10 mM Tris-HCl (pH 7.4), and 1 mM ethylenediaminetetra-acetic acid (EDTA) using a Tissue Tearor (Fisher). Tissue and cells lysates 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 (Millipore, Bio

Figure 3.1: Substrates used to detect PAF-AH activity.
(**A**) C_{16}-2-*thio*-PAF; (**B**) Bodipy FL C_{11}-PAF (B-PAF)

A



B

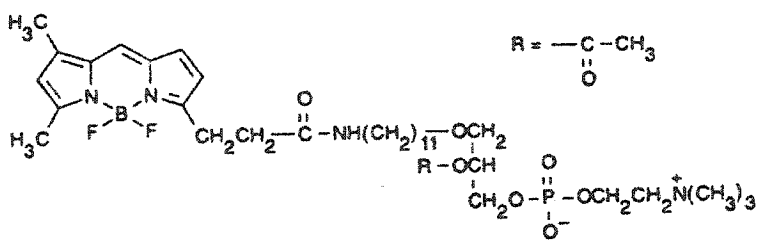
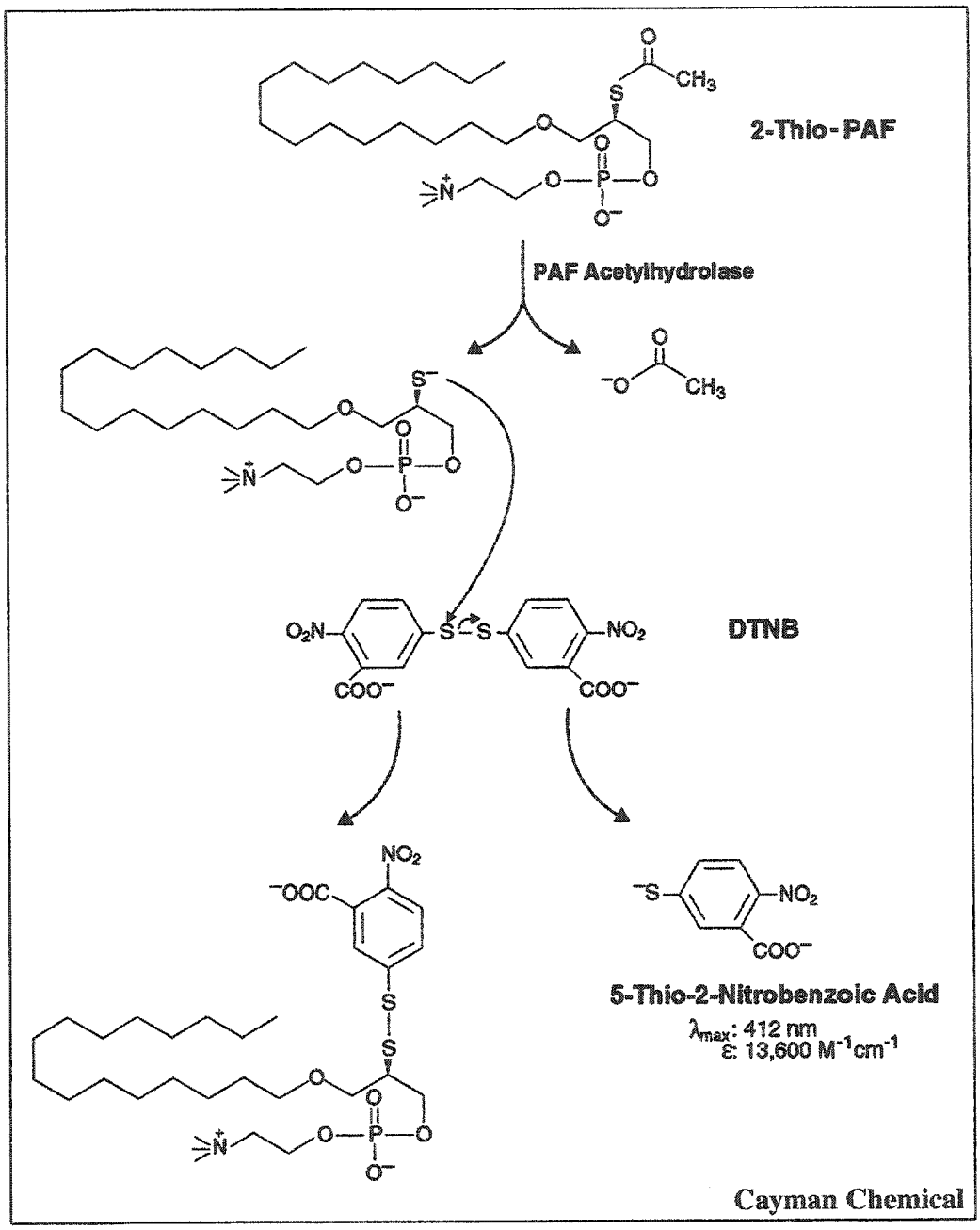


Figure 3.2: The Cayman Chemical PAF acetylhydrolase Assay kit reaction. The assay uses C_{16}-2-*thio*-PAF, which serves as a substrate for PAF-AHs (plasma and intracellular). Upon hydrolysis of the acetyl-thioster bond at the *sn*-2 position by PAF-AH, free thiols are detected using DTNB at 405 nM. This assay cannot be used to study the kinetics of intracellular PAF-AH activity given that DTNB has been shown to inhibit PAF-AH II (Hattori et al., 1995a).


2-Thio-PAF
PAF Acetylhydrolase
DTNB
5-Thio-2-Nitrobenzoic Acid
λmax: 412 nm
ε: 13,600 M-1cm-1
Cayman Chemical

Process Division). Protein content was determined using a Bradford Protein Assay DC kit according to the protocol provided by the manufacturer (Bio-Rad Laboratories). Protein (50 µg) was incubated with C_{16}-2-thio-PAF (Fig 3.1A) substrate for 30 minutes at room temperature. Hydrolysis of the thioester bond at the sn-2 position was detected by conjugation with DTNB (Ellman's reagent) at 405 nM (Fig 3.2). Control reactions included samples incubated without lysate or media and samples incubated without substrate.

Internalization assay (live cell imaging)

PC12-AC cells (5 X 10^4 cells/well) were plated in complete media overnight in a 24-well-plate (VWR) coated with 0.1% gelatin. Cells were washed with 10 mM phosphate buffered saline (PBS, 10 mM sodium phosphate, 137 mM sodium chloride) and incubated with 1 µM B-PAF (Fig 3.1B) in RPMI 1640 containing 0.1% BSA (Sigma). B-PAF was custom synthesized for our laboratory by Molecular Probes. At each time point (0, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120 min), incubation media was removed, cells were washed with PBS or 200 mM Na acetate (pH 4.5) containing 25 mM NaCl, and 1% BSA to separate cells surface B-PAF (acid-sensitive) from internalized B-PAF (acid-resistant) (Ohshima et al., 2002). Live cell imaging under phase and fluorescence of identical cell fields was performed using a DMIL 20X inverted microscope (Leica) equipped with a QICAM digital camera (Quorum Technologies) and captured using Openlab software v3.14 (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 v 3.14 software. Time-lapse animations were reconstructed using AfterEffects v 5.5 (Adobe).

Lipid extraction and thin layer chromatography (TLC)

PC12-AC cells (1 x 10^5 cells/cm^2) were plated onto 10 cm diameter tissue culture plates (VWR), and 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 serum-free media containing 0.1% BSA, for 0, 5, 15, 30, 45, 60, and 75 minutes. A number of experiments included the addition of DFP (0.1 mM and 1 mM; Sigma) or the addition of a PAF antagonist (FR 49175, 10 μM; Biomol) or vehicle (EtOH, 0.1%; PBS, 0.1%) 15 minutes before treatment with B-PAF (1 μM). Cells were maintained in media throughout the experiment, and 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 by scraping, using a cell lifter (Fisher) in acidified methanol. Lipids were extracted from the extracellular milieu and cytosolic fractions by the Bligh and Dyer method (Bligh and Dyer, 1959) and were developed on TLC plates (20 X 20 cm Silica gel 60; Whatman, VWR) 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 ultraviolet (UV) light using AlphaImager-1220 software (Alpha Innotech Corporation Fisher Scientific). Fluorescence intensity was determined by densitometry using the advanced measurement module of Openlab v3.14.

Cell death assays

PC12-AC cells (1 x 10^5 cells/cm^2) were plated overnight in complete media in 6 cm diameter tissue culture plates (VWR). Cells were treated in serum-free media containing 0.025% BSA with DTNB (1 mM) and/or DFP (0.1 mM and 1 mM), a potent inhibitor of serine proteases and acetylcholinesterase known to inhibit PAF-AH activity, or PAF antagonists (FR 49175, CV 6209, CV 3988; Biomol) or vehicle (EtOH, 0.1%; PBS, 0.1%), 15 minutes prior to treatment with PAF (1 μM; Biomol). Cell cultures were incubated at 37°C with 1 μM PAF and maintained in media for 24 h. Cell survival was assessed by hemocytometer cell counts of Trypan Blue-excluding cells.

Statistical analysis

Data were analyzed using one-way factorial ANOVA tests. [Cell survival with PAF antagonists]: Following detection of a statistically significant difference in a given series of treatments, *post hoc* Dunnett's t-tests were performed in order to determine which treatment condition differs significantly from a single control condition (*$p < 0.05$, **$p < 0.01$, significant reduction in cell survival compared to vehicle treatment; †$p < 0.05$, †† $p < 0.01$, significant protection

relative to PAF-treated cultures). [Cell survival with PAF-AH inhibitors]: Following detection of a statistically significant difference in a given series of treatments, *post hoc* Tukey tests were performed in order to determine which treatment conditions differs significantly from another treatment (**$p < 0.01$, significant reduction in cell survival).

3.4 Results

3.4.1 Functional PAF-AH activity is detected in PC12-AC lysates and serum-containing media but not serum-free media.

To establish whether PAF is metabolized by PAF-AH isoforms in PC12-AC cells or culture media, *in vitro* activity was assayed using C_{16}-2-thio-PAF (Fig 3.1A). Cytosolic PAF-AH activity in PC12-AC cell lysates was equivalent to enzymatic activity detected in mouse brain homogenates (Table 3.1). Plasma PAF-AH activity, present in mammalian serum, was detected in cell-free complete media containing 10% HS and 5% NCS (Table 3.1) and minimally in low serum media containing 0.5% HS (Table 3.1). PAF-AH activity was not detected in serum-free media or conditioned RPMI/0.1% BSA media harvested from PC12-AC cells (Table 3.1).

3.4.2 Internalization of PAF in PC12-AC cells by quantitative time-lapse fluorescence microscopy.

The kinetics of PAF internalization were analyzed at single-cell level by quantitative time-lapse fluorescence microscopy using B-PAF (Fig 3.1B). In

Table 3.1: Cytosolic and plasma PAF-AH activity in PC12-AC cells, mouse brain lysate, and tissue culture media.
Mouse brain and PC12 cytosolic fractions were prepared as described in materials and methods. The catalytic activity of samples containing 50 µg of cytosolic protein was measured using the PAF-AH assay kit (Cayman Chemical). Catalytic activity was also determined in complete media, low serum media, serum-free RPMI and PC12-AC conditioned media. Results reported as Means ± SEM (n= 2-12 replicates conducted in over 1-5 individual experiments).

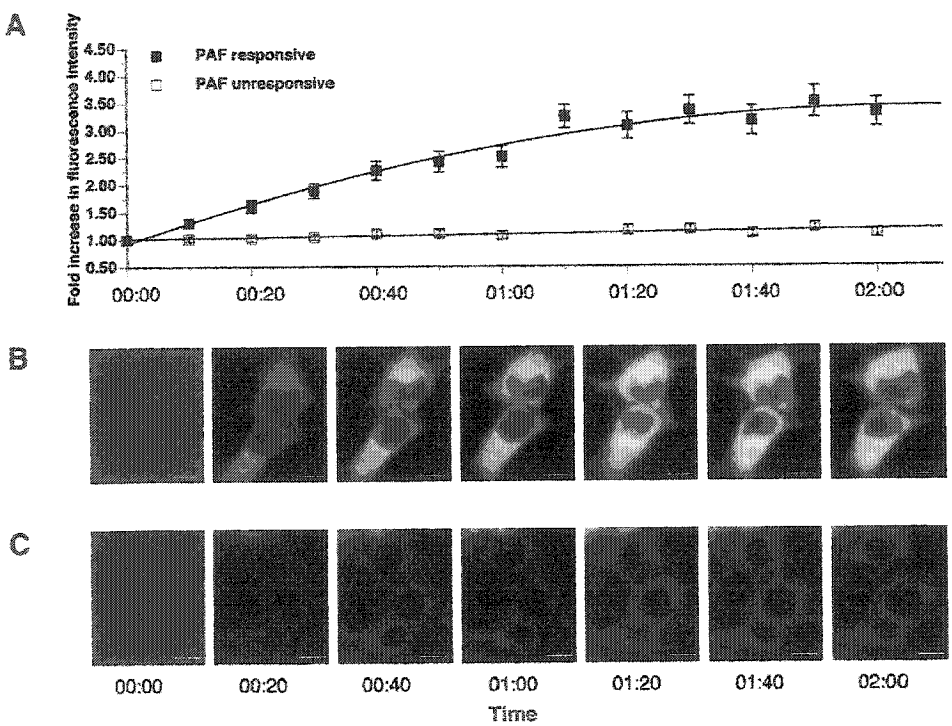
	Positive control mouse brain lysate	PC12-AC lysate	Complete media	Low Serum media	Serum-free media	PC12-AC conditioned serum-free media + 0.1% BSA
PAF-AH activity (nmol/min/µg)	1.97 ± 0.44 n=8	1.83 ± 0.28 n=12	3.36 ± 0.10 n=2	0.06 ± 0.04 n=3	0.01 ± 0.00 n=2	0.00 ± 0.00 n=3

time-lapse microscopic studies, cultures were incubated for up to 120 min with 1 μM B-PAF in RPMI 1640 containing 0.1% BSA at 37°C. Every 10 min, the B-PAF containing media was removed and cells were washed with PBS or with a sodium acetate buffer (pH 4.5) containing 1% BSA to strip uninternalized ligand bound to cell surface proteins at the plasma membrane (Ohshima et al., 2002). Time-lapse analysis of cell-associated fluorescence revealed two populations of cells (Fig 3.3 and Appendix A, Quick-time movie). No differences in the rate of internalization or in the percentage of cells internalizing B-PAF were detected between PBS and acidic pH wash conditions, and data were combined for quantitative analysis (Fig 3.3A). Sixty percent of the PC12-AC population were PAF-unresponsive over the 120 min test period with little to no increase in cell-internalized fluorescence detected over time (Fig 3.3A,C). Forty percent of cells in the PC12-AC cultures were PAF-responsive (Fig 3.3A,B). The most pronounced linear increase in cell-associated fluorescence was observed over the first 40 min of exposure (r^2=0.98) with linear internalization continuing for up to 70 min (r^2=0.97, Fig 3.3A,B). Incorporation plateaued between 80-120 min after B-PAF administration (Fig 3.3A,B).

3.4.3 Internalization and metabolic fate of PAF in PC12-AC cells by lipid extraction *and TLC.*

Internalization kinetics of exogenous B-PAF were confirmed on a population basis by lipid extraction and TLC using B-PAF (Fig 3.1B). PC12-AC

Figure 3.3: Quantitation and characterization of B-PAF internalization in PC12 cells.
(**A**) Two populations of PC12-AC cells were identified by live cell imaging. (**A,C**) PAF-unresponsive cells (**A,B**) PAF-responsive cells. (**A**) Results reported as Means ± SEM (n= 16-25 per data point). (**B,C**) Scale bars: 5 µm. A QuickTime movie of PAF internalization is provided on the CD ROM included in Appendix A.

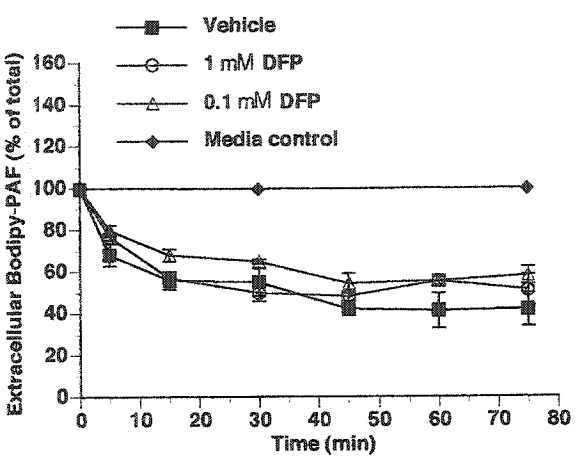

A
Fold increase in fluorescence intensity
4.50
4.00
3.50
3.00
2.50
2.00
1.50
1.00
0.50
PAF responsive
PAF unresponsive
00:00
00:20
00:40
01:00
01:20
01:40
02:00
B
C
00:00
00:20
00:40
01:00
01:20
01:40
02:00
Time

cultures were treated with B-PAF for up to 75 min at 37°C as described for internalization assays. Lipids in the extracellular media and lipids internalized in the cell cytosol were extracted immediately after ligand exposure (0 min) or following 5, 15, 30, 45, 60, and 75 min of treatment using a modified acidic methanol/chloroform extraction protocol. This treatment removes both labile PAF and PAF bound to carrier proteins at the plasma membrane in acid-resistant conformation (Ammit and O'Neill, 1997). This treatment could not be applied to live cells imaged over time due to the cytotoxicity associated with the acidified methanol wash. Fluorescent products were identified by TLC, and lipid yields were determined by analysis of fluorescence intensity. Most of the B-PAF recovered at 0 min (99.75$\pm$0.09) was present in the extracellular fraction (Fig 3.4A, Vehicle; Fig 3.5A, Extracellular). Maximal internalization was observed over the first 45 min of incubation (Fig 3.4A, Vehicle; Fig 3.5A, Cytosolic) with 42% of the recovered B-PAF detected in the extracellular fraction and 48% detected in the cytosolic fraction (Fig 3.4A, Vehicle; Fig 3.5A, Extracellular).

Cleavage of B-PAF by PAF-AH activity resulted in a fluorescent *lyso* lipid (B-*lyso*-PAF) that could be distinguished by a shift in mobility when run on a TLC plate. Initial conversion of B-PAF to B-*lyso*-PAF, the immediate metabolite of PAF, was evident between 5 and 15 min of incubation (Fig 3.4B, Vehicle). Linear metabolism was observed between 15 and 75 min (r^2=0.97, Fig 3.4B, Vehicle). To control for possible non-specific degradation over time, conversion of B-PAF to B-*lyso*-PAF in cell-free RPMI 1640 containing 0.1% BSA over time was

Figure 3.4: Metabolic fate and isoform contribution.
PC12 cells were pre-incubated with the PAF-AH inhibitor DFP at 1 mM and 0.1 mM or with PBS (vehicle) for 15 minutes. Following these treatments, 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**) The relative fluorescence of extracellular B-PAF in each fraction is expressed as the percentage of the total. DFP had no effect on the rate or extent of B-PAF internalization. (**B**) B-PAF degradation was measured by the percentage of total B-*lyso*-PAF over the percentage of the total fluorescence. 0.1 mM DFP inhibited PAF-AH activity by 38%, and 1 mM DFP elicited a further 17% reduction effectively inhibiting 55% of cytosolic PC12-AC PAF-AH activity. (**A,B**) Media control was done in cell free media at 0, 30 and 75 minutes to show the absence of PAF-AH activity in the media. Data represent the means of duplicate samples and are representative of two independent experiments.

A


Vehicle
1 mM DFP
0.1 mM DFP
Media control
Extracellular Bodipy-PAF (% of total)
160
140
120
100
80
60
40
20
0
0
10
20
30
40
50
60
70
80
Time (min)

B

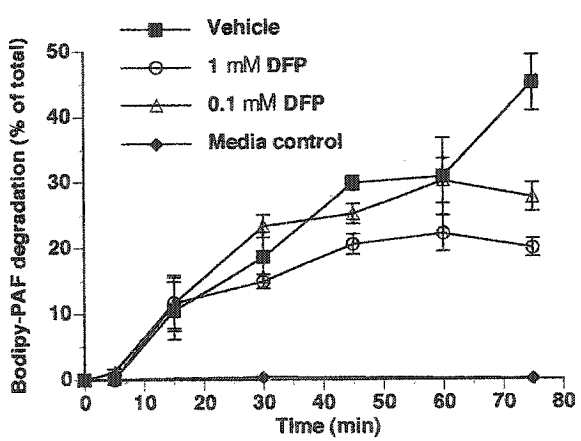
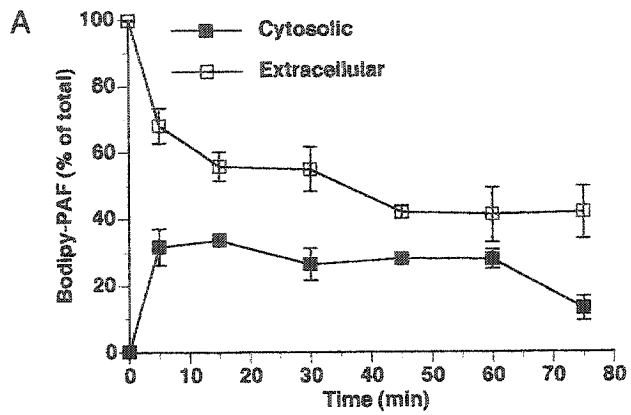
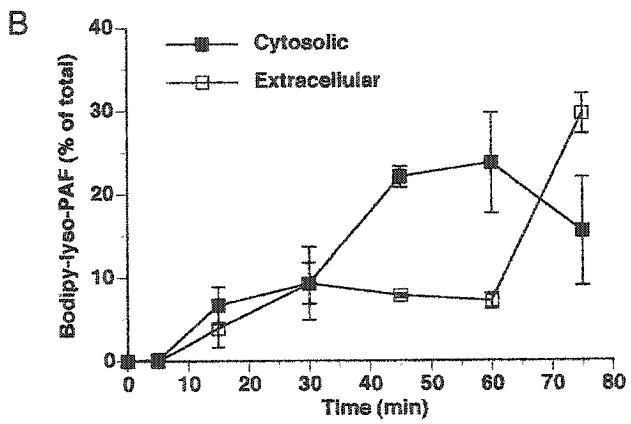
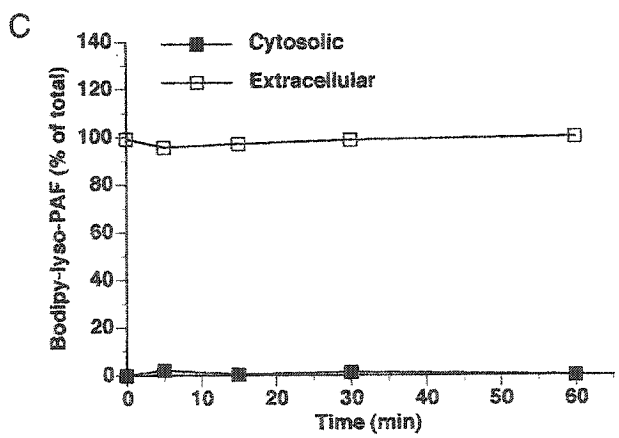

Vehicle
1 mM DFP
0.1 mM DFP
Media control
Bodipy-PAF degradation (% of total)
50
40
30
20
10
0
0
10
20
30
40
50
60
70
80
Time (min)

Figure 3.5: Compartmentalization of B-PAF and B-*lyso*-PAF to the cell cytosol or to the extracellular milieu.
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**) The relative fluorescence of B-PAF or (**B**) B-*lyso*-PAF in each fraction is expressed as the percentage of the total. (**C**) To establish whether B-*lyso*-PAF is internalized in PC12-AC cells, cultures were treated with 1 μM B-*lyso*-PAF for up to 60 min, and extracellular and cytosolic compartmentalization was assessed. (**A,B**) Data are the means of duplicate samples and are representative of two independent experiments.


A
Cytosolic
Extracellular
Bodipy-PAF (% of total)
Time (min)
0
20
40
60
80
100
0
10
20
30
40
50
60
70
80


B
Cytosolic
Extracellular
Bodipy-lyso-PAF (% of total)
Time (min)
0
10
20
30
40
0
10
20
30
40
50
60
70
80


C
Cytosolic
Extracellular
Bodipy-lyso-PAF (% of total)
Time (min)
0
20
40
60
80
100
120
140
0
10
20
30
40
50
60

assessed. No degradation over the 75 min test period was observed (Fig 3.4A,B, Media Control).

Compartmentalization of B-PAF and B-*lyso*-PAF to the cell cytosol or to the extracellular milieu was analyzed by fractionation and TLC. Maximal cytosolic B-PAF levels were reached within 5 min of exposure (Fig 3.5A, Cytosolic). Intracellular levels remained constant for up to 60 min followed by a 40% decrease in cellular B-PAF (Fig 3.5A, Cytosolic). Steady-state B-PAF levels (Fig 3.5A, Cytosolic) during active B-PAF internalization (Fig 3.3A, PAF-responsive) resulted from sustained metabolism of B-PAF to B-*lyso*-PAF by PC12-AC cells (Fig 3.4B, Vehicle). Between 5 and 30 min, 50% of the recovered B-*lyso*-PAF was identified in the extracellular fraction; 50% was retained in the cytosolic fraction (Fig 3.5B). No further increases in extracellular B-*lyso*-PAF were detected between 30 and 60 min, with PAF metabolites accumulating in cell cytosol (Fig 3.5B). A 4-fold increase in extracellular B-*lyso*-PAF was detected between 60 and 75 min (Fig 3.5B, Extracellular) corresponding with a decline in both cytosolic B-PAF and B-*lyso*-PAF (Fig 3.5A,B, Cytosolic). To establish whether B-*lyso*-PAF is internalized in PC12-AC cells, cultures were treated with 1 μM B-*lyso*-PAF for up to 60 min, and extracellular and cytosolic compartmentalization was assessed (Fig 3.5C). Exogenous B-*lyso*-PAF added directly to culture media was not internalized by PC12-AC cells (Fig 3.5C).

3.4.4 PAF-AH I activity predominates in PC12-AC cells.

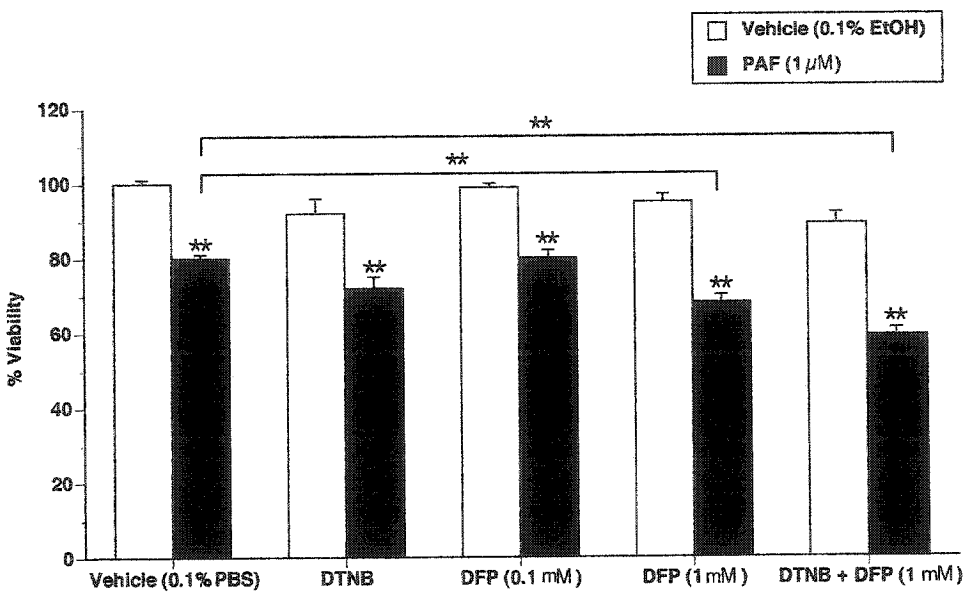
To provide pharmacological insight into the dominant PAF-AH isoform in PC12-AC cells, cultures were treated with the active serine-blocking reagent DFP. DFP is an active serine-blocking agent that has been used to inhibit activity of cytosolic PAF-AH isoforms in concentration-dependent fashion (Hattori et al., 1995a; Hattori et al., 1996; Manya et al., 1999). Acetylhydrolase activity mediated by a) PAF-AH I $\alpha 2/\alpha 2$ homodimers are reportedly resistant to DFP treatment, b) PAF-AH I $\alpha 1/\alpha 1$ homodimers or $\alpha 1/\alpha 2$ heterodimers are inhibited by 0.1 mM DFP, and c) PAF-AH II is inhibited by 1 mM DFP (Hattori et al., 1995a; Hattori et al., 1996; Manya et al., 1999). In the present study, DFP had no effect on the rate or extent of B-PAF internalization (Fig 3.4A, 0.1 mM DFP, 1 mM DFP). After 75 min of treatment, 0.1 mM DFP inhibited PAF-AH activity by 38% (Fig 3.4B, 0.1 mM DFP). Increasing the DFP concentration to 1 mM elicited a further 17% reduction effectively inhibiting 55% of cytosolic PC12-AC PAF-AH activity (Fig 3.4B, 1 mM DFP). 45% of the cytosolic PAF-AH activity was resistant to DFP within the 75 min treatment period. Given that the intracellular DFP concentrations must reach pharmacological efficacy, percent inhibition was confirmed by evaluating *in vitro* activity of cell lysates directly treated with DFP by Scott Ryan, a M.Sc. student in our laboratory (data not shown). As expected, the same trend was respected, but a more efficient inhibition was observed in cell lysates over time, revealing that 28% (as compared to 45% with the lipid extraction and TLC assay) of cytosolic activity was DFP-resistant, representing $\alpha 2/\alpha 2$ PAF-AH I homodimer activity. Taken together, these experiments provide pharmacological evidence suggesting that PAF-AH I activity predominates over

PAF-AH II activity, and that PAF-AH I enzymatic activity elicited by DFP-sensitive PAFAH I α1/α1 or α1/α2 homo or heterodimers in PC12-AC cells is likely greater than the DFP-resistant α2/α2 homodimers.

3.4.5 Inhibition of cytosolic PAF-AH activity increases PAF-mediated cytotoxicity.

To determine whether inhibition of cytosolic PAF-AH isoforms compromises PC12-AC survival following PAF challenge, cultures were treated with PAF (1 μM) or vehicle (0.1% EtOH) in the presence of DFP, DTNB, and/or vehicle (PBS) (Fig 3.6). DFP had no effect on the survival of vehicle-treated cells at 0.1 mM and little effect on the survival of vehicle-treated cells at 1 mM (Fig 3.6, Vehicle). The 38% inhibition of PAF-AH activity in 0.1 mM DFP-treated cells (Fig 3.4B) did not affect PC12-AC susceptibility to PAF-mediated death (Fig 3.6, PAF). Inhibition of PAF-AH I α1/α1, PAF-AH I α1/α2, and PAF-AH II activity by 1 mM DFP (Fig 3.4B) enhanced PAF-mediated cell death by an additional 11% ($p<0.01$, Fig 3.6, PAF). To ensure that PAF-AH II activitiy was completely inhibited, PC12-AC cells were also treated with a combination of 1 mM DFP and 1 mM DTNB. This combination of an active serine-blocking and a sulfhydryl-blocking reagent has been reported to completely abolish activity of purified PAF-AH II (Hattori et al., 1995a). PAF-treated cell survival was compromised when cells were pretreated with the combination of 1 mM DTNB and 1 mM DFP (Fig 3.6). However, DTNB/DFP-treated cell survival was not significantly different from what was observed following DFP treatment (absence of DTNB).

Figure 3.6: Inhibition of cytosolic PAF-AH activity increases PAF-mediated cytotoxicity in PC12 cells.
PC12 cultures were exposed for 24 h to PAF (1 μM) or vehicle (0.1% EtOH) in the presence of DTNB (1 mM), DFP (0.1 mM or 1 mM), a combination of DTNB (1 mM) with DFP (1 mM), and/or vehicle (PBS), in 0.025% BSA serum-free media. Partial inhibition of PAF-AH by the presence of 1 mM DFP enhanced PAF-mediated cell death. Cell survival was assessed by Trypan Blue exclusion assay. Data are expressed as percent survival of vehicle-treated cultures. Results are reported as Mean ± SEM of n=10-30 per condition. [**$p<0.01$, significant reduction in cell survival; ANOVA, *post hoc* Tukey test *t*-test].


Vehicle (0.1% EtOH)
PAF (1 μM)
% Viability
120
100
80
60
40
20
0
**
**
**
**
**
**
Vehicle (0.1% PBS)
DTNB
DFP (0.1 mM)
DFP (1 mM)
DTNB + DFP (1 mM)

These data indicate that PAF-mediated cell death is exacerbated by inhibition of cytosolic PAF-AH isoforms.

3.4.6 Stimulation of cytosolic PAF-AH activity protects cells from PAF-mediated death.

The abilities of three different PAF antagonists (CV 6209, CV 3988, and FR 49175) to block PAF-mediated cytotoxicity were examined. PAF-mediated cytotoxicity was inhibited by FR 49175 (0.5-10 μM) (Fig 3.7C) but not by CV 6209 (Fig 3.7A) or CV 3988 (Fig 3.7B).

The ability of the PAF antagonist FR 49175 to reduce PAF-mediated cell death in the absence of PAFR by stimulating PAF-AH activity was assessed in more detail by lipid extraction and TLC. FR 49175 (10 μM) did not affect the rate or extent of B-PAF internalization over the 75 min exposure period (Fig 3.8A) but accelerated the temporal kinetics of B-PAF degradation and release of cytosolic B-*lyso*-PAF (Fig 3.8B,C). In vehicle-treated cultures, steady-state intracellular B-PAF levels dropped 75 min after ligand exposure (Fig 3.8B), corresponding to a release of B-*lyso*-PAF into the extracellular milieu (Fig 3.8C). In FR 49175-treated cells, reduced cytosolic B-PAF levels were observed within 45 min of exposure (Fig 3.8B) corresponding to the release of B-*lyso*-PAF at this earlier time point (Fig 3.8C).

Figure 3.7: PC12 cell survival is afforded by the PAF antagonist FR 49175, but not CV 6209 or CV 3988, following challenge with PAF.
PC12 cells were exposed for 24 h to PAF (1 μM) or vehicle (0.1% EtOH) in the presence of different concentrations of PAF antagonists in low serum media. PAF-induced cell loss was inhibited by (**C**) FR 49175 (0.5-10 μM), but not (**A**) CV 6209 or (**B**) CV 3988. Cell survival was assessed by Trypan Blue exclusion assay. Data are expressed as percent survival of vehicle-treated cultures. Results are reported as Mean ± SEM of n=5-47 cultures per data point. [**$p<0.01$, significant reduction in cell survival compared to vehicle treatment; † $p<0.05$, †† $p<0.01$, significant protection relative to PAF-treated cultures; ANOVA, *post-hoc* Dunnett's *t*-test].

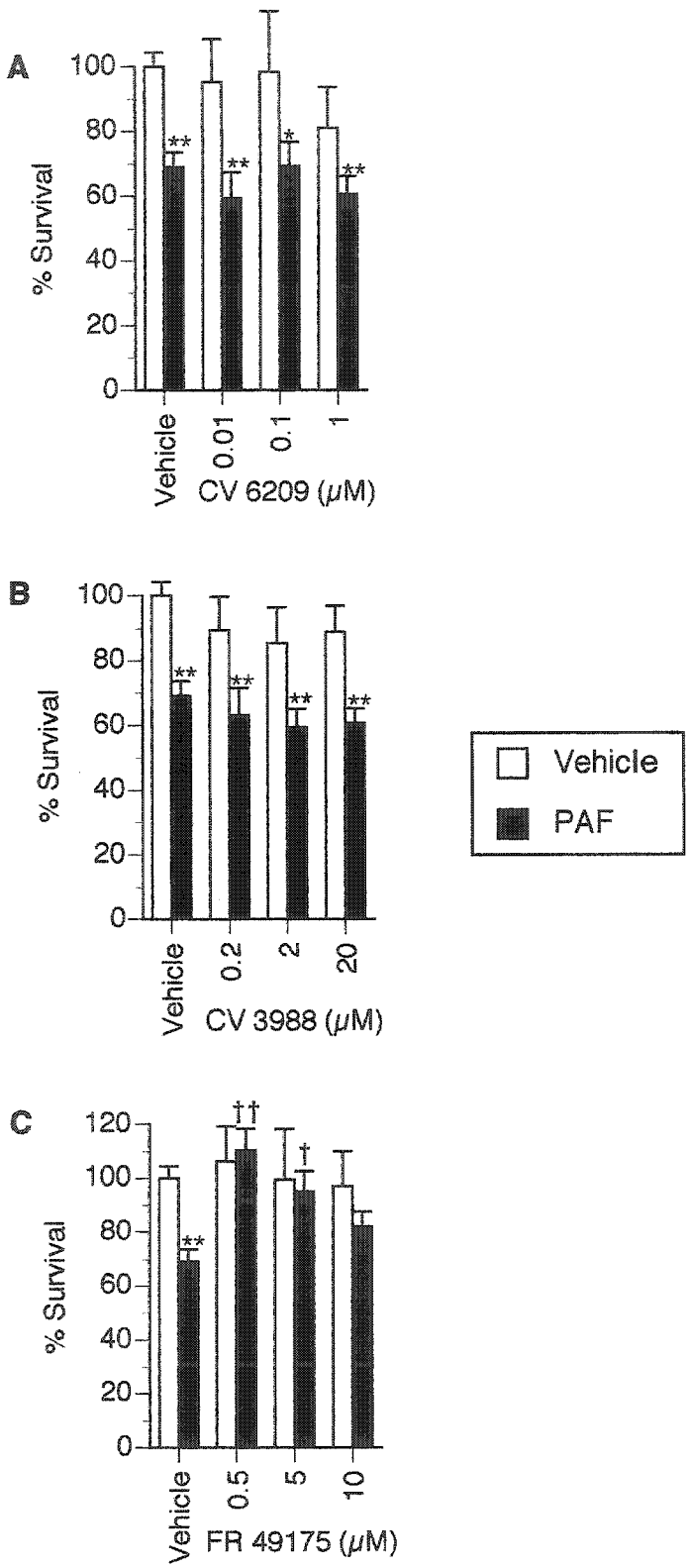
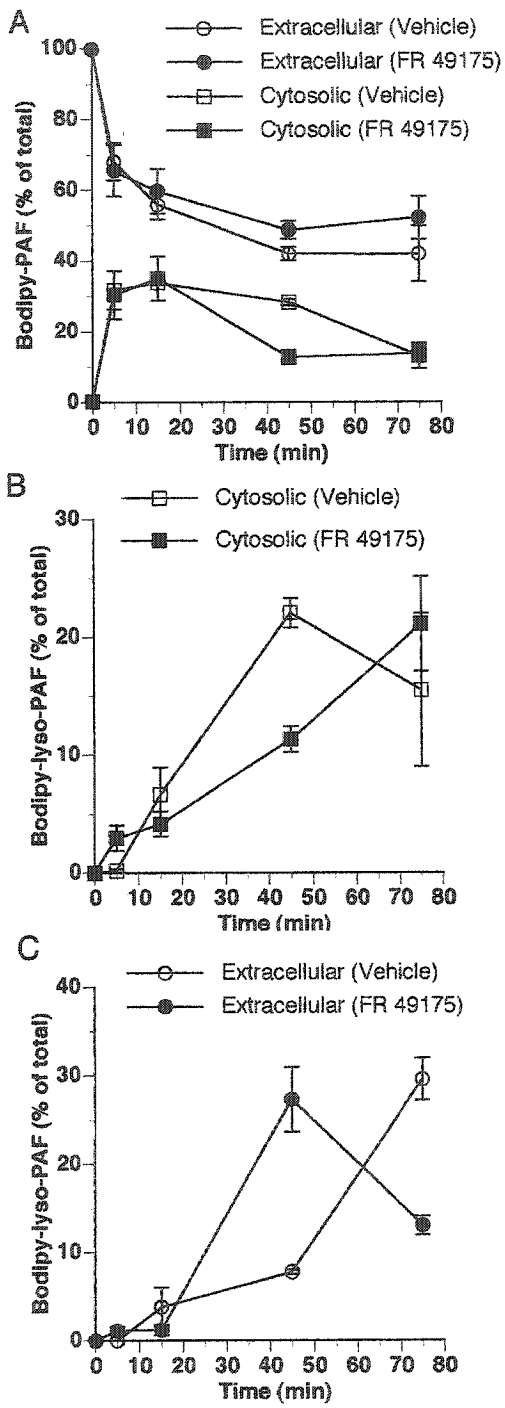

A
% Survival
100
80
60
40
20
0
**
**
*
**
Vehicle
0.01
0.1
1
CV 6209 (μM)
B
% Survival
100
80
60
40
20
0
**
**
**
**
Vehicle
0.2
2
20
CV 3988 (μM)
Vehicle
PAF
C
% Survival
120
100
80
60
40
20
0
**
††
†
Vehicle
0.5
5
10
FR 49175 (μM)

Figure 3.8: Stimulation of cytosolic PAF-AH activity by the PAF antagonist FR 49175.
PC12 cells were pre-incubated with the antagonist FR 49175 (10 μM) or with PBS (vehicle) for 15 minutes. Following these treatments, cells were incubated with 1 μM B-PAF at 37°C for 0, 5, 15, 45 and 75 minutes. At each time point, lipids were extracted from the extracellular and cytosolic fractions, and were separated by TLC. (**A-C**) The relative fluorescence of extracellular or cytosolic B-PAF or B-*lyso*-PAF in each fraction is expressed as the percentage of the total. (**A**) FR 49175 had no effect on the rate or extent of B-PAF internalization (Extracellular). (**B**) In FR 49175-treated cells, reduced cytosolic B-PAF levels were observed within 45 min of exposure as compared to 75 min in vehicle-treated cells, (**C**) corresponding to the release of B-*lyso*-PAF at this earlier time point. Data represent the means of duplicate samples and are representative of two independent experiments.


A
Extracellular (Vehicle)
Extracellular (FR 49175)
Cytosolic (Vehicle)
Cytosolic (FR 49175)
Bodipy-PAF (% of total)
100
80
60
40
20
0
0 10 20 30 40 50 60 70 80
Time (min)
B
Cytosolic (Vehicle)
Cytosolic (FR 49175)
Bodipy-lyso-PAF (% of total)
30
20
10
0
0 10 20 30 40 50 60 70 80
Time (min)
C
Extracellular (Vehicle)
Extracellular (FR 49175)
Bodipy-lyso-PAF (% of total)
40
30
20
10
0
0 10 20 30 40 50 60 70 80
Time (min)

3.5 Discussion

The concentrations of PAF in tissues or plasma are governed by the equilibrium between its biosynthesis and degradation. Both intracellular and plasma PAF appears to undergo rapid turnover through the actions of specific hydrolases which inactivate the lipid autacoid through a hydrolytic cleavage of the *sn*-2 ester bond, releasing free acetate and the immediate PAF metabolite *lyso*-PAF (Derewenda and Ho, 1999).

To establish whether PAF is metabolized by the cytosolic PAF-AH isoforms detected in Chapter 2 in PC12-AC cells, *in vitro* activity was assayed using C_{16}-2-thio-PAF. These data mark the first report of functional PAF-AH activity in PC12 cells. Results from Chapter 2 demonstrate that PC12-AC cells express PAF-AH II and all three subunits of PAF-AH I, but not the secreted plasma PAF-AH. In this Chapter, these data were confirmed at the functional level in PC12-AC cell lysates and by analyzing serum-free and conditioned media.

To provide insight into the kinetics of cytosolic PAF-AH activity in PC12-AC cells, time-lapse analysis of cell-associated fluorescence revealed that PAF is most rapidly internalized in a sub-population of PC12 cells over the first 40 min of exposure, with linear internalization continuing for up to 70 min. The observation that only 40% of the PC12-AC population was PAF-responsive over the 120 min treatment period is consistent with earlier data demonstrating that internalization of certain PAF analogues is largely dependent on the state of cellular activation (Bratton et al., 1992). Furthermore, given the fact that PC12 cells lack the known

G-protein coupled PAFR, these data indicate that PAF internalization mechanisms can occur in the absence of PAFR. This conclusion is consistent with earlier studies demonstrating that PAFR-independent internalization is accelerated by binding to the PAFR and subsequent endocytosis of ligand/receptor complexes (Gerard and Gerard, 1994; Ohshima et al., 2002). Previous data have suggested that the route of PAFR-independent internalization of PAF is enhanced transbilayer movement (flipping) across the plasma membrane, occurring as a result of changes in membrane physical properties accompanying cellular activation (Bratton et al., 1992).

PAF-AH activity in cells, tissues or biological fluids is usually detected with a radiochemical assay based on the chromatographic separation of labeled acetate enzymatically released from [$_3$H-acetyl]PAF (Blank et al., 1983). The purpose of the present study was to develop a sensitive technique to detect intracellular PAF-AH activity by fluorometric quantitation of B-PAF and B-*lyso*-PAF in a continuous assay. In the present study, a fluorescence-based assay for PAF-AH was developed based on previous studies using a Bodipy fluorophore attached at the *sn*-2 position, originally synthesized by (Hendrickson et al., 1999). However, the original assay was restricted to the plasma PAF-AH since the cytosolic enzyme has a more strict specificity for an acetyl group at the *sn*-2 position of PAF (for a review see (Stafforini et al., 1997)). The B-PAF used in our studies was custom synthesized for our laboratory by Molecular Probes, and can be used for cytosolic PAF-AH activity studies since the fluorophore is attached at the end of the *sn*-1 position of PAF.

Thus far, kinetic studies on PAF-AH activity have been conducted primarily on the plasma isoform. Previous studies showed that exogenously added PAF (10^{-9} M) has a half-life of 5 min in the plasma of normal individuals (Yoshida et al., 1996). Data from our studies on the kinetics of cytosolic PAF-AHs indicate that B-PAF internalization in PC12-AC cells occurs within the first 45 min of exposure, and that catabolism to B-*lyso*-PAF is initiated within 15 min of ligand internalization, and continues for at least 75 min. B-*lyso*-PAF is compartmentalized by PC12-AC and released into the extracellular milieu in three phases: phase 1) active release of half of the metabolized PAF is observed within the first 30 min of ligand exposure. Released PAF metabolites are presumably bound to albumin or other carrier protein in a protected configuration given that fractionation into a labile extracellular pool is only observed using a protocol capable of detecting secreted PAF in acid-resistant conformation (Ammit and O'Neill, 1997), and not using an acidic pH shown to strip uninternalized PAF from cell surface receptors (Ohshima et al., 2002); phase 2) between 30 and 60 min, B-*lyso*-PAF accumulates in the cell cytosol; phase 3) after 60 min of incubation, cytosolic B-*lyso*-PAF is released into the extracellular milieu. Other studies have examined whether the receptor-mediated PAF internalization has a significant role on intracellular PAF metabolism, and demonstrated that PAFR in macrophages significantly activates PAF degradation by enhancing internalization of receptor-bound PAF and the release of PAF-AH (Ohshima et al., 2002). When PAFR internalization was blocked by concanavalin A (ConA), which is known to inhibit the internalization of the PAFR (Le Gouill et al., 1997),

the metabolic conversion of PAF to *lyso*-PAF increased from $t_{1/2} \approx$ 20 min to $t_{1/2} \approx$ 40-50 min (Ohshima et al., 2002). PAFR-independent internalization in macrophages isolated from PAFR-knockout mice (Ohshima et al., 2002) is comparable to the rate of internalization observed in PAFR-negative PC12-AC cells in the present study.

Treatment with PAF-AH inhibitors was used to establish which PAF-AH isoform predominates in PC12 cells and to determine if the inhibition of cytosolic PAF-AH activity would increase PAF-mediated cytotoxicity. Serine-blocking agents are generally potent inhibitors of PAF-AHs (Chroni and Mavri-Vavayanni, 2000). Intracellular hydrophobic PAF-AHs (from bovine liver and mouse platelet) have been shown to be inhibited by PMSF, DTNB, and DFP (Hattori et al., 1995a; Karpouza and Vakirtzi-Lemonias, 1997). DFP is a serine-blocking agent inhibiting serine proteases and acetylcholinesterase. As previously mentioned, acetylhydrolase activity mediated by a) PAF-AH I $\alpha 2/\alpha 2$ homodimers are reportedly resistant to DFP treatment, b) PAF-AH I $\alpha 1/\alpha 1$ homodimers or $\alpha 1/\alpha 2$ heterodimers are inhibited by 0.1 mM DFP, and c) PAF-AH II is inhibited by 1 mM DFP (Hattori et al., 1995a; Hattori et al., 1996; Manya et al., 1999). Based on these studies, two different DFP concentrations were used as a pharmacological means of distinguishing between PAF-AH I and PAF-AH II enzymatic activity. In the present study, DFP had no effect on the rate or extent of B-PAF internalization, but had a partial inhibition of 38% in PAF degradation with 0.1 mM DFP, and a further 17% inhibition with 1 mM DFP, effectively inhibiting 55% of cytosolic PC12-AC PAF-AH activity. As mentioned previously,

a more efficient inhibition was obtained using an *in vitro* PAF-AH assay, since pharmacological inhibition of PAF-AH in living cells is not as optimal as in cell lysates. Taken together, these data suggest that relative enzymatic activities are PAF-AH I $\alpha1/\alpha2$[5] > PAF-AH I $\alpha2/\alpha2$ > PAF-AH II in PC12 cells, and provide strong evidence that PAF-AH I activity protects PC12 cells from pathological PAF challenge.

The PAF-AH I complex from bovine brain has previously been shown to be easily dissociated into catalytic $\alpha1/\alpha2$ heterodimer and the β subunit (Derewenda and Ho, 1999). Interestingly, labeling with radioactive inhibitor [^{3}H]DFP has shown that the $\alpha1$ subunit is significantly more active than the $\alpha2$ subunit (Hattori et al., 1993). However, when the two α subunits are overexpressed separately in *E coli*, the proteins invariably form stable homodimers, both of which are equally catalytically competent (Hattori et al., 1993). A 38% reduction in PAF-AH activity did not render PC12 cells more susceptible to PAF-mediated cytotoxicity, suggesting that catabolic activities from DFP-resistant PAF-AH I $\alpha2/\alpha2$ and from PAF-AH II are sufficient to prevent supplemental reduction in cell survival. However, inhibition of PAF-AH I $\alpha1/\alpha1$, PAF-AH I $\alpha1/\alpha2$, and PAF-AH II activities generated a significant enhancement of PAF-mediated cell death in PC12 cells. These data demonstrate that PAF-mediated cell loss is exacerbated by inhibition of cytosolic PAF-AH isoforms.

Three PAF antagonists, two PAF analogs (CV 3988 and CV 6209) and a structurally unrelated analog (FR 49175) have been screened for their ability to

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[5] $\alpha1/\alpha1$ homodimers have not been detected *in vivo* to date (Manya et al., 1998).

inhibit PAF-induced PC12-AC cell death. PAF-induced cytotoxicity in PC12-AC cells was attenuated by FR 49175, but not CV 6209 and CV 3988. These data confirm our previous report demonstrating that CV 3988 does not exhibit cytoprotective activity in the absence of PAFR (Brewer et al., 2002). CV 6209 has been shown to exhibit a binding profile consistent with a single binding site (Marcheselli et al., 1990). CV 3988 has proven specificity for PAFR (Dupré et al., 2001). FR 49175 has been shown to have the lowest affinity for PAFR and to exhibit the least inhibitory activity on PAFR-mediated effects (Dupré et al., 2001). In keeping with these PAFR-specific profiles, CV 6209 and CV 3988 did not inhibit cell death in PAFR-negative PC12 cells. In the absence of PAFR in PC12 cells, we hypothesized that the effects of FR 49175 act downstream of receptor activation. We provide direct evidence that FR 49175 accelerates the kinetics of PAF degradation and the release of cytosolic B-*lyso*-PAF. Given the fact that FR 49175 did not affect the rate or extent of B-PAF internalization over the 75 min exposure period, it is likely that this PAF antagonist is not acting on a plasma-bound protein or altering the physicochemical properties of the plasma membrane in PAFR-negative cells. FR 49175 is a derivative of the fungal metabolite gliotoxin (Okamoto et al., 1986). Other gliotoxin derivates have been shown to exert a diverse range of biological activities, including antimicrobial, antifungal and antiviral properties, as well as DNA-damaging and immunomodulating activity (Beaver and Waring, 1994; Nicholas et al., 2003). These activities primarily hinge on the presence of the disulfide bond that can form mixed disulfides with free thiols present in protein targets, or form ROS in

the presence of reducing agents, such as glutathione (Nicholas et al., 2003). A recent study done on kinetics inhibition by natural product inhibitors revealed that a dithiadiketopiperazine gliotoxin related to FR 49175 is a non-competitive enzymatic inhibitor that can bind to free enzyme and enzyme–substrate complexes (Nicholas et al., 2003). Thus, FR 49175 represents a potential therapeutic agent to block PAF-mediated cytotoxicity by modulating PAF-AH activity.

In summary, these data build upon our mechanistic understanding of how PAF-mediated cytotoxicity is regulated in cells. The kinetics of cytosolic intracellular PAF-AHs has revealed that these enzymes have an important role to play in PAF degradation and in protection against PAF-mediated cell death. Furthermore, we provide evidence that PAF-AH activity can be accelerated by at least one PAF inhibitor and thereby protect cells from PAF-induced cell loss. These data point to a novel pathway that can be pharmacologically manipulated to promote cell survival following apoptotic challenge.

CHAPTER 4: ELABORATION OF TOOLS TO DETERMINE THE EFFECT OF FUNCTIONAL LIS1 AND MUTANT LIS1 OVEREXPRESSION ON PAF-AH ACTIVITY AND APOPTOTIC SUSCEPTIBILITY.

4.1 Introduction

The enzymatic activity of PAF-AH I is regulated in multiple ways by switching the composition of the α1 and α2 catalytic subunits, and by manipulating the regulatory β subunit (LIS1) (Manya et al., 1998; Manya et al., 1999). The β subunit has been shown to strongly accelerate the enzymatic activity of the α2/α2 homodimer but to suppress the activity of the α1/α1 homodimer, and to have little effect on the α1/α2 (Manya et al., 1999). All human *Lis1* mutations examined to date abolish or reduce the capacity of LIS1 protein to interact with the α1 and α2 catalytic subunits. As reviewed in Chapter 1, mutations in the *Lis1* gene coding for the regulatory 45 kD subunit (β) of trimeric brain PAF-AH I are the genetic determinant of MDS, a developmental brain anomaly characterized by type 1 lissencephaly (Reiner et al., 1993; Hirotsune et al., 1998). Lissencephaly is a severe brain malformation characterized by abnormal positioning of cortical layers resulting from defective neuronal migration (Hirotsune et al., 1998). Afflicted individuals present with mild to profound developmental learning and memory deficits, experience recurring epileptic seizures, and face a reduced life expectancy (Vallee et al., 2001). *Lis1* loss of function may prevent the PAF-AH I complex from recognizing its substrate, thereby leading to abnormal levels of PAF during cerebral

development, and subsequent impairment of neuronal migration (Clark et al., 1995; Stafforini et al., 1996). Alternatively, as suggested by the findings of Manya et al (1999), *Lis1* mutation may abrogate regulation of catalytic activity of the two homodimers thereby disrupting PAF homeostasis.

4.2 Objectives

In order to gain molecular insight into the role of LIS1 in regulating the anti-apoptotic action of PAF-AH I at the physiological and pathophysiological levels in the CNS, two mammalian expression vectors expressing a) wild-type (WT) *Lis1* and b) a truncated *Lis1* mutant were constructed: pCDM8/Flag-LIS1WT and pcDM8/Flag-LIS1Δ144. The truncated protein is the result of a missense mutation identified in MDS patients. To facilitate expression in both PC12 cells and terminally differentiated primary neuronal cultures for future study, two adenoviral expression vectors were generated using the pAdTrack-CMV adenoviral vector: pAdTrack/LIS1WT and pAdTrack/LIS1Δ144. Adenoviral vectors were sent to the NRI Adenoviral Core Facility for packaging into 293T cells, titring, and verification of protein expression. These tools will be used by other members of the Bennett laboratory to determine the effect of overexpression of functional or mutant *Lis1* on cell viability and PAF-AH I catalytic activity. Moreover, PAF-AH expression in postnatal rat primary cerebellar, cortical, and hippocampal cultures has also been characterized to further elucidate the role of LIS1 in the brain. Together, the PC12 and primary neuronal models and the adenoviral tools generated in this thesis will permit a

closer examination of the specificities of the α1 and α2 catalytic activities, the anti-apoptotic actions, and the regulatory effects of LIS1.

4.3 Materials and Methods

Mammalian expression constructs

WT rat *Lis1* was amplified from adult Wistar rat brain mRNA template by RT-PCR as described in Chapter 2 for PAF-AH II. PCR primers corresponding to the 5'- and 3'- complete coding sequence of rat *Lis1* were prepared containing an *Eco*RI site (Sense: 5' GAATTCACAGCCAAACTGGTGCTGTC-3') and a *Xba*I site (Antisense: 5'-TCTAGAACTCAATCAACGGCACTCCC-3'), compatible for insertion into the pCDM8/Flag vector (kindly provided by Dr. N. Gerard, Harvard Medical School). The sense primer used has been designed to mutate the normal initiating methionine of LIS1 to a leucine (ATG→CTG), placing the *Lis1* sequence downstream of the initiating ATG of the Flag sequence in pCDM8/Flag. For the creation of the mutant rat *Lis1* coding sequence, primer sequences corresponding to the 5'- and 3'-coding sequence of the mutant rat *Lis1* were prepared using the same sense primer as the *Lis1 WT* containing *Eco*RI site (Sense: 5' GAATTCACAGCCAAACTGGTGCTGTC-3'). The antisense primer sequence contains a *Xba*I site (Antisense 5'-GGCCTCTAGACCCTTGAGA GTTCACTCAAA-3'), and was designed to introduce a non-sense mutation by PCR at position 430 (C→T) of the complete rat *Lis1* coding sequence, resulting in a truncated protein of 144 amino acids, described clinically as a mutation producing a grade 2 MDS phenotype (Pilz et al., 1998). All restriction

endonucleases and ligation reagents were obtained from Promega. Two µl of the cDNA RT reaction mix from Wistar rat brain tissue were PCR amplified in a total volume of 50 µl containing 1X PCR buffer (20 mM Tris-HCl (pH 8.4), 50 mM KCl), dNTPs; (200 µM each, Promega), 5 U of *Taq* DNA polymerase, 1 mM $MgCl_2$ for LIS1WT and 1.5 mM $MgCl_2$ for LIS1Δ144, and 20 pmol per primer for LIS1WT, and 25 pmol per primer for LIS1Δ144. The PCR reactions were brought up to a final volume of 50 µl with NF-H_2O (Promega) and amplified in a GeneAmp PCR System 2400 (Applied Biosystems): 94°C for 5 minutes, 30-35 cycles of 94°C for 30 seconds, 55°C for 60 seconds (LIS1Δ144) or 58°C for 30 seconds (LIS1WT), and 72°C for 2 minutes (LIS1Δ144) or 72°C for 45 seconds (LIS1WT), followed by a final incubation at 72°C for 7 minutes. Control reactions are as described in Materials and Methods of Chapter 3. The LIS1WT PCR amplicon, the LIS1Δ144 PCR amplicon and the pCDM8/Flag-PAFR vector were digested with *Eco*RI and *Xba*I according to the recommendations provided by the manufacturer. All restriction enzyme digests were agarose gel purified (Quiagen) and combined in standard ligation reactions containing T4 DNA ligase enzyme with varying vector: insert ratios (including a control reaction for vector recircularization), according to recommendations of the manufacturer, and incubated overnight at 4°C. The ligation reactions were diluted and transformed into *E. coli* MC1061/P3 ultracompetent cells (Invitrogen). Antibiotic resistant colonies were selected and their plasmids purified. Restriction enzyme digest analyses of purified pCDM8/Flag-LIS1WT and pCDM8/Flag-LIS1Δ144 plasmids were performed according to the protocol provided by the manufacturer, followed

by direct DNA sequencing on both strands with the T7 forward primer and their respective antisense primer. Sequencing analyses and primer syntheses were performed at the BRI (University of Ottawa).

Adenoviral expression constructs

The adenoviral expression vector pAdTrack-CMV (kindly provided by Dr. Ruth Slack, University of Ottawa) was used to create the pAdTrack/LIS1WT and the pAdTrack/LIS1Δ144 constructs. The Flag-LIS1WT gene and the Flag-LIS1Δ144 gene were excised from the pCDM8/Flag-LIS1WT and pCDM8/Flag-LIS1Δ144 plasmids by restriction enzyme digestion with *Hin*dIII and *Xba*I for subsequent ligation into the multiple cloning site of the pAdTrack-CMV vector, also digested with *Hin*dIII and *Xba*I. All restriction enzyme digests were agarose gel purified and combined in standard ligation reactions containing T4 DNA ligase enzyme with varying vector: insert ratios (including a control reaction for vector recircularization) and incubated overnight at 4°C. Transformations were performed using JM109 High Efficiency Competent Cells (Promega). Standard plasmid miniprep procedures were used on antibiotic resistant colonies for screening and isolation of recombinant plasmid DNA (Promega). Based on these screening results, large-scale recombinant plasmid DNA preparations were performed (Qiagen). Restriction enzyme digest analyses of purified pAdTrack/Flag-LIS1WT and pAdTrack/Flag-LIS1Δ144 plasmids were performed to verify inserts, followed by direct DNA sequencing on both strands. Sequencing analyses were performed at the BRI (University of Ottawa). These

constructs have been packaged by the NRI Adenoviral Core Facility (Dr. Ruth Slack, University of Ottawa).

PC12-AC cell cultures

PC12-AC cells were cultured as described in Chapter 2. Stock cultures were fed with complete media every 2-3 days and passaged when sub-confluent.

Primary cultures

Wistar rat pups (Charles Rivers) were deeply anesthetized with sodium pentobarbital on postnatal day 6 and 10 (P6 and P10) and euthanized by decapitation. Brains were removed and placed in a 10 cm diameter tissue culture plate containing artificial cerebrospinal fluid (ACF; 26 mM $NaHCO_3$, 124 mM NaCl, 5mM KCl, 2 mM $CaCl_2$, 1.3 mM $MgCl_2$, 10 mM D-glucose, 100 units/ml penicillin, 100 µg/ml streptomycin, pH 7.3). Whole brains were separated into cortical, hippocampal and cerebellar regions and placed in separated 10 cm diameter tissue culture plates containing ACF. Dissected tissues were minced into fine strips with a scalpel, transferred to single sterile 50 ml polypropylene tubes and centrifuged at 500 rpm for 5 min. Trypsin/EDTA (0.05% Trypsin, 0.53 mM EDTA) was added (5 ml) and tubes were lightly vortexed at the lowest vortexer/mixer setting. Tubes were capped and incubated for 10 min at 37°C in a hybridization oven with rotation. Plating media (Neurobasal medium, 10% heat inactivated fetal calf serum (FCS), 10% HS, 100 units/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine) was added (5 ml) and samples were

centrifuged at 500 rpm for 5 min. The pellet was incubated for 10 min at 37°C with 10x transcription buffer (Promega) and 5 U of RQ1-DNase (Promega) in a total volume of 500 µl. Plating media (10 ml) was added and tissue was dissociated by trituration through successive 10 ml, 5 ml, 1 ml, and pasteur pipettes. Cell number and viability was determined by Trypan Blue hemocytometer counts. Approximately 95% of all cells excluded the dye after the dissociation procedure. 5 ml of the cell suspension was plated per 10 cm diameter tissue culture plate containing coverslips pretreated with 100 mg/ml poly L-lysine for 2 h (air dried and washed with plating media before use) and maintained in a 5% CO_2/95% air atmosphere. Primary cultures were subsequently maintained in Neurobasal medium containing 100 units/ml penicillin, 100 µg/ml streptomycin, 1x B27 supplement, and 2 mM L-glutamine. To suppress glial contamination, P6 cerebellar cells were cultured in the presence of mitotic inhibitors (1 µM Cytosine b-D-arabinofuranoside (araC), 10 µM 5-fluoro-2'-deoxyuridine (FUdR), 10 µM uridine (Urd)). All culture reagents were obtained from Invitrogen. Mitotic inhibitors were obtained from Sigma. All animal manipulations were performed in compliance with approved institutional protocols and according to the strict ethical guidelines for animal experimentation established by the Canadian Institutes of Health Research.

RT-PCR analysis of cytosolic PAF-AH isoforms

Total RNA was isolated from primary culture cells 10 days after plating, using Trizol reagent (Invitrogen). RT of total RNA extracted from primary

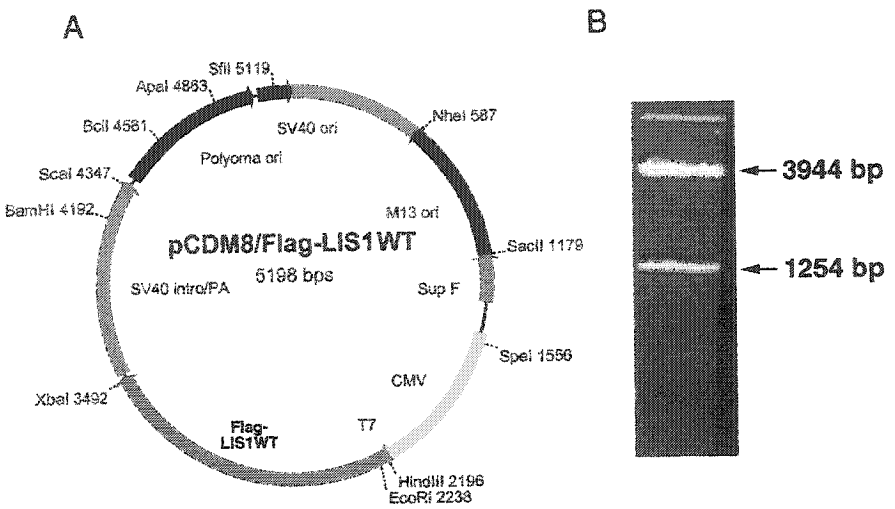
cultures and subsequent PCR analysis were performed as described in Chapter 2 Methods, using the same primer sequences as provided in Table 2.1.

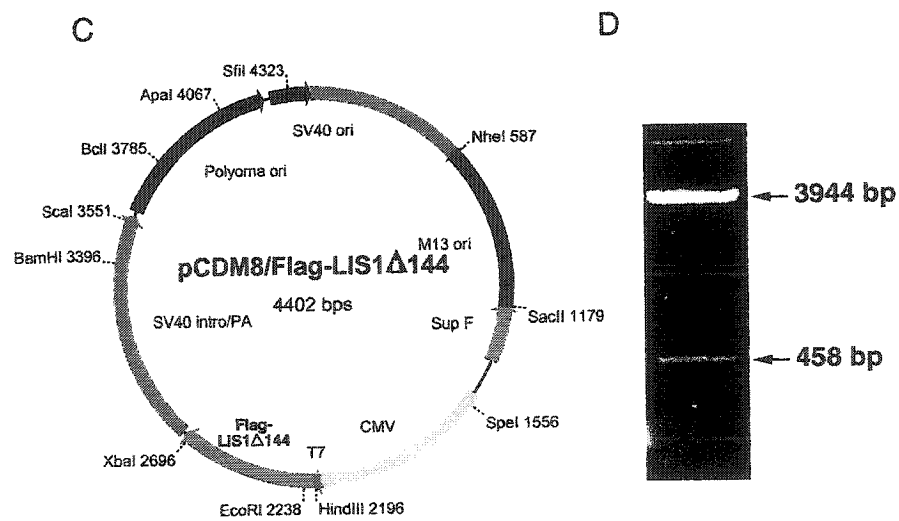
4.4 Results

4.4.1 Creation of rat LIS1 mammalian and adenoviral expression vectors.

The complete rat *Lis1* cDNA was isolated from rat brain tissue by PCR, using primers designed with *Eco*RI (N-terminus) and *Xba*I (C-terminus). The final product (with compatible ends) was sequenced on both strands and inserted into a pCDM8 vector containing a Flag-tagged sequence. The resulting plasmid is referred to as pCDM8/Flag-LIS1WT (Fig 4.1A). Restriction enzyme digestion confirmed insertion of *Lis1*WT (Fig 4.1B). The integrity of the pCDM8/Flag-LIS1WT plasmid and the appropriate orientation of the gene in respect to the cytomegalovirus (CMV) promoter were further confirmed by direct sequencing. When double-digested with *Eco*RI and *Xba*I restriction enzymes, the pCDM8/Flag-LIS1WT vector contained a 1254 bp fragment corresponding to the *Lis1* insert and a 3944 bp fragment corresponding to the pCDM8/Flag vector sequence (Fig 4.1B). The complete *Lis1* sequence, Flag-tagged at the N-terminus, was excised from the pCDM8/Flag-LIS1WT vector by restriction enzyme digestion with *Hind*III and *Xba*I and inserted into the pAdTrack-CMV adenoviral vector. The resulting plasmid is referred to as pAdTrack/LIS1WT (Fig

Figure 4.1: Generation of mammalian LIS1 WT and LIS1Δ144 expression vectors.
The coding region of the rat *Lis1* (LIS1WT) gene and the mutated rat *Lis1* (LIS1Δ144) gene were amplified from rat brain RNA template by RT-PCR and inserted into the pCDM8/Flag expression vector. (**A**) The resulting WT construct is referred to as pCDM8/Flag-LIS1WT and expresses WT LIS1 as a Flag fusion protein. (**B**) Successful insertion of the WT LIS1 gene was confirmed by *Eco*RI/*Xba*I restriction endonuclease digest analysis. (**C**) The mutant construct is referred to as pCDM8/Flag-LIS1Δ144 and expresses LIS1Δ144 as a Flag fusion protein. (**D**) Successful insertion of the LIS1Δ144 gene was confirmed by restriction endonuclease digest analysis.


A
B
SfiI 5119
ApaI 4883
NheI 587
SV40 ori
BclI 4581
Polyoma ori
ScaI 4347
BamHI 4192
M13 ori
pCDM8/Flag-LIS1WT
5198 bps
SacII 1179
SV40 intro/PA
Sup F
SpeI 1556
CMV
XbaI 3492
Flag-LIS1WT
T7
HindIII 2196
EcoRI 2238
3944 bp
1254 bp

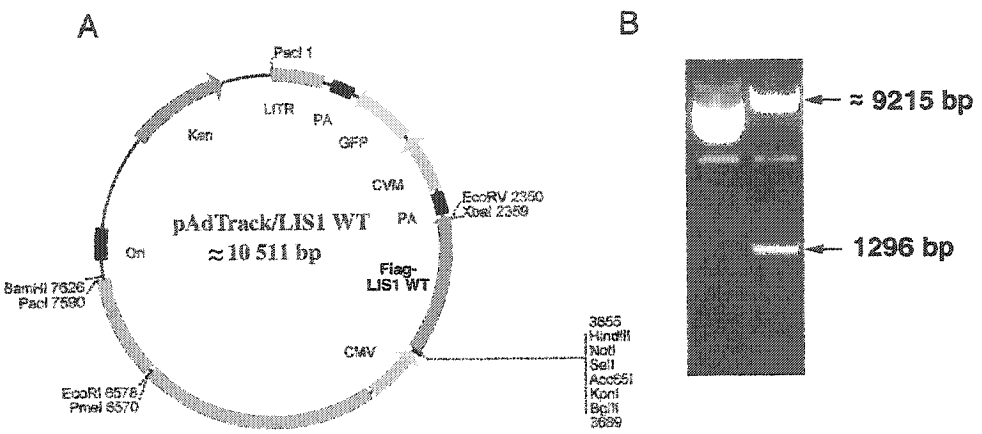

C
D
SfiI 4323
ApaI 4067
SV40 ori
NheI 587
BclI 3785
Polyoma ori
ScaI 3551
M13 ori
BamHI 3396
pCDM8/Flag-LIS1Δ144
4402 bps
SV40 intro/PA
Sup F
SacII 1179
SpeI 1556
Flag-LIS1Δ144
CMV
T7
XbaI 2696
EcoRI 2238
HindIII 2196
3944 bp
458 bp

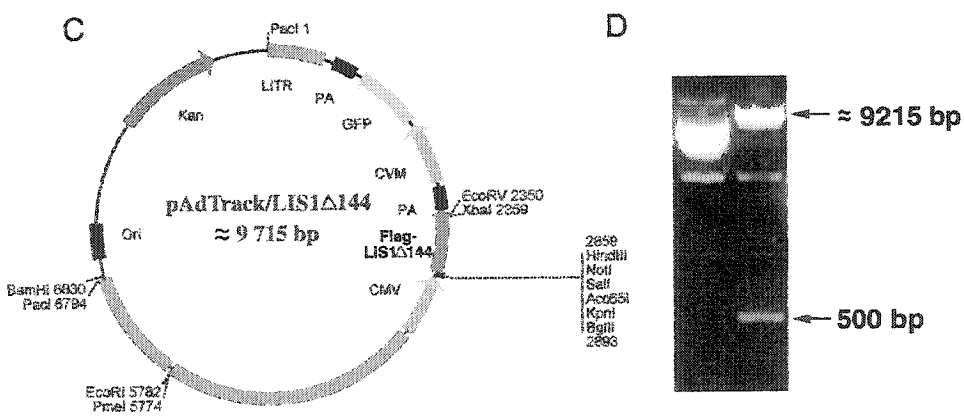
4.2A). Restriction enzyme digestion confirmed insertion of Flag-LIS1WT (Fig 4.2B). The integrity of the pAdTrack/LIS1WT plasmid and the appropriate orientation of the gene in respect to the CMV promoters were further confirmed by direct sequencing. When double-digested with *Hin*dIII and *Xba*I restriction enzymes, the pAdTrack/LIS1WT vector contains a 1296 bp fragment corresponding to the Flag-LIS1WT insert and an estimated 9215 bp fragment, corresponding to the pAdTrack-CMV vector sequence (Fig 4.2B). The estimated size is due to the fact that the pAdTrack-CMV vector is not completely sequenced. The adenoviral expression vector pAdTrack/LIS1WT was sent to the NRI Adenoviral Core Facility, for adenoviral construction and protein expression verification on transiently transfected 293T cells by immunocytochemistry and Western analysis using anti-Flag antibody M2 (Sigma) (data not shown).

4.4.2 Creation of mutant rat LIS1 mammalian and adenoviral expression vectors.

To create the mutant *Lis1*, a non-sense mutation was introduced by PCR at position 430 (C→T) of the complete rat *Lis1* coding sequence, resulting in a truncated protein of 144 amino acids described as a grade 2 MDS phenotypic mutation. The mutated rat *Lis1* cDNA was isolated from rat brain tissue by PCR, using primers designed with *Eco*RI (N-terminus) and *Xba*I (C-terminus). The final product (with compatible ends) was sequenced on both strands and inserted into a pCDM8 vector containing a Flag-tagged sequence. The resulting plasmid is referred to as pCDM8/Flag-LIS1Δ144 (Fig 4.1C). Restriction enzyme digestion

Figure 4.2: Generation of adenoviral LIS1 WT and LIS1Δ144 expression vectors.
(**A**) The Flag-LIS1WT cDNA was subcloned from pCDM8/Flag-LIS1WT vector into pAdTrack-CMV vector using existing *Hin*dIII and *Xba*I restriction enzymes. The resulting plasmid is referred to as pAdTrack/LIS1WT. (**B**) Successful insertion of the Flag-LIS1 WT gene was confirmed by *Hin*dIII/*Xba*I restriction endonuclease digest analysis. (**C**) The Flag-LIS1Δ144 fusion protein sequence was subcloned from pCDM8/Flag-LIS1Δ144 vector into pAdTrack-CMV vector using existing *Hin*dIII and *Xba*I restriction enzymes. The resulting plasmid is referred to as pAdTrack/LIS1Δ144. (**D**) Successful insertion of the Flag-LIS1Δ144 gene was confirmed by restriction endonuclease digest analysis.


A
B
PacI 1
LITR
PA
GFP
Kan
CVM
EcoRV 2350
XbaI 2359
pAdTrack/LIS1 WT
≈10 511 bp
PA
Ori
Flag-
LIS1 WT
BamHI 7626
PacI 7590
CMV
3655
HindIII
NotI
SalI
Acc65I
KpnI
BglII
3689
EcoRI 8578
PmeI 8570
≈ 9215 bp
1296 bp

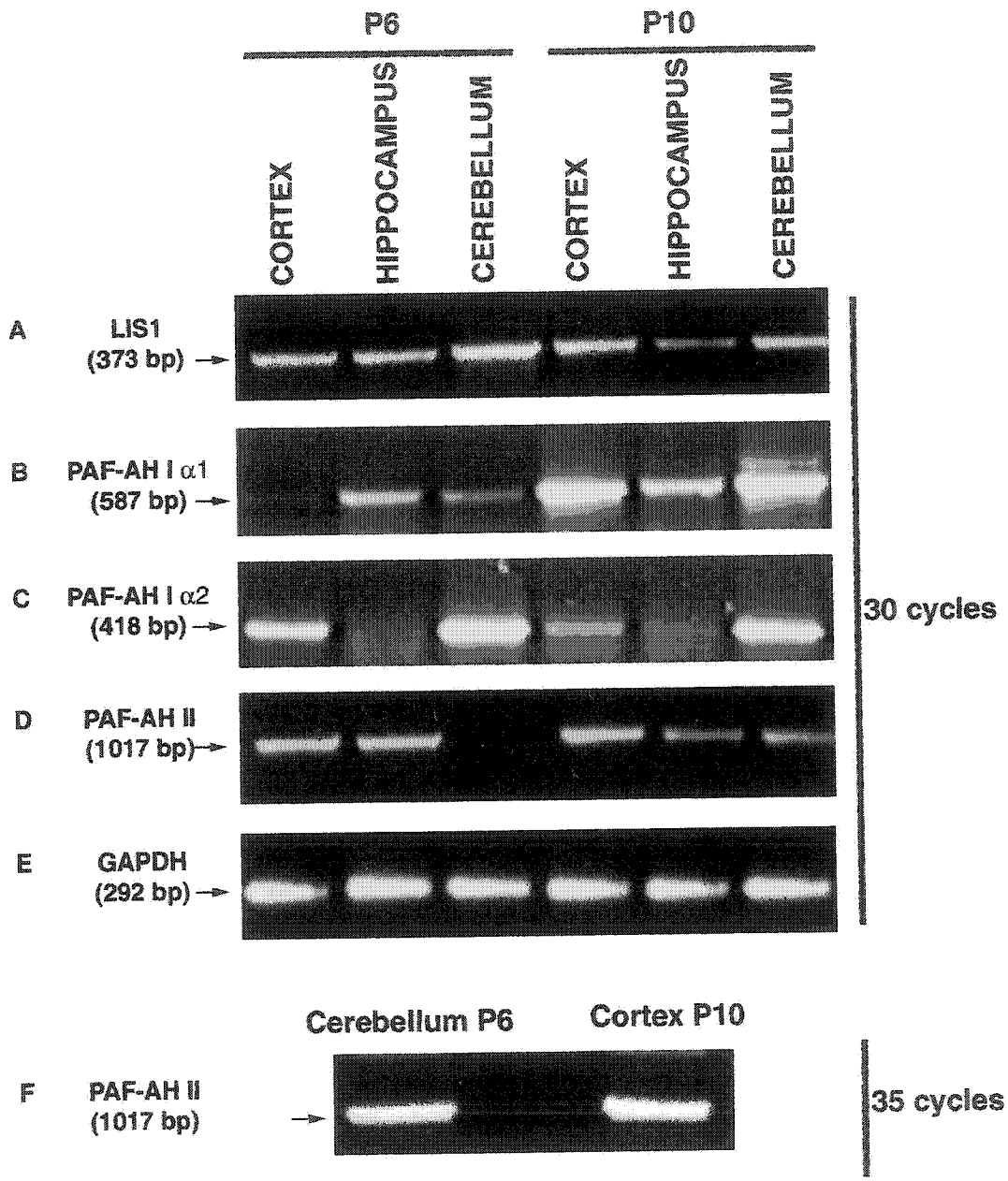

C
D
PacI 1
LITR
PA
GFP
Kan
CVM
EcoRV 2350
XbaI 2359
pAdTrack/LIS1Δ144
≈ 9 715 bp
PA
Ori
Flag-
LIS1Δ144
2659
HindIII
NotI
SalI
Acc65I
KpnI
BglII
2693
CMV
BamHI 8630
PacI 8794
EcoRI 5782
PmeI 5774
≈ 9215 bp
500 bp

confirmed insertion of LIS1Δ144 (Fig 4.1D). The integrity of the pCDM8/Flag-LIS1Δ144 plasmid and the appropriate orientation of the gene in respect to the CMV promoter were further confirmed by direct sequencing. When double-digested with *Eco*RI and *Xba*I restriction enzymes, the pCDM8/Flag-LIS1Δ144 vector contains a 458 bp fragment corresponding to the mutant *Lis1* insert, and a 3944 bp fragment corresponding to the pCDM8/Flag vector sequence (Fig 4.1D). The complete LIS1Δ144 sequence, Flag-tagged at the N-terminus, was excised from the pCDM8/Flag-LIS1Δ144 vector by restriction enzyme digestion with *Hin*dIII and *Xba*I and inserted into the pAdTrack-CMV adenoviral vector. The resulting plasmid is referred to as pAdTrack/LIS1Δ144 (Fig 4.2C). Restriction enzyme digestion confirmed insertion of Flag-LIS1Δ144 (Fig 4.2D). The integrity of the pAdTrack/LIS1Δ144 plasmid and the appropriate orientation of the gene in respect to the CMV promoters were further confirmed by direct sequencing. When double-digested with *Hin*dIII and *Xba*I restriction enzymes, the pAdTrack/LIS1Δ144 vector contains a 500 bp fragment corresponding to the Flag-LIS1Δ144 insert and an estimated 9215 bp fragment, corresponding to the pAdTrack-CMV vector sequence (Fig 4.2B). The adenoviral expression vector pAdTrack/LIS1Δ144 was sent to the NRI Adenoviral Core Facility for adenoviral construction and protein expression verification as described above (data not shown).

4.4.3 Expression of endogenous Lis1, PAF-AH I α1, PAF-AH I α2, and PAF-AH II mRNA transcripts in cortical, cerebellar and hippocampal primary cultures.

Expression of intracellular PAF-AH mRNAs was investigated by RT-PCR of cortical, cerebellar and hippocampal primary cultures from P6 and P10 Wistar rats. RT-PCR was performed using primers that recognize rodent transcripts for *Lis1* (Fig 4.3A), PAF-AH I α1 (Fig 4.3B), PAF-AH I α2 (Fig 4.3C) and PAF-AH II (Fig 4.3D,F), defined by amplicons of 373, 587, 418 and 1017 bp respectively. The "housekeeping" gene, GAPDH, was also amplified as an internal standard (Fig 4.3E). Several control reactions were carried out in parallel to exclude false positive and false negative results for each isoform analyzed (data not shown). Expression of *Lis1* subunit mRNA was observed in all tested cultures (Fig 4.3A). PAF-AH II mRNA expression was observed in all cultures with a 30-35 cycles PCR reaction (Fig 4.3D,F). PAF-AH I α1 transcript was observed in all cultures tested, but the level of expression appeared to be reduced in P6 cortical cultures (Fig 4.3B). High PAF-AH I α2 expression levels in the cerebellum were maintained at P6 and P10 (Fig 4.3C; cerebellum), whereas levels were reduced in primary hippocampal cultures (Fig 4.3C; hippocampus). Note that cultures were not treated with mitotic inhibitors and, as such, represent mixed neuronal/glial cultures (cerebellum and hippocampus) or primarily glial cultures (cortical) based on the previous immunocytochemical characterizations performed in our laboratory.

Figure 4.3: Rat primary mixed neuronal/glial cultures express all three PAF-AH I subunits (LIS1, α1, α2) and PAF-AH II mRNA.
RT-PCR analysis was performed for (**A**) *Lis1*, (**B**) PAF-AH I α1, (**C**) PAF-AH I α2 and (**D,F**) PAF-AH II, in primary cultures from P6/P10 rat brain (cortex, hippocampus and cerebellum), defining amplicons of 373, 587, 418 and 1017 bp respectively. The absence of genomic DNA contamination and reagent contamination was verified by performing control reactions in parallel (data not shown). (**E**) Template integrity of random-primed RT products was verified using GAPDH primers, defining a 292 bp amplicon.


P6
P10
CORTEX
HIPPOCAMPUS
CEREBELLUM
CORTEX
HIPPOCAMPUS
CEREBELLUM
A
LIS1
(373 bp)
B
PAF-AH I α1
(587 bp)
C
PAF-AH I α2
(418 bp)
D
PAF-AH II
(1017 bp)
E
GAPDH
(292 bp)
30 cycles
Cerebellum P6
Cortex P10
F
PAF-AH II
(1017 bp)
35 cycles

4.4.4 Expression of endogenous PAF-AHs mRNA transcripts in isolated neural cells of cerebellar primary cultures.

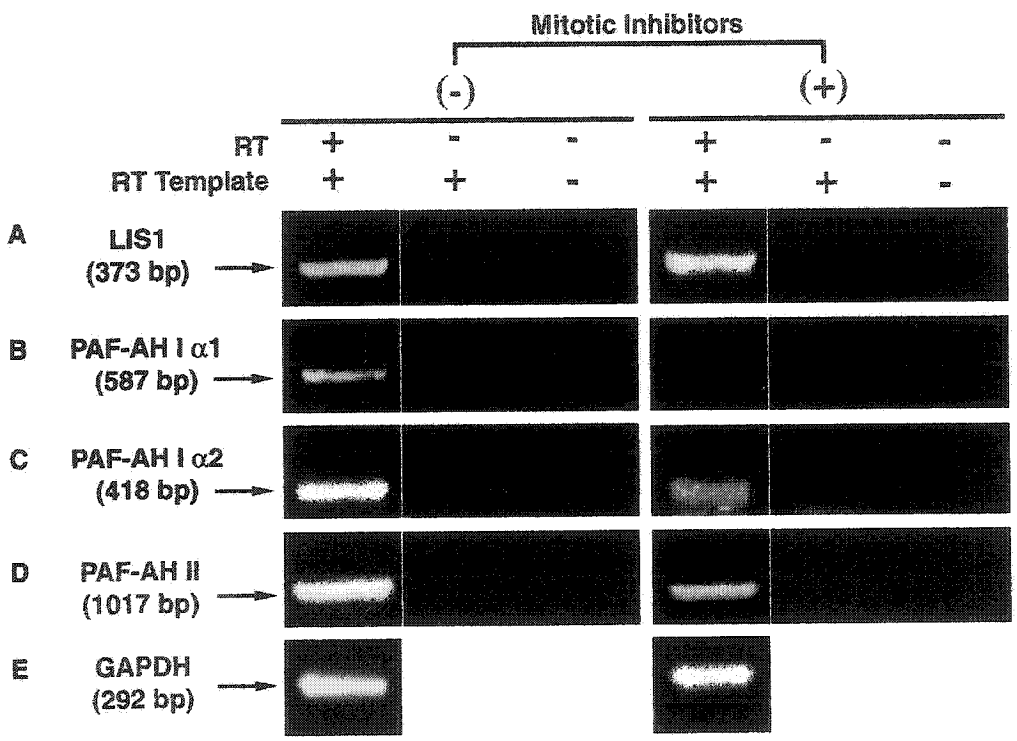
Expression of intracellular PAF-AH isoforms at the mRNA level was established by RT-PCR on P6 rat cerebellar pure neuronal cultures treated with mitotic inhibitors. RT-PCR was performed using primers that recognize rodent transcripts for *Lis1* (Fig 4.4A), PAF-AH I α1 (Fig 4.4B), PAF-AH I α2 (Fig 4.4C) and PAF-AH II (Fig 4.4D), defining amplicons of 373, 587, 418 and 1017 bp respectively. The GAPDH gene was also amplified as an internal control (Fig 4.4E). Pure neuronal cultures express mRNA transcripts for *Lis1* (Fig 4.4A), PAF-AH I α2 (Fig 4.4C), and PAF-AH II (Fig 4.4D), but do not express mRNA transcript for PAF-AH I α1 (Fig 4.4B).

4.5 Discussion

In Chapter 2 and 3, cytosolic PAF-AH isoforms were shown to exert anti-apoptotic activity. The goal of this study was to develop tools to elucidate the potential role of LIS1 in regulating this activity in PC12 cells and primary cultures.

As reviewed in Chapter 1, PAF-AH I specifically inactivates PAF lipids, playing an important role in brain development (Hattori et al., 1993). The heterotrimeric PAF-AH I complex is composed of two catalytic subunits, α1 and α2, and a non-catalytic β subunit (herein referred to as LIS1). Recent evidence has demonstrated that the LIS1 subunit likely regulates PAF-AH I enzymatic activity, and that optimal functioning of the LIS1 protein appears to be a critical requirement for normal development of the human brain cortex (Clark et al.,

Figure 4.4: Differential expression of PAF-AHs transcripts in primary neuronal cultures and mixed neuronal/glial cultures isolated from P6 cerebellum.
RT-PCR analysis of (**A**) *Lis1*, (**B**) PAF-AH I α1, (**C**) PAF-AH I α2 and (**D**) PAF-AH II, in mixed (mitotic inhibitors -) and pure (mitotic inhibitors +) neuronal cultures form P6 rat cerebellum, defining amplicons of 373, 587, 418 and 1017 bp respectively. The absence of genomic DNA contamination and reagent contamination has been verified by performing control reactions in parallel: No RT during the RT reaction, and no template during the PCR reaction. (**E**) Template integrity of random-primed RT products was verified using GAPDH primers, defining a 292 bp amplicon.


Mitotic Inhibitors
(-)
(+)
RT
RT Template
A
LIS1
(373 bp)
B
PAF-AH I α1
(587 bp)
C
PAF-AH I α2
(418 bp)
D
PAF-AH II
(1017 bp)
E
GAPDH
(292 bp)

1997; Manya et al., 1999). LIS1 is highly conserved between species showing only a single amino acid difference between mouse and human, three amino acid differences between human and bovine, 70% identity with Drosophila LIS1, and 42% homology with the nuclear migration protein NudF in *Aspergillus nidulans* (Peterfy et al., 1994; Hattori et al., 1995b; Morris et al., 1998; Liu et al., 1999). Because only one amino acid substitution is observed between the human and the rat β subunit, it was possible to create a mutation in the rat *Lis1* sequence that would correlate with a known human *Lis1* mutation.

A number of approaches have been used in earlier studies to define the function of LIS1 in cultured mammalian cells. Overexpression of WT LIS1 and application of *Lis1* antisense oligonucleotides have been demonstrated to produce a dramatic increase in mitotic index, accompanied by a variety of mitotic spindle abnormalities, implicating LIS1 in the control of cell division itself (Faulkner et al., 2000). However, no research has yet been conducted to look at the effect of LIS1 overexpression on PAF metabolism in a neuronal cell system. A previous report has revealed that the neuronal sensitivity to LIS1 dosage may result from abnormalities in neuronal or interkinetic migration and proliferation (Gambello et al., 2003). Recent data have demonstrated that *Lis1*-deficient cells have an increased susceptibility to cell death, suggesting a role for LIS1 in cell survival (Gambello et al., 2003). It is important to remember that LIS1 purifies as a component of PAF-AH I, and that the substrate, PAF, affects microtubule and neuronal migration in cultures (Bix and Clark, 1998; McNeil et al., 1999). It is hypothesized that LIS1 may regulate the activity or localization of the PAF-AH I

enzyme (Manya et al., 1999). The tools generated in this thesis will permit direct evaluation of this hypothesis in primary neuronal culture.

To address the role of *Lis1* mutation, an expression vector transducing a mutated form of *Lis1* has been created by the insertion of a missense mutation that results in a truncated protein of 144 amino acids (referred as LIS1Δ144 in the thesis). LIS1 amino acid sequence analysis has revealed that it contains seven WD40 repeats, recognized for multiple protein-protein interactions (Wang et al., 1995; Garcia-Higuera et al., 1996). Of the mimicked human *Lis1* mutations, all show loss of interactions with both of the α1 and α2 proteins (Sweeney et al., 2000). The mutant LIS1 that will be produced by the adenoviral vector generated in this study is lacking the second WD40 repeat (Cardoso et al., 2002). This repeat has been shown to be critical for the binding of the LIS1 subunit to the α1 and α2 catalytic subunits, to form the trimeric PAF-AH I complex (Sweeney et al., 2000). Thus, it is hypothesized that the mutant will be unable to associate with the catalytic subunits. Purification of this protein and *in vitro* assay with recombinant α1 and α2 protein will permit direct determination as to the effect of this mutation on the regulation of α1/α2 and α2/α2 catalytic activity.

During this project's design, there was no commercially LIS1 antibody available. To facilitate detection and functional studies of the *Lis1* gene product, two epitope-tagged expression vectors have been constructed: pCDM8/Flag-LIS1WT and pCDM8/Flag-LIS1Δ144. These constructs allow proteins to be expressed ectopically as fusion proteins with a Flag epitope tag attached at the

amino (N) terminus of expressed LIS1 proteins. The Flag epitope has been extensively used, and previous studies have demonstrated that the presence of the Flag peptide does not alter the characteristics of the studied molecule (Burioni et al., 2001). However, even if protein functions are not altered by a N-terminus tag, it should be noted that a previous report has indicated that N-terminal Flag affinity tag reduced protein expression, whereas the same tag fused to the carboxy (C)-terminus had no effect (Lee and Altenberg, 2003). Therefore, it is hypothesized that the level of LIS1 expression (functional or mutant) would be greater if the Flag peptide had been fused at the C-terminus. However, it is predicted that the presence of the Flag epitope at the N-terminus of LIS1 WT and LIS1Δ144 sequences will not interfere in the function of these proteins.

Under even the most optimal transfection conditions, plasmid-mediated gene transfer *in vitro* is highly cell type- and protocol-specific, with relatively low transfection efficiencies. Therefore, the Flag-LIS1WT and the Flag-LIS1Δ144 have respectively been inserted into an adenoviral system (pAdTrack-CMV vector), to produce the pAdTrack/LIS1WT plasmid and the pAdTrack/LIS1Δ144 plasmid. These constructs have been sequenced to identify proper initiating and terminating sequences in the predicted reading frame. Recombinant adenoviral vectors have a number of advantages over other means of gene transfer, one of which is their very high gene transfer efficiency (Kremer and Perricaudet, 1995). Adenoviral systems are also efficient vectors for neuronal gene therapy due to their ability to infect post-mitotic cells, their high efficacy of cell transduction, and

their low pathogenicity (Barkats et al., 1998). The two adenoviral constructs generated in this study will be used to undertake future studies in primary cell cultures.

PC12-AC cells can be differentiated to a neuronal-like phenotype with the use of nerve-growth factor (NGF), and can acquire many of the properties characteristic of sympathetic neurons. However, these cells are unable to pursue glial or oligodendrocyte lineage. While the PC12 cell model was proven to be a convenient cell system for the characterization of PAF-AHs enzymatic activity, research on the implication of LIS1 as part of the PAF-AH I complex will require a PAF-sensitive system that is more representative of the CNS. This idea is supported by the observation that Lis1 RNA and protein are greatly enriched in the CNS, particularly in neurons, during embryonic development in drosophila and rat (Smith et al., 2000). Because our preliminary experiments have demonstrated that rat brain tissue exhibits endogenous *Lis1*, PAF-AH I α1, PAF-AH I α2, and PAF-AH II mRNA transcripts, (Fig 2.3; positive control; Chapter 2), primary cultures prepared from P6 and P10 cortex, hippocampus and cerebellum rat brains were tested for PAF-AHs mRNA expression. RT-PCR analysis revealed that these mixed neuronal and glial cultures express PAF-AH II and all three subunits of PAF-AH I (*Lis1*, α1, α2). In order to identify the cell type(s) that expressed each subunit, 95% pure neuronal cultures from P6 rat cerebellum[6] were generated in the presence of mitotic inhibitors, and analyzed

[6] The protocol used in this thesis was demonstrated to generate cultures containing >95% pure neurons as determined by immunocytochemistry by other members of the Bennett laboratory.

by RT-PCR. An unexpected finding was that no transcript for PAF-AH I α1 was detected in pure neuronal cultures from P6 rat cerebellum, suggesting that the α1 subunit is mostly expressed by non-neuronal cells in postnatal Wistar cerebellum (i.e. oligodendrocytes and astrocytes). The PAF-AH I catalytic composition has been shown to change from α1/α2 heterodimers in developing embryos to α2/α2 homodimers in adult brain (Manya et al., 1998). While these data are consistent with this down-regulation in α1 expression observed in postnatal tissue, expression in glia was surprising. It has previously been demonstrated that the α1 subunit appears to be expressed specifically in neurons of neonatal brains between embryonic day 19 (E19) and P7 (Manya et al., 1998). The α1 subunit has been shown to be specifically expressed in migrating neurons in the embryonic and postnatal stages, whereas the α2 expression level is almost constant from the fetal stages through adulthood (Albrecht et al., 1996; Manya et al., 1998). Consistent with these data, expression of the α2 and LIS1 subunits was found to be fairly universal in all cerebellar cultures tested. Our results suggest that the α1subunit is not expressed by neurons and is minimally expressed by non-neuronal cells in P6 rat cerebellar primary cultures. Based on a recent report demonstrating that the LIS1 subunit strongly accelerates the enzymatic activity of the α2/α2 homodimer but rather suppresses the activity of the α1/α1 homodimer (Manya et al., 1998), we speculate that PAF-AH I enzymatic activity will be increased by the overexpression of *Lis1*. Using the novel PAF-AH kinetic assay and adenoviral vectors generated in this thesis, another student in the Bennett laboratory will test this hypothesis.

CHAPTER 5: GENERAL DISCUSSION AND CONCLUSIONS

The studies presented in this thesis were designed to add to the existing understanding of how intracellular PAF-AHs regulate the kinetics of PAF-mediated cell death following pathophysiological challenge. It has been hypothesized that sustained exposure to elevated concentrations of PAF is a principle mediator of neuronal death in ischemia, encephalitis, epileptic seizure, meningitis, and HIV-1 dementia (Bazan et al., 1995; Bazan, 1998; Birkle et al., 1998; Perry et al., 1998; Langley et al., 1999; Schifitto et al., 1999; Fukuda et al., 2000). While PAF can be metabolized into other biologically active phospholipids, most of PAF effects have been recognized to be receptor-mediated (for a review see (Prescott et al., 2000)). A single G-protein coupled PAFR has been cloned (for a review see (Ishii and Shimizu, 2000)). However, we have recently established that PAF elicits apoptosis in PC12-AC cells in the absence of the known PAFR (Brewer et al., 2002). To provide insight into PAFR-independent apoptosis, the PC12 cell model was used in this thesis to examine the role of intracellular PAF-AHs in regulating PAF-mediated cell death. Previous studies from our laboratory have established that PC12 cells are PAF-sensitive but do not express PAFR (Brewer et al., 2002). In this study, we demonstrate that the extent of cell loss was exacerbated by pharmacological blockade of PAF-AH catalytic activity, repeated PAF treatment, or administration of a PAF-AH resistant analog, suggesting that intracellular PAF-AHs play a cytoprotective role by limiting the kinetics of PAF-mediated death signaling. In support of this hypothesis, this study is the first to characterize functional PAF-AH activity in

PC12-AC lysates, and marks the first report of endogenous PAF-AH I (LIS1, α1, α2) and PAF-AH II expression in the PC12 cell line.

To further elucidate the role of intracellular PAF-AHs in PAF catabolism and protection against PAF-mediated cell death, we used a fluorescent PAF analog, B-PAF, to develop a novel assay quantifying intracellular PAF-AHs kinetics in living cells. In these series of experiments, we demonstrated that PAF is rapidly internalized over the first 40 min of exposure in PC12 cells. This observation is important in that it indicates that PAF internalization can occur in the absence of the PAFR. This is consistent with previous data suggesting that PAF internalization can be mediated by transbilayer movement (flipping) across the plasma membrane, as well as by expression of a PAF-specific transglutaminase, promoting transbilayer incorporation of exogenous ligand and regulated release of newly synthesized PAF (Bratton et al., 1992). B-PAF degradation into B-*lyso*-PAF is initiated within 15 min of ligand internalization, and continues for at least 75 min. The PAF-AH enzymes responsible for this hydrolysis were identified as PAF-AH I (α1, α2, and LIS1 subunits) and PAF-AH II. Because the rat PAF-AH II sequence had not previously been deposited with Genbank, we confirmed transcript identity by cloning the full-length cDNA (Accession number AY225592). The release of B-*lyso*-PAF from PC12-AC into the extracellular milieu is represented by a three phase process: phase 1) active release of half of the metabolized PAF is observed within the first 30 min of B-PAF exposure; phase 2) between 30 and 60 min, B-*lyso*-PAF accumulates in the

cell cytosol; phase 3) after 60 min of incubation, cytosolic B-*lyso*-PAF is released into the extracellular milieu.

To establish whether cytosolic PAF-AHs activity regulates PAF-mediated apoptosis, we analyzed whether pharmacological inhibition of enzymatic activity compromised PC12 cell survival. Concentration-dependent effects observed following treatment with the serine protease inhibitor DFP provide pharmacological evidence consistent with the conclusion that PAF-AH I activity predominates over PAF-AH II activity, and that PAF-AH I enzymatic activity elicited by DFP-sensitive PAFAH I $\alpha 1/\alpha 1$ or $\alpha 1/\alpha 2$ homo or heterodimers in PC12-AC cells is likely greater than the DFP-resistant $\alpha 2/\alpha 2$ homodimers. Moreover, PAF-mediated cell death was increased by inhibition of cytosolic PAF-AH isoforms, suggesting that sustained exposure to PAF or a defect in PAF catabolism can be detrimental following a pathophysiological insult.

A series of PAF antagonists have been screened for their ability to reduce deleterious effects elicited by PAF. We provide direct evidence that FR 49175 attenuates PAF-mediated cell death in PAFR-negative PC12 cells. It also accelerates the kinetics of PAF inactivation to *lyso*-PAF and the release of cytosolic *lyso*-PAF, without affecting the rate or extent of PAF internalization over the 75 min exposure period in PC12 cells. This structurally unrelated PAF analog has been shown to have a low affinity for PAFR and to exhibit only a slight inhibitory activity on PAFR-mediated effects (Dupré et al., 2001). Using PAFR-negative PC12-AC cells, we show that this PAF antagonist has PAF inhibitory activity in addition to acting on PAFR. We hypothesized that the

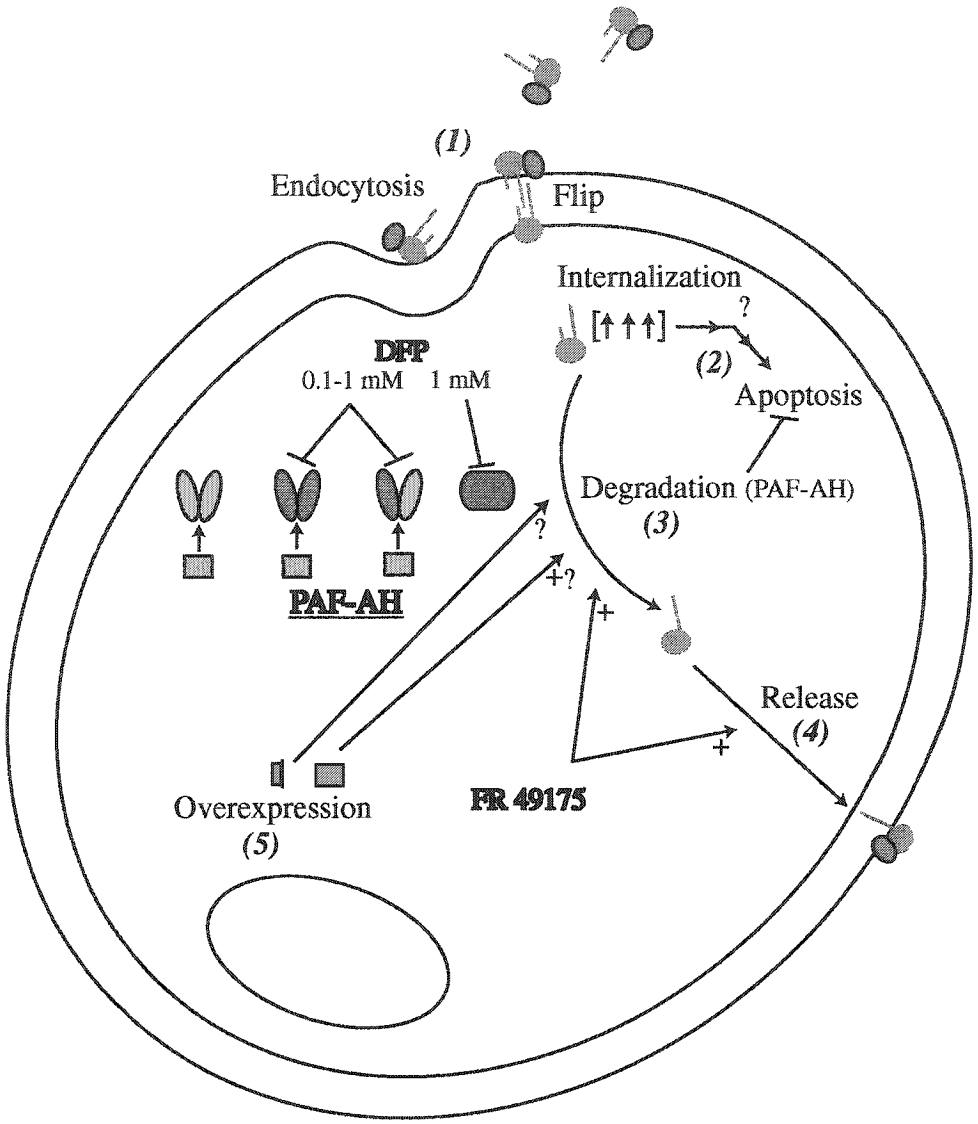
presence of FR 49175 impacts downstream of receptor activation, by likely binding to free enzyme and enzyme–substrate complexes. Therefore, FR 49175 represents a potential therapeutic agent to block PAF-mediated cytotoxicity, by modulating PAF-AH activity. According to previous reports showing that exogenous administration of plasma PAF-AH inhibits PAF-mediated neurotoxicity (Ogden et al., 1998; Hirashima et al., 2000), intracellular PAF-AHs may represent potential targets that can be pharmacologically manipulated to promote cell survival following pathological challenge.

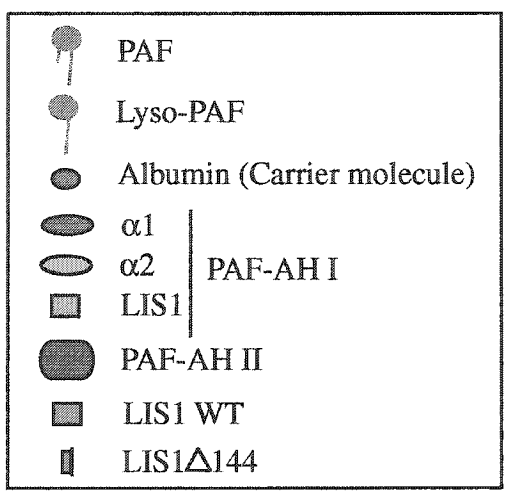
The PAF-AH I complex has received much attention in part because the subunit that modulates enzymatic activity (the β subunit) is the product of the *Lis1* gene. Mutations in this subunit cause MDS, a disorder characterized by severe brain abnormalities (Hattori et al., 1994a). Recent evidence has demonstrated that the β subunit likely regulates PAF-AH I enzymatic activity, and that optimal functioning of the LIS1 protein appears to be a critical requirement for normal development of the human brain cortex (Clark et al., 1997; Manya et al., 1999). The LIS1 subunit of the PAF-AH I complex appears to be an essential player as reflected by the deleterious consequences of its malfunctioning. No research has yet been conducted to evaluate the effect of *Lis1* overexpression on PAF metabolism in a neuronal cell system. By deciphering the precise details of the LIS1 protein under normal and abnormal conditions, we will gain additional insight into how intracellular PAF-AHs regulate the kinetics of PAF-mediated cell death. To achieve this goal, two adenoviral vectors driving expression of WT *Lis1* or a MDS-relevant mutant have been constructed to permit closer evaluation

of the regulatory effects of this PAF-AH subunit on PAF-mediated cytotoxicity.

In summary, the fact that precise regulation of PAF levels seems to be a key factor during and after brain development suggests that careful control of its degradation must play a key role in such mechanisms. The precise role and function of intracellular PAF-AHs in the brain is still a subject of investigation. However, based on the data generated in this thesis, we propose the following model by which PAF triggers apoptotic death in the absence of PAFR (refer to Fig 5.1). (1) When extracellular PAF levels rise following certain conditions of trauma and injury, it can rapidly be internalized by surrounding cells. The route of PAFR-independent internalization of PAF is possibly enhanced transbilayer movement (flipping) across the plasma membrane that may be occurring as a result of changes in membrane physical properties. It is also hypothesized that PAF binds to a novel PAF binding protein located at the plasma membrane, followed by subsequent endocytosis of ligand/binding protein complexes based on unpublished data from our laboratory. It can finally be speculated that PAF enters the cells through a PAF-specific transglutaminase promoting transbilayer incorporation of exogenous ligand. (2) Presence of acute levels of PAF or sustained exposure to active ligand is known to mediate a reduction in cell survival. We have previously demonstrated that this cell death occurs through an apoptotic pathway (Brewer et al., 2002) and have recently established that this pathway is, in part, caspase-3 dependent (Bonin et al. submitted). (3) Intracellular PAF-AH activities, controlled by two distinct isoforms designated PAF-AH I (α2/α2, α1/α1, α1/α2) and PAF-AH II, have been reported to regulate

Figure 5.1: Proposed model by which PAF triggers apoptotic death in the absence of PAFR (for comments see text).


(1)
Endocytosis
Flip
Internalization
[↑↑↑]
?
(2)
Apoptosis
DFP
0.1-1 mM
1 mM
Degradation (PAF-AH)
(3)
PAF-AH
?
+?
+
Release
(4)
+
Overexpression
(5)
FR 49175


PAF
Lyso-PAF
Albumin (Carrier molecule)
α1
α2
LIS1
PAF-AH I
PAF-AH II
LIS1 WT
LIS1Δ144

the intracellular PAF concentrations, by degrading PAF into its inactive metabolite *lyso*-PAF. Inhibition of intracellular PAF-AHs by the serine-blocking agent DFP has the effect of preventing adequate degradation of PAF. Thus, PAF accumulates within the cells and renders them more susceptible to death by apoptosis. Therefore, it is likely that PAF-AHs limit the extent of PAF-mediated apoptotic signaling in PC12 cells, by regulating the kinetics of PAF degradation. PAF-induced cytotoxicity in PC12-AC cells can pharmacologically be attenuated by the PAF antagonist FR 49175. We proposed that by binding to free intracellular enzyme and enzyme–substrate complexes, FR 49175 accelerates the kinetics of PAF degradation and the release of cytosolic *lyso*-PAF. (4) Following catabolism of PAF by intracellular PAF-AHs, *lyso*-PAF is produced and released into the extracellular milieu in three phases. It could be suggested that these three phases of PAF release correspond to the respective translocation of the intracellular PAF-AHs (PAF-AH I α1/α2, PAF-AH I α2/α2 and PAF-AH II), given that it has been demonstrated that PAF-AH II can translocate between cytosol and membrane in response to a redox state of the cells. Following their release from the cytosolic fraction, PAF metabolites remain cell associated, presumably bound to albumin or other carrier proteins in a protected configuration at the plasma membrane. (5) Based on a recent report demonstrating that the LIS1 subunit strongly accelerates the enzymatic activity of the α2/α2 homodimer but rather suppresses the activity of the α1/α1 homodimer, we predict that PAF-AH I enzymatic activity will be increased by the overexpression of LIS1 WT. Thus, overexpressing LIS1 WT should accelerate

the kinetics of PAF degradation and promote protection against PAF-mediated apoptosis. Conversely, we speculate that a mutation in *Lis1* will lead to a loss of function of the regulatory subunit of the PAF-AH I complex and to abnormal regulation of intracellular PAF levels. These hypotheses will be further evaluate by other students in the Bennett laboratory.

Overall, the kinetics of cytosolic intracellular PAF-AHs have revealed that these enzymes have an important role to play in PAF degradation to *lyso*-PAF, leading to protection against PAF-mediated cell death. New discoveries continue to add interesting components to the puzzle and the next decade holds great promise.

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Bonin F, Ryan SD, Bullock P, Migahed L, Mo F, Arai H, Franks DJ, and Bennett SAL; Cytosolic platelet activating factor I and II protect PC12 cells from apoptotic loss and can be pharmacologically targeted to promote cell survival. (Submitted to JBC).

Poster Presentations

Bonin F, Franks D, Bennett S.A.L. (2002) Development of an *in vitro* system to study LIS1-mediated signaling. (Society for Neuroscience 32nd Annual meeting; Abstract 235.1; Orlando, USA)

Bonin F, Franks D, Bennett S.A.L. (2002) Functional platelet activating factor acetylhydrolases expressed by PC12 cells regulate platelet activating factor-induced apoptosis. (Canadian Federation of Biological Sciences; Montreal, Canada)

Bonin F, Franks D, Bennett S.A.L. (2002) Molecular mechanisms of LIS-1 signaling in PC12 cells. (BMI Graduate Research Day; University of Ottawa, Canada)

Bonin F, Brewer C, Chen J, Bennett SAL (2001). Effect of platelet activating factor receptor expression on nerve growth factor-induced differentiation of PC12 cells. (Society for Neuroscience 31st Annual meeting; Abstract 31: 793.16; San Diego, USA) and (Ottawa Life Sciences Research Conference; Ottawa, Canada)

Bonin F, Bennett S.A.L., Franks D. (2001). L'influence de la stimulation du récepteur du facteur d'activation plaquettaire (PAF) sur la prolifération et la différenciation des précurseurs neuronaux. (UQTR, Department of chemistry-biology: B.Sc.in medical biology ; Trois-Rivières, Canada)

Lecture Presentations

A time to live: Platelet activating factor receptor-GPCR activation inhibits growth arrest associated with neuronal differentiation (February 2002); Minisymposia on Cell Signaling and Neuronal Survival (Louisiana State University Neuroscience Center of Excellence, New Orleans, USA)

Techniques in Cellular Regulation (January 2002); Guest lecturer for the fourth year Biochemistry course « Cellular Regulation and Control » (BCH 4125) (University of Ottawa, Canada)

Employment, Teaching Experience and Volunteer work

Sept. 2003 - Present	**Neurochem Inc.** Research assistant in Molecular Biology
Apr. 2003	**Provincial Expo-Sciences finals** *Volunteer judge*
	University of Ottawa *Teaching Assistant*
Jan. 2002 - Mai 2002	-Biochemistry Laboratory (BCH 2736)
Sep. 2001 - Jan. 2002	-Principles of Chemistry (CHM 1710)
June 1995 – Jan. 2001	**McDonald's Restaurant** *Trainer (Chef d'équipe)*

Computer Skills

Microsoft Word, WordPerfect, Excel, Powerpoint, Improvision Openlab, Adobe Photoshop, Adobe illustrator, Adobe After Effect, DeltaGraph, Clone Manager, Acrobat, EndNote.

Awards/Scholarships

2003	Scottish Rite Charitable Foundation of Canada scholarship ($ 6750)
2001	Premiers research excellence recruitment summer scholarship ($ 4000)
1999	Best Employee of the year from McDonald's Restaurant
1998	Distinction Award for implication in Ronald McDonald Children's charities
1996	Les Caisses Desjardins scholarship (300 $)
1996	Ronald McDonald scholarship (300 $)
1996	Distinction Award for grades in English

**** References are available on request**