Activation of Phospholipases A_2 and D of a Human Neuroblastoma Cell Line (LA-N-2) by N-Dodecyl-L-lysine Amide (Compound 24), a Putative G Protein Activator: Characteristics of Inhibition by (-)-Nicotine

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A Thesis Submitted to the Faculty of Graduate Studies In Partial Fulfillment of the Requirements for the Degree of Master of Science

Department of Pharmacology and Therapeutics

University of Manitoba

May, 2002

THE UNIVERSITY OF MANITOBA

FACULTY OF GRADUATE STUDIES

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BY

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A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirements of the degree of

MASTER OF SCIENCE

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Acknowledgements

I would like to acknowledge and thank my advisor Dr. Daniel Sitar for his insight, guidance and friendship during my tenure as a graduate student in the Department of Pharmacology and Therapeutics. Conducting medical research is interesting and challenging and the skills and lessons I learned will be of great value to me while I persue future endeavours.

I would like to thank Dr. Fiona Parkinson for her advice, encouragement and expertise related to the molecular biological aspects of my research. In addition, I would like to thank Yie Wei Zhang for her technical assistance. Her pleasant demeaner and enthusiastic nature were appreciated. Cudo's are also extended to the other members of the Parkinson laboratory, past and present, for sharing their expertise on various issues.

I would also like to thank Dr. Grant Hatch for his interest in my research and hence, for generating some interesting ideas and contributing his knowledge of lipid biochemistry. Thank you to William Taylor for lending me, on a moments notice, reagents/catalogs and offering technical advice regarding chromatography.

In addition, I would also like to sincerely thank Dr. Donald Smythe and Dr. Wayne Lautt for their wisdom and insight. Our discussions were time well spent.

I would also like to thank Dr. Deepak Bose, Dr. Ratna Bose and Dallas Legare for their valuable assistance in preparing presentations.

Thank you to all the faculty members, technicians and fellow graduate students who enriched my time here in graduate school.

Thank you to the University of Manitoba and the Department of Pharmacology and Therapeutics for Financial Support.

Last, but not least, my greatest appreciation is directed towards my partner Diane Fejes and our son Owen for their support of my endeavours, wherever they lead me. Thanks for being there!

This Thesis is Dedicated to my son Owen
May he appreciate the pursuit of knowledge as much as I have

Abstract

Compound 24, an alkyl-substituted amino acid amide, was used as a tool in an attempt to determine the mechanism/location of nicotine inhibition of the amyloid beta peptide (AβP) induced stimulation of phospholipases, previously reported by our laboratory and thought to occur through a pertussis toxin (PTX)-sensitive G protein in a human LA-N-2 neuroblastoma cell line. Compound 24, previously reported to stimulate PTX-sensitive G proteins in cell membranes and membrane protein fractions, stimulated phospholipase A_2 (PLA_2) and phospholipase D (PLD) activities in LA-N-2 cells. The chosen indicator of PLA_2 activity was arachidonic acid (AA), while that for PLD activity was phosphatidylethanol (PE)

The phospholipase activations were not inhibited by (-)-nicotine unless cells were pre-treated with PTX. The PLA_2 and PLD stimulations following PTX pre-treatment may result from multiple stimulation mechanisms operating in the presence of compound 24. Inhibition of AβP induced phospholipase stimulations by (-)-nicotine may occur primarily where AβP interacts with the cell membrane, possibly through a non-cholinergic nicotinic receptor.

We determined that the α4 subunit is not expressed and the α7 subunit of the nicotinic receptor is expressed in very small quantities in LA-N-2 cells. These findings suggest that these nicotinic receptor subunits are not likely involved in our cell culture model of (-)-nicotine inhibition of AßP induced stimulations of PLA_2 and PLD. Following exposure to PTX, plasma membrane permeability changes are postulated to occur, resulting in intracellular inhibition by (-)-nicotine. Ultimately (-)-nicotine may have the potential to exert inhibitory effects at multiple levels, depending on the composition of the environment bathing the cell.

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Abbreviations

α-Btx	Alpha Bungarotoxin
AβP	Amyloid Beta Peptide
AA	Arachidonic Acid
ABAD	Amyloid Beta Peptide Binding Alcohol Dehydrogenase Dehydrogenase
AD	Alzheimer's Disease
AMPA	Alpha-Amino-3-Hydroxy-5-Methyl-4-Isoxazole Propionic Acid
Apaf-1	Apoptotic Protease Activating Factor
APOE	Apolipoprotein E
APP	Amyloid Precursor Protein
BBB	Blood Brain Barrier
BSA	Bovine Serum Albumin
CNS	Central Nervous System
COX	Cyclooxygenase
COX-2	Cyclooxygenase 2
CTX	Cholera Toxin
Dab	3, 3-Diaminobenzidine tetrahydrochloride
DEPC	Diethyl Pyrocarbonate
DG	Diglycerides

DNTPs	Deoxy-nucleotide Triphosphates
DTT	Dithiothreitol
ε2	Epsilon 2
ε3	Epsilon 3
ε4	Epsilon 4
EDTA	Ethylenediaminetetraacetic Acid
EGB761	Ginkgo Biloba Extract 761
EP	Endocytic Pathway
ER	Endoplasmic Reticulum
FBS	Fetal Bovine Serum
GAPDH	Glyceraldehyde Phosphate Dehydrogenase
HNE	Hydroxynonenal
IPA	Indole-3-Propionic Acid
LA-N-2	Los Angeles Neuroblastoma 2 Human Cell Line
LDLR	Low Density Lipoprotein Receptor
LOX	Lipoxygenase
LRP	Low Density Lipoprotein Receptor Related Protein
LTP	Long Term Potentiation
MAC	Membrane Attack Complexes
MAO	Monoamine Oxidase
MAP	Microtubule Associated Proteins
M-CSF	Macrophage-Colony Stimulating Factor

MSRA	Methionine Sulfoxide Reductase A
NAC	Non-Amyloid Component of Alzheimer's Disease Amyloid
NAChR	Nicotinic Acetylcholine Receptor
NBQX	2, 3-Dihydroxy-6-Nitro-7-Sulfanoyl Benzo (f) Quinoxaline
NFκβ	Nuclear Factor Kappa Beta
NFT	Neurofibrillary Tangles
NMDA	N-Methyl-D-Aspartate
NOS	Nitric Oxide Synthase
NSAIDS	Non-Steroidal Anti-inflammatory Drugs
NTR	Neurotrophin Receptor
p75 NTR	p75 Neurotrophin Receptor
PA	Phosphatidic Acid
PC	Phosphatidylcholine
PCR	Polymerase Chain Reaction
PE	Phosphatidylethanol
PHF	Paired Helical Filaments
PKA	Protein Kinase A
PKC	Protein Kinase C
PLA_2	Phospholipase A_2
PLC	Phospholipase C
PLD	Phospholipase D

PtdEtOH	Phosphatidylethanol
PTX	Pertussis Toxin
RAGE	Receptor for Advanced Glycation End Products
ROS	Reactive Oxygen Species
RT	Reverse Transcriptase
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
TBARS	Thiobarbituric Acid-Reactive Substances
TLC	Thin Layer Chromatography
TNFα	Tumour Necrosis Factor Alpha
VLDLR	Very Low Density Lipoprotein Receptor

General Introduction

Alzheimer's Disease

Alzheimer's disease (AD), discovered in 1907 by Alois Alzheimer and characterized by neuritic (senile) plaques, neurofibrillary tangles (NFT) and inflammation, culminates in the loss of cholinergic neurons in the hippocampus, cerebral cortex and basal forebrain nuclei (Mesulam, 1999). Reductions in neurotransmitter release are also observed with the serotonergic and noradrenergic neurons in the median raphe and locus ceruleus (Smith, 1998). Memory loss, which gradually becomes severe, is an inevitable consequence of AD. Additional manifestations include impairments in attention, language, perception, reasoning and comportment. It is postulated that AD may not be a disease, but a failure to keep up with the increasingly more burdensome work of plasticity (Mesulam, 1999).

Alzheimer's disease is the most common form of dementia among the elderly and the fourth leading cause of death in those over 65 years of age (Swartz et al, 1999). Old age, apolipoprotein E (APOE) genotype, family history of dementia and Down's syndrome are confirmed risk factors for AD, with old age being the strongest risk factor (Snowdon et al, 1996; Jorm, 1997; Smith, 1998; Skoog, 2000).

Unconfirmed risk factors include ethnicity, head trauma, aluminum exposure, history of depression, hypothyroidism, inactivity, advanced maternal age, poor written linguistic ability and exposure to electromagnetic fields (Jorm, 1997). Head injury is predicted to be a risk factor when it is widespread and chronic (Mesulam, 1999). Lack of written linguistic ability may

predispose individuals to developing AD. Nuns, residing in convents in the United States, had their written linguistic abilities assessed through an examination of autobiographies they wrote in their 20's. Those nuns judged to have poor written linguistic abilities, comprised of low idea density and low grammatical complexity, developed senile plaques and NFT in late life. Therefore, it was concluded that low linguistic ability in early life correlated well with poor cognitive function and AD in late life (Snowdon et al, 1996; Skoog, 2000). Presently, it is unclear whether poor written linguistic ability is an early symptom of AD or a potential contributing factor (Skoog, 2000).

The pathogenic processes characterizing AD undoubtedly occurred for many years prior to overt clinical signs and symptoms (Kanfer et al, 1998). Symptoms may subtly occur for years or decades preceeding the diagnosis of dementia. The pre-clinical phase of AD consists of verbal memory impairment, possibly reflecting hippocampal damage followed by impairment of brain areas governing language, spatial orientation, attention, concentration and psychomotor speed (Skoog, 2000). Biological markers for the pre-clinical stage include high plasma concentrations of the 42 amino acid amyloid beta peptide, AβP (1-42) and hippocampal atrophy measured by magnetic resonance imaging (Skoog, 2000). As AD progresses, the hippocampus degenerates further and becomes isolated, culminating in short-term memory impairment. The CA1 and subiculum regions of the hippocampus show the greatest degeneration. In the final stages of AD, the patient is completely debilitated and requires constant supervision (Smith, 1998).

Alzheimer's disease is presently incurable and can only be definitively diagnosed through post-mortem examination using histopathological confirmation, by biopsy at autopsy. Probable

AD is diagnosed through clinical exam, mental status assessment and neuropsychological tests. Possible AD is diagnosed when the typical clinical syndrome is present (Cummings, 1998). The Canadian Study of Health and Aging estimated that in 1991, 160, 000 Canadians met the criteria for AD. If the current trend continues, by 2031 the number of Alzheimer's cases may triple, while the population increases by an estimated factor of 1.4 (Swartz et al, 1999). Therefore it is imperative that new treatments or a cure be discovered in a timely fashion.

The Cholinergic Hypothesis

The consistent finding of reduced cholinergic functioning in AD led to the development of the cholinergic hypothesis. Cholinergic innervation of the cerebral cortex arises from the nucleus basalis of Meynert, a limbic structure that maintains an unusually high level of plasticity into late adulthood (Mesulam, 1999). In the basal forebrain, basalis of Meynert, during AD, there is generally a depletion of cholinergic neurons and senile plaques and NFT are present, suggesting that a specific sub-cortical lesion may be responsible for the cholinergic deficit seen with AD (Smith, 1998).

The cholinergic hypothesis has been disputed because there are individuals with AD showing no neuronal loss in the basalis of Meynert and still others with considerable basal forebrain neuronal loss manifesting very limited dementia. In addition, the cholinergic system is not exclusively affected by AD. Reductions in neurotransmitter release are seen with serotonergic and noradrenergic neurons in the median raphe and locus ceruleus (Smith, 1998).

Despite ongoing debate regarding the validity of the cholinergic hypothesis, cholinesterase inhibitors like Donepezil, Rivastigmine and Galantamine have been developed and are licensed for use in Canada. They represent the best AD treatment approach presently available. Cholinesterase inhibitors are thought to slow cognitive decline by inhibiting acetyl and butyryl cholinesterases, synaptic enzymes that break down acetylcholine, ultimately prolonging the lifetime of acetylcholine in the synaptic cleft. It is presently unknown which of the cholinesterase inhibitors is the most effective because comparison trials have not been conducted (Gauthier, 2002).

Excitatory Amino Acids

The large pyramidal neurons are among the most susceptible to degeneration and death, as indicated by the presence of NFT. These neurons utilize aspartate and glutamate, excitatory amino acids that can be neurotoxic following chronic depolarization. Presently it is uncertain whether excitotoxicity plays an important role in AD. Senile plaques and NFT have been discovered in the corticocortical association areas, leading to the suggestion that AD may progress along the excitatory amino acid connecting pathways (Smith, 1998).

The Physiological Manifestations of Alzheimer's Disease

Neurofibrillary Tangles

Neurofibrillary tangles are major microscopic lesions, found predominantly in the axons and dendrites of the large pyramidal neurons in the hippocampal and frontotemporal regions of the cerebral cortex and in the surrounding extracellular space (Smith, 1998). Some researchers consider NFT the primary pathogenic mechanism in AD (Epstein, 1999). The components of NFT can be divided into the following six groups: 1) cytoskeletal elements: tau, neurofilaments, high-molecular weight microtubule-associated protein MAP2, vimentin and tropomyosin, 2) protease-related elements: ubiquitin, alpha $_1$ antichymotrypsin, alpha $_1$ antitrypsin, cathepsins B and D, trypsin and elastase, 3) proteoglycans: heparan, chondroitin and keratin sulfate, 4) inflammatory molecules: cytokines, acute phase proteins and complement molecules, 5) amyloidogenic related molecules: AβP, amyloid precursor protein (APP), presenilins and APOE, 6) serum related molecules: P-component (Smith, 1998).

Microtubules, composed of tubulin dimers, are important for cell structure, vesicle and organelle movement and cell division. The non-tubulin proteins associated with tubulin microtubules are known as microtubule associated proteins (MAP) (Roses, 1995). Tau, which is required for microtubule assembly, is the MAP that forms insoluble paired helical filaments (PHF) when hyperphosphorylated (Smith, 1998; Mesulam, 1999). Paired helical filaments ultimately form NFT, comprised of two axially opposed helical filaments with a diameter of 10 nm. Experimental evidence suggests that the parallel components form a twisted ribbon-like

structure as opposed to separate filaments (Smith, 1998). Tau is highly phosphorylated, ubiquitinated, glycosylated and glycated when forming paired helical filaments. The increased tau phosphorylation represents one of the earliest neuronal changes prior to the development of NFT and this phosphorylation purportedly interferes with tau's ability to assemble microtubules, ultimately compromising neuronal transport and function, eventually destroying the synapse and neuron (Smith, 1998; Mesulam, 1999).

Nearly all individuals over 60 have at least a few NFT in their brains, confined to the limbic and paralimbic structures such as the hippocampus, the nucleus basalis of Meynert, the amygdala and the entorhinal-transentorhinal cortex, important areas responsible for memory functioning. This is known as the low limbic stage of NFT distribution. The next level is known as the high limbic stage of NFT distribution, which may be associated with mild cognitive impairment, senescent forgetfulness and pre-clinical AD. At this level, the NFT become more numerous in the limbic areas and then proceed to form clusters in the adjacent fusiform and inferotemporal gyri. Neurofibrillary tangles also appear in additional paralimbic areas like the temporal pole, insula, orbitofrontal cortex and parolfactory gyrus. After reaching high densities within the limbic, paralimbic and inferotemporal areas the NFT emerge and accumulate in the pre-frontal and parietotemporal association areas involved in language, attention and perception (Mesulam, 1999). Finally, NFT distribution proceeds to the low and high neocortical stages, associated with mild and severe dementia respectively. At these stages, cognitive functions in addition to memory become compromised. Some people appear to maintain cognitive function despite manifesting high densities of limbic and neocortical tangles. The explanations available

include a compensatory effect of neuronal reserve capacity and/or a decline from an exceptionally high baseline, without apparent dementia (Mesulam, 1999).

High levels of neuroplasticity, associated with processes leading to AD, are postulated to contribute to increased expression and phosphorylation of tau, eventually promoting its polymerization into NFT. Neurofibrillary tangles would initially appear in the limbic/paralimbic areas where neuronal plasticity is high. The resulting cytoskeletal disfunction in the limbic/paralimbic neurons would lead to dendrite degeneration and loss of axonal projection target synapses. Adjacent neurons would then have to increase their plasticity to replace lost connections. Due to high demands on these neurons, the process would be largely ineffective, resulting in NFT formation. The plasticity burden would eventually be shifted to cortical neurons, resulting in increased plasticity demands culminating in NFT formation and cell death (Mesulam, 1999).

Protease and Protease Inhibitors

Proteolytic dysfunction in relation to AD has been studied due to the discovery of a number of protease related molecules found associated with senile plaques and NFT. These molecules include alpha $_1$-antichymotrypsin, alpha $_1$-antitrypsin, cathepsins B and D, trypsin, elastase and ubiquitin. Proteases and their inhibitors can cause AD-like changes in vitro, resulting in tau and ubiquitin accumulations in neurons. Also, the presence of the Kunitz protease inhibitor domain on several APP transcripts can result in the formation of inhibitory complexes with certain proteases, suggesting a molecular link between AD and proteolytic

imbalance. Ubiquitin was discovered to be associated with neurofibrillary tangles. It is suggested that when neurons become stressed with the accumulation of abnormally phosphorylated proteins, a cytoprotective ubiquitination response ensues (Smith, 1998).

APP and the Generation of AβP

The APP is a receptor-like trans-membrane protein encoded on chromosome 21 and expressed in humans as four major isoforms, containing 695, 717, 751 and 771 amino acid residues, with neurons expressing the more abundant 695 amino acid residue isoform (Smith, 1998; Selkoe, 1999; Gandy et al, 2000; Harkany et al, 2000). Purportedly, a significant fraction of APP is associated with the cytoskeleton, possibly through interactions with tau (Smith, 1998). The APP is highly conserved in mammalian evolution, suggesting important physiological roles, but presently these roles are unclear. Proposed physiological functions include release of peptide ligands, autocrine cell growth regulator, extracellular protease regulator, cell contact adhesion, cytoskeletal homeostasis and cell surface receptor (Smith, 1998). The APP is reported to play a role in promoting neuronal plasticity by influencing cell-substrate interactions during neurite extension, promoting the formation and maintenance of synapses in the CNS, modulating long-term potentiation (LTP) and protecting neurons from excitotoxic and oxidative insults (Mesulam, 1999).

Alpha/Gamma Secretase Cleavage of the APP

When the APP is cleaved under physiological conditions by alpha secretase, the formation of amyloidogenic products is precluded (Mesulam, 1999; Selkoe, 1999). Only a limited number of APP molecules in most cell types undergo alpha secretory cleavage, potentially leaving many APP molecules subjected to beta and gamma secretase cleavage and resulting in AβP formation (Selkoe, 1999). Alpha secretase activity cleaves the AβP sequence between amino acids 16 and 17, generating an N-terminal fragment, the soluble ectodomain fragment (sAPPalpha) and a smaller membrane embedded C-terminal fragment (Checler, 1995; Selkoe, 1999; Gandy et al, 2000; Kumar-Singh et al, 2000). Recently, sAPPalpha has been reported to promote neuronal plasticity and is postulated to be neuroprotective (Mesulam, 1999).

The smaller C-terminal fragment is cleaved primarily at the plasma membrane by a gamma secretase, producing p3 and p7 fragments (Checler, 1995; Selkoe, 1999; Gandy et al, 2000). The p3 fragment is reported to be non-fibrillar, relatively insoluble and highly aggregatable. Presently, it is debatable whether the p3 fragment is neurotoxic. Additionally, if drugs are designed to block the beta and/or gamma secretases, then the alpha secretase pathway may become increasingly used, resulting in the production of the potentially neurotoxic p3 fragment. It has been hypothesized that the p3 fragment lacks domains for APOE binding and microglial and complement activation (Kumar-Singh et al, 2000).

Beta/Gamma Secretase Cleavage of the Amyloid Precursor Protein

Alternatively, during AD or under experimental conditions mimicking AD, APP processing appears to shift towards a pathological processing pathway. Cleavage by beta secretase at the N-terminus of the AβP sequence releases sAPPbeta, and a membrane bound C-terminal fragment retaining the intact AβP. The beta secretase cleavage is thought to begin in the constitutive secretory pathway, i.e. the trans-Golgi network, or within endosomes upon internalization (Gandy et al, 2000). The membrane bound C-terminal domain then undergoes gamma secretase cleavage leading to the release of AβP. Gamma secretase cleavage liberates a series of AβPs ranging from 39 to 43 amino acids in length.
(Checler, 1995; Harkany et al, 2000).

Path of Secretase Activity

The path of secretase activity appears to depend on the neuronal differentiation state, with the differentiated phenotype associated with diminished basal utilization of the non-amyloidogenic alpha secretase cleavage pathway. Also, the type of secretase used by the neuron depends on whether the process of regulated cleavage is occurring. Regulated cleavage occurs when signals are transduced by neurons, resulting in increased alpha secretase activity and decreased beta secretase activity (Gandy et al, 2000).

Changes in APP Expression and Turnover Resulting in Increased AβP concentration

Acute and chronic brain injuries influence APP expression, cellular ion homeostasis and metabolite supply corresponding to a rise in proteolytic enzyme activity. An aggravation of beta and gamma secretase processing of APP leading to abundant generation of AβP may result. In addition, following inflammation, activated microglia increase the expression of APP and the release of full length AβP. The above observations along with the reduced prevalence of AD following long lasting anti-inflammatory medication support the hypothesis that glial activation resulting from inflammatory mechanisms and acute brain trauma may initiate AβP production in the brain and exacerbate neurotoxic cascades leading to late life dementia (Harkany et al, 2000).

In addition, alterations in cholesterol regulation by APOE and its receptor may be responsible for an abnormal turnover and mis-sorting of synaptic proteins such as APP. Another hypothesis suggests that protein synthesis occurs following stress-induced synaptic damage, resulting in an imbalance of synaptic proteins, increased amyloid formation and dementia (Iwai, 2000).

Senile Plaques

The process of plaque formation reportedly takes many years and deposition of AβP in the brain is an inevitable consequence of aging (Halliday et al, 2000). Amyloid beta peptide comprises the majority of plaque forming material, with (AβP 1-40) being the most common component (Halliday et al, 2000). Longer (AβP 1-42) and shorter (AβP 1-28) forms have also

been discovered (Golde et al, 2000; Halliday et al, 2000;). Plaques also contain APOE, non-AβP component of AD amyloid (NAC), dystrophic synapses and neurites, tau proteins, reactive microglia, unprocessed APP, alpha $_1$ antichymotrypsin, IgG complement proteins and AβP plus glycosaminoglycans in a complex bundle (Seilheimer et al, 1997; Smith, 1998; Swartz et al, 1998; Bodles et al, 2000; Serpell, 2000). Neuritic plaques are localized within synapses between adjacent neurons, predominantly in the hippocampus and frontotemporal regions of the cerebral cortex (Smith, 1998). The number of senile plaques is reported to be highest in the amygdala (Sigurdsson et al, 2000).

Amyloid generically describes a heterogeneous class of tissue protein precipitates that possess a common beta pleated sheet secondary structure sensitive to the dye Congo red (Gandy et al, 2000). Amyloid beta peptide is found mainly in alpha helical form in solution (Smith, 1998; Chauhan et al, 2000; Halliday et al, 2000). In healthy individuals, soluble AβP monomers are found in sub-nanomolar concentrations in the cerebrospinal fluid and plasma (Seilheimer et al, 1997; Selkoe, 1999). In AD, soluble AβP concentration may be a thousand times higher (Seilheimer et al, 1997). It is the fibrillar, or mature form of AβP, which forms by nucleation, and whose formation is promoted by metal cations like Zn^{2+} and Al^{3+}, that ultimately adopts the neurotoxic beta pleated sheet conformation, forming diffuse and neuritic plaques. Congo Red and rifampicin bind to AβP monomers and therefore inhibit the formation of fibrils (Rymer and Good, 2001).

Experimental evidence suggests that AβP (1-42) aggregates more rapidly into amyloid fibrils than does AβP (1-40) and constitutes the bulk of AβP deposited within the AD brain parenchyma (Smith, 1998; Selkoe, 1999; Golde et al, 2000). It is also suggested that AβP (1-42)

is more insoluble and therefore more neurotoxic (Mesulam, 1999). Immunohistochemistry has shown that AβP (1-40) contributes to the formation of dense compact neuritic plaques (Kumar-Singh et al, 2000).

Diffuse Plaques

The diffuse plaques, which are the earliest to form, remain diffuse and do not form classical neuritic plaques. Diffuse plaques are relatively heterogeneous in size, with smaller plaques being more common. Diffuse plaques are composed of primarily AβP (17-42), a species not found in neuritic plaques (Golde et al, 2000). The average size of diffuse deposits is 20 μm in diameter. Within diffuse plaques there are no signs of reactive glial cells or degenerating neurites (Smith, 1998). In addition, diffuse plaques are not associated with extensive neuritic dystrophy and are present in the brains of non-demented individuals. Therefore, it has been inferred that deposition of AβP (17-42) is not pathogenic or relatively non-pathogenic compared to longer AβP forms (Golde et al, 2000).

Amyloid Fibrillogenisis

Following release into the extracellular space, monomeric AβP self aggregates, ultimately forming 10-200 μm diameter senile plaques (Seilheimer et al, 1997; Smith, 1998; Chauhan et al, 2000; Sigurdsson et al, 2000). During fibrillogenesis, transient but discrete AβP dimers, tetramers and protofibrils exist between the two stable states of AβP, the monomeric protein and

the fibril. Immediate seeding of metastable AβP solutions is promoted by alpha synuclein and negatively charged surfaces (Harkany et al, 2000).

Intermediary protofibrils appear early during fibrillogenesis and disappear when fibrils appear. The sudden change is reported to be unpredictable and likely involves a conformational change followed by the lateral association and winding of protofibrils (Harkany et al, 2000). Protofibrils exhibit beta sheet structure and may trigger AD (Bodles et al, 2000; Serpell, 2000). Interestingly, it is suggested that fibrils may be neuroprotective, due to the aggregation and inactivation of the purportedly neurotoxic protofibrils (Lansbury, 1999).

The presence of truncated AβP species in diffuse and senile plaques led to the hypothesis that the AβP (1-40) and (1-42) termini are unnecessary for plaque formation. The amino acids 25 to 35 were designated the active center of AβP (Harkany et al, 2000; Serpell, 2000). Others argue that the N and C terminals are functionally important, because when lipoproteins, complement factors and zinc bind to the termini, peptide folding and fibrillogenesis are modulated (Harkany et al, 2000). It is also hypothesized that AβP deposition may be influenced by C-terminal variation, while the biological response to deposited AβP may be influenced by the amino terminus (Golde et al, 2000).

AβP (17-21), the central hydrophobic cluster, is reported to be essential for AβP - AβP binding, while the beta turn of the AβP (26-29) region and hydrophobic interactions in the (29-42) domain are important for stabilization of AβP in a fibrillar state (Harkany et al, 2000; Serpell, 2000; Sigurdsson et al, 2000). Also, the presence of a methionine residue at position 35 of AβP, the carboxy-terminal amino acids from valine-40 to alanine-42, the amino terminal

region and the region from lysine-16 to phenylalanine-20 are apparently significant for the promotion and stabilization of AβP aggregation (Seilheimer et al, 1997; Harkany et al, 2000).

Neurochemical factors contribute to the structural transitions of distinct molecular forms of AβP, and to the stabilization, extension and dissolution of AβP aggregates. Electron microscopy has revealed that the number and morphology of fibers in-vitro depends on peptide concentration, pH, buffer ions and staining procedure (Seilheimer et al, 1997). For example, acidic pH promotes the interaction of AβP with essential membrane components. It has also been demonstrated that glycosaminoglycans and gangliosides accelerate the random to beta sheet conformational transition of monomeric AβP, and the lateral aggregation of pre-formed AβP fibrils (Harkany et al, 2000). Aggregation of AβP appears to increase in the presence of proteoglycans like chondroitin and keratin sulfate, which are associated with senile plaques and NFT. In addition, proteoglycans appear to protect AβP from proteolysis and phagocytosis, preventing senile plaque removal (Smith, 1998).

The Non-AβP Component of Alzheimer's Disease Amyloid (NAC)

The NAC is purported to be neurotoxic. It is derived from a precursor protein known as alpha-synuclein, which consists of 140 amino acids and is associated with pre-synaptic terminals in the cerebral cortex, striatum and hippocampus. Alpha-synuclein may play a role in synaptic vesicle mobilization. The hydrophobic 35 amino acid NAC promotes amyloidogenesis in vitro by binding to the 25-35 region of AβP and is inherently amyloidogenic, resulting in fibril formation (Bodles et al, 2000; Iwai, 2000). In addition, it has been determined that NAC

amyloid formation can be promoted by AβP in vitro (Bodles et al, 2000). The NAC appears to reside in senile plaque cores, but not in diffuse plaque cores. The physiological function of NAC is unknown, but it may play an important role in neuronal plasticity (Iwai, 2000).

Full-length NAC and truncated NAC (1-18) were found to be toxic to human neuroblastoma SH-SY-5Y cells and NAC (3-18) and NAC (1-18) were found to be toxic to rat pheochromocytoma PC12 cells due to the formation of beta sheet structures (Bodles et al, 2000). Alternately, NAC may protect neurons by promoting the formation of large amyloid deposits, which some researchers postulate are less toxic than protofibrillar AβP. Ultimately, NAC appears to play important roles in amyloid formation and synaptic change (Iwai, 2000).

Uncertainty Surrounding Senile Plaque Involvement in the Alzheimer's Disease Pathology

Despite evidence supporting the involvement of senile plaques in AD, there is uncertainty surrounding senile plaque formation and its involvement in AD. One hypothesis is that remnants of dead neurons, which contained neurofibrillary tangles, are the basis for the formation of senile plaques (Smith, 1998). In addition, senile plaque formation may illicit neuronal death, but this is disputed due to observations that plaques appear at the wrong time and place with respect to dementia (Mesulam, 1999; Harkany et al, 2000). Initial memory loss appears to relate to loss of limbic function, yet the initial plaques appear in the association neo-cortex rather than limbic areas. Also, extensive plaques have been observed in non-demented elderly individuals, although this could be related to an individual's higher average baseline of brain functioning.

Transgenic animals over-expressing APP did not form NFT and there appears to be no correlation between plaque density and NFT (Mesulam, 1999).

AβP From the Vasculature

It is presently unknown whether APP derived AβP is generated exclusively in the brain by neurons/glia or if some penetrates the brain from the circulation (Harkany et al, 2000). Evidence suggests that the blood-brain barrier (BBB) is impaired as a result of aging or vascular alterations observed in neurodegenerative disorders. The BBB could also be disturbed through head trauma, hypertension, atherosclerosis and stroke (Kuo et al, 1999). Some investigators believe that senile plaques are derived exclusively from the circulation or vascular endothelial cells; others believe it originates in the brain; and still others theorize that it comes from both (Smith, 1998; Kuo et al, 1999).

Over 90% of those with AD display congophilic angiopathy or cerebrovascular amyloid deposits. The cerebrovascular amyloid is practically identical to the senile plaque amyloid and is deposited in the vessel walls of mainly the leptomeningeal and cortical arterioles (Smith, 1998). Amyloid beta peptide (1-40) is the predominant constituent of congophilic angiopathy (Golde et al, 2000; Kumar-Singh et al, 2000). These deposits are known to cause hemorrhage and possible entry of amyloid into the brain parenchyma through a compromised BBB. Cerebrovascular amyloid deposits are also found in the normal aging brain, albeit in lower concentrations (Smith, 1998).

APP and AβP Trafficking

Intracellular APP trafficking occurs predominantly in neurons, but also in microglia and astrocytes and follows a constitutive secretory pathway. Alpha secretase processing is believed to occur intracellularly, likely in a late compartment of the constitutional secretory pathway and maximally at acidic pH. In PC12 cells alpha secretase is thought to exert its actions in the trans-Golgi compartment. Gamma secretase processing, on the other hand, is thought to occur at the plasma membrane, resulting in the liberation of the p3 and p7 fragments (Checler, 1995).

Alpha secretase candidates include cathepsin B, the multicatalytic proteinase complex or a 105-120 kDa metalloproteinase isolated from human brain. Candidates for a membrane bound alpha secretase include endopeptidase 3.4.24.11, a calcium activated, dithiothreitol-sensitive metalloproteinase present in rat brain cortex and an integral membrane metallopeptidase recently detected in crude membranes prepared from H4 human neuroglioma cells (Checler, 1995).

It appears that APP can be internalized and AβP generated in acidic endosomes/lysosomes. Clathrin-mediated endocytosis of APP can occur followed by rapid recycling of the APP to the plasma membrane for further processing (Checler, 1995; Selkoe, 1999). The best evidence for re-internalization comes from an experiment in which APP on the plasma membrane was radioiodinated and allowed to internalize at 37 °C. In 15 to 30 minutes, radioiodinated AβP was released from the cells, which could only have arisen from the radiolabeled APP. During internalization, AβP 1-42 may be generated in the endoplasmic reticulum (ER) and Golgi (Selkoe, 1999).

Wortmannin, a phosphatidylinositol kinase inhibitor, is effective in reducing extracellular AβP accumulation in vitro and reducing extracellular AβP accumulation, senile plaque number and area occupied by senile plaques in a Tg2576 mouse model. The mechanism of action is currently unknown, but wortmannin may be influencing APP trafficking, resulting in decreased secretion of AβP (Haugabook et al, 2000).

AβP's Purported Effects on Neurons

The mechanisms by which AβP mediates its neurotoxic effects include membrane depolarization, increased sensitivity to excitotoxins, alterations in calcium homeostasis, activation of receptors affecting cellular homeostasis, direct disruption of membrane integrity, oxidation, activation of complement or a combination of the above (Smith, 1998; Martinez-Senac et al, 1999; Gandy and Petanceska, 2000). Primate neurons are more sensitive to AβP toxicity than rodent neurons and aged neurons in general are more sensitive to AβP toxicity than young neurons (Golde et al, 2000).

In vitro aggregation/toxicity occurs with AβP (1-42) days/weeks after incubation at a concentration of >100 μM (Seilheimer et al, 1997). In vitro toxicity with AβP (25-35) is reported to occur immediately upon dissolution and the conformational change to β-sheet within 2 hours of dissolution (Bodles et al, 2000). Beta sheet formation requires dissolution of the respective AβP in the presence of $CaCl_2$ or $MgCl_2$ (Rymer and Good, 2001). In addition, biphasic actions have been induced by AβP on neurons in culture. Concentrations in the pM to nM range promote neuronal differentiation and increase neuronal viability in a neurotrophin-like

manner. Micromolar concentrations of AβP are neurotoxic and induce necrotic or apoptotic cell death in a dose dependent fashion. Low AβP concentrations are potentially associated with receptor- mediated signal transduction and higher AβP concentrations are predicted to be associated with long-lasting lipid-protein and protein-protein interactions. In vivo investigations using rodents found neurotoxic properties associated with μM AβP concentrations on basal forebrain cholinergic neurons, resulting in significant impairment of cognitive function. The concentration-dependent action of AβP on cholinergic function is thought to parallel neuropathological changes. An increasing plaque burden/ AβP concentration is correlated with the decline and disfunction of the basal forebrain cholinergic system during the progression of AD (Harkany et al, 2000).

Alterations in Neuronal Membrane Physicochemical Properties

A growing number of studies indicate that AβP may alter the physicochemical properties of neuronal membranes including membrane fluidity, permeability to ions and non-electrolytes and lipid peroxidation. It has been reported that AβP forms cation channels when incorporated into the lipid bilayer. Therefore, some effects may be mediated by direct interactions between AβP and membrane lipids (Martinez-Senac et al, 1999). A study conducted by Martinez-Senac and associates discovered that AβP (25-35) accumulates at the lipid/water interphase forming beta fibrils, which potentially perturbs phospholipid headgroup interactions. This effect is purportedly greater if the head group is negatively charged. Lipid/water interface perturbation by AβP may activate membrane-associated enzymes like phospholipases. Therefore inhibition

of the electrostatic interaction between AβP and the phospholipid bilayer will potentially prevent AβP toxicity (Martinez-Senac et al, 1999).

An additional study provides evidence that AβP (25-35) alters membrane properties in a concentration dependent fashion, possibly resulting in an increase in neuronal calcium conductance. Experiments were conducted using mouse brain membranes and human lymphocyte membranes. Low μM concentrations of AβP (25-35) and AβP (1-40) were found to induce small membrane fluidity changes in mouse brain membranes, which may alter properties and functions of membrane associated ion channels. Higher μM concentrations of AβP (25-35) and AβP (1-40) were found to have additional and more pronounced effects on membrane fluidity by potentially disrupting membrane structure. Exposure of human lymphocytes to 1-10 μM AβP (25-35) resulted in pronounced effects on calcium signaling. Membrane destabilization and aggregation occurred at >1 μM AβP (Muller et al, 1995).

Fresh globular AβP (1-40) administered to cultured, aged human, AD-free fibroblasts induced rapid structural modifications, including cytoskeletal organization, retraction of cellular processes and loss of cell-cell contacts, within minutes of incubation. Eventual cellular degeneration resulted and was prevented by an anti AβP antibody, zinc and Tris. In the presence of extracellular calcium, a sustained increase in intracellular calcium and cellular degeneration resulted. When cells were exposed to a calcium-free extracellular medium, cellular degeneration did not occur. This study suggests that toxicity does not require fibrillar AβP, calcium is involved in neurodegeneration and this effect can be mediated, possibly through calcium permeable pores (Zhu et al, 2000).

In addition to interactions of AβP with the plasma membrane, it is postulated that AβP might illicit sustained membrane permeability changes in the mitochondria, Golgi complex and endoplasmic reticulum (ER). Experimental data suggests that AβP and the C-terminal fragments of APP are capable of forming calcium selective cation pores in lipid bilayers. Longer fragments appear capable of forming channels, but the shorter neurotoxic fragments such as AβP (31-35), AβP (34-39) and AβP (33-35) probably do not (Harkany et al, 2000).

Disruption in calcium homeostasis and free radical formation are reported to be the consequence of indiscriminate intercalation of AβP into the plasma membrane. It was discovered via small angle X-ray diffraction that AβP (25-35) is lipophilic and inserts into the hydrophobic membrane core of liposomes, resulting in increased electron density without perturbing the width of the bilayer (Kanfer et al, 1999). Also, AβP (25-35) decreased the fluidity of mixed membranes prepared from several regions of rat brain. Core fluidity decreased in a concentration-dependent fashion when hippocampal membranes were incubated with AβP (1-42) and AβP (25-35), with a more pronounced decrease occurring with AβP (1-42). Membranes from AD patients and controls were affected by AβP (1-42) and AβP (25-35) in the same fashion and to the same extent. Therefore AβP appears to aggregate and accumulate in the plasma membrane during AD in a stable fashion, altering membrane fluidity (Eckert et al, 2000).

In addition to membrane fluidity changes and contrary to Kanfer et al's 1999 observations, plasma membrane thinning corresponding to a 30% decrease in moles of cholesterol appears to occur in AD, which can affect coupling and uncoupling of G proteins, contributing to signal transduction deficits. Thinner membranes can alter calcium handling responses to exogenous stimuli and can potentially expose abnormal protease cleavage sites on

the trans-membrane portion of APP, resulting in AβP accumulation and cellular damage (Roth et al, 1995). An additional study also determined that the cholesterol content of AD membranes is reduced compared to controls, but statistical significance was not reached (Eckert et al, 2000).

Immune System Activation

Interestingly and indirectly, AβP in vitro appears to enhance astroglial and microglial secretion of IL-1beta, a cytokine. In vitro, AβP stimulates the proliferation and morphological transformation of microglia and IL-1beta enhances AβP cytotoxicity in PC-12 cells. Therefore, activation of microglia in vitro appears to promote neuronal cell injury (Harkany et al, 2000). In addition, senile plaque complement proteins activated by AβP potentially contribute to the AD pathology by injuring or killing neurons. Complement is a branch of the humoral immune system involved in host defence. Antibody and/or antibody-independent activation of complement can lead to inflammation, opsonization and cytolysis (Emmerling et al, 2000).

Over 20 different proteins comprise the alternative and classical complement pathways, with both pathways terminating in membrane attack complexes (MAC) and subsequent lytic pore formation. Following cellular injury, cytokine-mediated recruitment and activation of immune cells to the site of complement activation occurs. Amyloid beta peptide interaction with Clq, a component of the complement, leads to increased amyloid aggregation. Glia, microglia, astrocytes and nerve cells can synthesize components of the complement system. Complement protein mRNA for the classical pathway is upregulated in AD compared to control. The brain responds to a chronic complement attack during AD by increasing expression of defence proteins

like clusterin and lactoferrin. Ultimately, complement activation has led to cell death in nerve cell cultures (Emmerling et al, 2000). In the cerebellum, where AβP deposits appear to cause no injury, plaques are reportedly free of associated complement, while in the forebrain, complement associates with plaques, injuring the surrounding cells (Gandy and Petanceska, 2000).

The non-steroidal anti-inflammatory drugs (NSAIDS) that inhibit cyclooxygenase 2 (COX-2), celecoxib and rofecoxib, are being investigated as potential AD treatments. It has been determined that celecoxib and rofecoxib block neurotoxicity resulting from chronic, low-level brain inflammation or from AβP in cell culture. It is presently unknown how they affect the complement (Emmerling et al, 2000). It is postulated that NSAIDS work to reduce inflammation in three different ways: 1) inhibition of the nuclear factor kappa beta (NFκβ) activator IKKβ, 2) inhibition of (COX-2) and 3) activation of PPARγ, a receptor, which inactivates NFκβ, thus blunting inflammation (Versteeg et al, 1999).

A prospective population-based cohort study, conducted in the Netherlands, on patients using NSAIDS, including Sulindac and Ibuprofen, for rheumatoid arthritis and osteoarthritis, suggested that the long-term use of NSAIDS reduces the risk of AD development. Future studies should attempt to determine if the benefits of NSAIDS therapy outweigh the risks (Veld et al, 2001).

The potential exists for the development of a new drug designed to act as a β-sheet breaker. A study demonstrated that a five-residue peptide called iAβ5 can induce AβP (1-42) fibril disassembly in pre-existing AβP deposits in a rat model and the effect is reproduceable and specific. The central hydrophobic cluster (17-21) is important for fibril formation and iAβ5 potentially binds to that region, destabilizing the interaction between AβP monomers/oligomers.

The subsequent exposure of potential cleavage sites may allow proteolysis and removal of AβP. Also, iAβ5 bound to AβP may act as an immune complex, subsequently reducing neurotoxic IL-1β secretion. Enhanced phagocytosis may result in the subsequent removal of AβP. Compounds with the properties exhibited by iAβ5 may therefore be able to reduce the size and or number of amyloid plaques in AD brains (Sigurdsson et al, 2000).

Receptor Mediated Neurotoxicity

There is evidence that AβP binds to receptors including the serpin receptor for advanced glycation end products (RAGE), non-selective low affinity neurotrophin receptor (p75 NTR) and glutamate receptors. It is difficult to understand how AβP binds to receptors for structurally dissimilar agonists and therefore it is hypothesized that AβP inserts into the plasma membrane causing a perturbation that deceives membrane proteins into triggering various intracellular responses (Kanfer et al, 1999; Pillot et al, 1999). This hypothesis is supported by an experiment in which aggregated AβP (25-35) perturbed lysosomal membranes, resulting in membrane current induction and neuronal calcium homeostasis disruption (Hirakura et al, 1998).

Studies have indicated that AβP may induce excitotoxic neuronal damage in a dual fashion: directly, by the formation of calcium permeable transmembrane pores and by it's regulatory action on glutamate receptors, or indirectly, through potentiating glutamate excitotoxicity. The excitotoxicity model implicates both neurons and glial cells. In response to multiple stressors, neuronal APP expression increases and if accompanied by beta and gamma secretase processing, AβP liberation occurs. The accumulating AβP activates microglia,

resulting in neurotoxic inflammatory cytokine release, glutamate release and the impairment of astroglial glutamate uptake. The increase in extracellular AβP and glutamate concentrations ultimately leads to neuronal excitotoxicity. Amyloid beta peptide potentially forms calcium permeable plasma membrane channels and acts on the N-methyl-D-aspartate (NMDA) receptor, while glutamate activates Alpha-Amino-3-Hydroxy-5-Methyl-4-Isoxazole Propionic Acid (AMPA) and NMDA receptors, eliciting sustained membrane depolarization. Intracellular calcium then reaches pathological concentrations, inducing cell death predominantly through apoptosis (Harkany et al, 2000).

Apoptosis is characterized by plasma membrane blebbing, cell shrinkage, loss of plasma membrane phospholipid symmetry, activation of one or more caspases, mitochondrial membrane depolarization, mitochondrial oxyradical production, mitochondrial calcium overload, chromatin condensation and DNA fragmentation (Mattson et al, 1998). Apoptotic protease activating factor (Apaf-1), a cysteine aspartate protease, transmits apoptotic signals from mitochondrial damage to activate caspase 9, which then activates caspase 3, ultimately resulting in neuronal cell death (Yuan and Yankner, 2000). The inhibition of caspases has resulted in a protective effect against AβP induced neurotoxicity (Copani et al, 1999).

Brain APP expression is inducible and there is strong evidence that APP processing is regulated by neuronal activity, is under the control of various neurotransmitter receptors and plays a protective role by rescueing neurons from excitotoxic stimulation. Inducible APP expression can occur during neuronal excitotoxicity associated with focal ischemia and NMDA receptor stimulation and with immunolesion-activated astro- and microglia. When alpha secretase processing occurs, neuroprotection may result (Harkany et al, 2000).

In vitro experimental evidence suggests that neurons begin a cell cycle in response to AβP. They complete the G_1 phase, enter S phase and are blocked from entering the G_2 phase and eventually die. This observation suggests that cell cycle activation may be a requirement for neuronal death in AD. The process of apoptosis may occur through activation of P53 by cyclin dependent kinase (Cdk) activity. Amyloid beta peptide may also induce apoptosis through interactions with the RAGE, which can mediate free radical production, the neurotrophin receptor (p75 NTR), which can induce neuronal cell death and the APP, which can also induce neuronal cell death. In addition, tumour necrosis factor alpha (TNFα) can also induce apoptosis. It is difficult to assess the contribution of apoptosis in AD neuronal loss due to the chronic nature of the disease. Therefore, at any given time only some apoptotic neurons are detectable, whereas other non-apoptotic neuronal degeneration is also occurring, leading one to conclude that there are multiple mechanisms of neuronal degeneration operating (Yuan and Yankner, 2000).

The involvement of the p75 NTR receptor in AD pathogenesis is predicted since upregulation of neurotrophin receptor expression has been demonstrated in AD, and p75 NTR expression correlates with cellular AβP sensitivity. The hypothesis is supported by in vitro evidence that p75 NTR binds AβP in a time and dose dependent fashion, resulting in the activation of NFκB signaling followed by neuronal apoptosis. It has been determined that non-aggregated AβP (1-40) can induce apoptosis in rat cortical neurons and this was purportedly induced by the C-terminal fusogenic domain of AβP, which displays membrane-destabilizing properties. Cytotoxicity appears to result from a direct interaction between the non-aggregated peptide and the neuronal plasma membrane (Pillot et al, 1999).

Freshly dissolved AβP (25-35) stimulated 35_S GTP gamma S binding, a functional measure of G-protein coupled receptor activation. The experiment used rat hippocampal membranes and concluded that AβP monomers and oligomers may activate G-protein coupled receptors. The receptor interactions are postulated to elicit abrupt changes in neuronal function.

Oxidative Stress

The free radical theory of aging indicates that the accumulation of reactive oxygen species (ROS) during aging results in damage to major cellular components, including the nucleus, mitochondrial DNA, membranes and cytoplasmic proteins (Christen, 2000). The breakdown of the lipid component is termed lipid peroxidation (Harkany et al, 2000). Irreversible damage or even cell death can occur with free radicals present. The features of oxidative cellular damage include enhanced disorganization of the plasma membrane and intracellular organelles, DNA fragmentation, altered catabolic pathways of peptides, destabilization of intracellular ion homeostasis, decrements of repair mechanisms and decline in the expression of antioxidant enzymes (Christen, 2000).

Free radical damage can accumulate over time. Neurons appear to be particularly vulnerable to free radical damage, because glutathione levels are low, membrane polyunsaturated fatty acid levels are high, and brain metabolism requires large quantities of oxygen. Free radicals likely involved in the AD pathology include the free hydroxyl radical, the superoxide radical, hydrogen peroxide and peroxynitrite, formed from the combination of superoxide and nitric oxide (Christen, 2000).

Oxidative damage to neurons increases as aging occurs and the process is enhanced during the progression of AD (Harkany et al, 2000). Oxidative stress associated with neurons, neurofibrillary tangles and senile plaques includes advanced glycation end products, nitration, advanced lipoperoxidative end products, carbonyl-modified neurofilament proteins and free carbonyls. Oxidation and resulting protein cross-linking may explain why the lesions are not removed, even following ubiquitination or neuronal death. It is hypothesized that abundant lysine residues found in tau and neurofilaments are sensitive to oxidative stress and consequently are important to the AD pathology (Smith, 1998).

When senile plaques are present, they are surrounded by microglia, the macrophages of the brain. The microglia, when activated produce free radicals. Also, neurofibrillar tangles and senile plaques contain iron, which is critical to the initiation of free radical formation (Smith, 1998).

When AβP was added to cultured hippocampal neurons, 4-hydroxynonenal (HNE), a cytotoxic product of lipid peroxidation was formed. AβP-associated free radical lipid peroxidation of arachidonic acid leads to the formation of HNE, which then forms adducts and alters the structure and function of transmembrane ion-motive ATPases and key transport proteins, leading to irreversibly large increases in intracellular calcium and cell death. Vitamin E protects biomolecules and brain-membrane proteins from AβP (25-35) induced oxidation, but not against the effects of HNE. Blunting the AβP-associated free radical oxidative stress prior to lipid peroxidation is a potential therapeutic strategy. In addition, HNE toxicity has reportedly been prevented by the endogenous antioxidant glutathione (Subramaniam et al, 1998).

AβP acts as a free radical following distortions of its electronic structure. Also, due to its lipophilic nature, AβP can interact with the membrane initiating receptor-mediated pro-inflammatory signaling pathways. Firstly, it was hypothesized that radicalization is critical to AβPs neurotoxicity, with methionine at position 35 considered a requirement for radicalization. The resulting reactive peptide species can inactivate oxidation-sensitive glutamine synthetase and creatine kinase enzymes and induce a transient increase in the superoxide scavenging enzyme, superoxide dismutase, as well as initiate lipid peroxidation. Removal of methionine at position 35 ilicits a severe reduction of the above activities. The high affinity binding of AβP with negatively charged surfaces, both on the cell surface and intracellularly, may alter the turnover of lipids and peptides by modifying the local micro-homeostasis in a manner permissive for abnormal enzymatic degradation of membrane components. For example, HNE is neurotoxic in a dose dependent manner and even in low concentrations increases neuronal vulnerability to AβP toxicity and exacerbates oxidative stress by affecting mitochondrial integrity and function (Yan et al, 2000).

AβP also increases oxidative stress by binding to RAGE and methionine sulfoxide reductase A (MSRA) receptors on all central nervous system (CNS) cell types, resulting in AβP translocation into the cytosol, followed by action at presently unidentified recognition sites on intracellular organelles, like the ER and mitochondria. Mitochondrial damage ultimately results in energy depletion and production of cytotoxic ROS. In addition, calcium uptake and calcium regulation of the mitochondria and ER become compromised, leading to an increased internal calcium concentration. Amyloid beta peptide can activate NFκB in both nerve and glial cells through RAGE and p75 NTR receptors, which use free radicals like hydrogen peroxide as

intracellular messengers. NFκB then stimulates the expression of nitric oxide synthase (NOS), cell-surface recognition molecules and inflammatory cytokines. Nitric oxide production increases followed by production of peroxynitrite, a strong oxidizing and nitrating agent, ultimately contributing to AβP toxicity (Yan et al, 2000).

In addition, AβP binding to RAGE and subsequent activation of NFκB results in enhanced expression of macrophage-colony stimulating factor (M-CSF). In addition, RAGE mediated NFκB activation results in a positive feedback loop with increased expression of the RAGE receptor followed by increasing NFκB activation. The most potent RAGE mediated cellular stress occurred at lower AβP concentrations ($10^{-9} - 10^{-6}$ M), whereas at higher concentrations RAGE independent modulation of cellular function occurred (Yan et al, 2000).

Also, neuronal NOS exhibits calcium and calmodulin dependence, and becomes activated downstream of excitotoxic stimulation. Therefore, a potential link exists between excitotoxicity and oxidative stress in AβP toxicity because the regulation of NOS expression is dependent on both ROS and Ca/calmodulin (Harkany et al, 2000). Also, binding of AβP to neuronal RAGE induces the production of M-CSF, by an NFκB dependent pathway. Macrophage colony stimulating factor then interacts with its microglial receptor, c-fms, a tyrosine kinase, initiating transformation of microglia and release of APOE and potentially cytotoxic inflammatory cytokines like TNF-alpha (Harkany et al, 2000; Yan et al, 2000). Also, RAGE binds soluble AβP and promotes fibrillogenesis (Yan et al, 2000).

A potential intracellular target of AβP is the amyloid beta peptide binding alcohol dehydrogenase (ABAD) receptor on the ER and mitochondria. Binding of AβP to intracellular ABAD is speculated to occur at sites remote from protective mechanisms; thus resulting cellular

perturbations are predicted to be severe and sustained. Amyloid beta peptide binding alcohol dehydrogenase acts as an alcohol dehydrogenase in the ER and mitochondria, as well as being involved in mitochondrial beta-oxidation of fatty acids. In AD there is decreased glucose transporter activity at the BBB, suggesting that maintenance of neuronal energy homeostasis might be accomplished through upregulation of fatty acid beta-oxidation or other metabolic pathways. It is proposed that ABAD is a component of that compensatory pathway and its interaction with AβP results in the generation of ROS and toxic aldehydes constituting a protective host response gone awry in AD (Yan et al, 2000). The mechanism of AβP toxicity mediated through ABAD is indicative of the potential toxicity resulting from soluble intracellular and extracellular AβP.

Oxidative stress upregulates the production of APP and AβP, suggesting a possible positive feedback loop between oxidative stress and AβP deposition (Smith, 1998). The brain attempts to protect itself against oxidative insults through activating NFκB and through expressing antioxidant enzymes like superoxide dismutase and heme oxygenase-1 (Smith, 1998; Harkany et al, 2000). In addition to vitamin E, indole-3-propionic acid (IPA) and melatonin, which are found in the body under physiological conditions, have been tested as free radical scavengers. The free radical scavenging properties of IPA were found to surpass those of melatonin, the most powerful known natural hydroxyl radical scavenger, in E-18 fetal rat primary hippocampal neurons and in SK-N-SH human neuroblastoma cells. The mechanism is not related to that of vitamin E, which possesses a reactive hydroxyl group, enabling it to donate a hydrogen atom, thereby reducing free radicals like peroxyl radicals that promote radical chain reactions. Because of their high reactivity, chain-breaking antioxidants like vitamin E are highly

reactive and autoxidize in the presence of transition metals, increasing the formation of primary radicals like hydroxyl radicals. Melatonin and IPA are endogenous electron donors, primarily detoxifying hydroxyl radicals, initiators of radical chain reactions. The hydroxyl radicals are the most reactive of all the oxygen-derived free radicals. Melatonin and IPA are therefore far superior to other antioxidant compounds such as vitamin E. (Chyan et al, 1999).

It is presently unknown whether oxidative stress is a primary or secondary event in the pathogenesis of AD. There is evidence however, that the early events in AD are related to oxidative damage (Smith, 1998). In addition, AβP is thought by most researchers to be a major generator of free radicals. Although some research has shown contradictory findings, it is suggested that both AβP and NFT are cellular compensations for increased oxidative stress and serve antioxidant functions (Smith et al, 2000). Despite differences of opinion on the source of free radicals in AD, researchers agree that free radicals play a significant role in the pathology.

Genetics

There is evidence for a significant genetic component in a substantial proportion of individuals with AD. It is presently unknown if there are genetic linkages to abnormal tau activity, but ample evidence exists linking genetics to abnormal amyloid processing in AD (Mesulam, 1999). The risk of developing AD increases nearly 3.5 times when a parent or sibling has it (Jorm, 1997). Also, there are families that display autosomal dominant transmission of the disorder, the more aggressive familial AD (Smith, 1998).

The APP Mutations

Approximately 1 % of familial Alzheimer's disease cases are linked to at least eight missense APP mutations, located on chromosome 21 (Epstein, 1999; Kumar-Singh et al, 2000). These mutations affect APP metabolism in two ways. The Swedish double mutation, located near the beta secretase cleavage site, increases the production of both AβP (1-42) and AβP (1-40), while mutations near the gamma secretase cleavage site result in increased AβP (1-42) production (Kumar-Singh et al, 2000). The development of AD occurs with almost 100% penetrance in those who express a mutated APP. The mechanism by which a mutation results in the development of AD is unknown, although APP mutations increase the amount of AβP produced and/or the proportion of AβP (1-42) (Smith, 1998).

The Austrian T714I mutation involves amino acid 43 of AβP, located directly at the gamma 42 secretase cleavage site. This mutation increases the amount of AβP (1-42) while decreasing AβP (1-40) production, results in early age of AD onset, rapid disease progression and death. It is generally believed that AβP (1-40) is needed for maturation of non-fibrillar pre-amyloid AβP (1-42) into the purportedly neurotoxic fibrillar amyloid. The formation of typical cored plaques in these individuals was retarded. This study determined that the mostly immature non-fibrillar pre-amyloid plaques are essential to the AD pathology, challenging the assumption that amyloid fibril accumulation underlies neuronal disfunction in AD (Kumar-Singh et al, 2000).

The Presenilin Mutations

Mutations in the presenilin 1 and presenilin 2 genes, located on chromosomes 14 and 1 respectively, are associated with 70% of early-onset familial AD cases. Over 50 mutations in these genes have been discovered (Epstein, 1999). The gene products of presenilins 1 and 2 are similar trans-membrane proteins of 463 and 448 amino acids, with between 6 and 9 hydrophobic membrane spanning domains. The mutations occur in positions homologous between the two proteins and within or near the trans-membrane domains. Presently, the functions of presenilins 1 and 2 are unknown, although it is hypothesized that they may act as receptors, ion channels, protein processors or function in cellular trafficking (Smith, 1998; Epstein, 1999).

In AD, presenilin mutations are thought to affect APP processing, increasing the quantity of AβP, possibly through direct action or as a cofactor in the gamma secretase cleavage of AβP (Smith, 1998; Epstein, 1999). Additionally, presenilin mutations are thought to influence the position and/or efficiency of the APP cleavage or lead to increased production of the more neurotoxic AβP (1-42) (Mesulam, 1999; Bothwell and Giniger, 2000). The APP and presenilin mutations occurring in AD are thought to either increase the net rate of AβP release or subtly alter the cleavage site, resulting in the production of the more amyloidogenic AβP (1-42) (Bothwell and Giniger, 2000). It is postulated that presenilins may even be gamma-secretases (Yuan and Yankner, 2000).

The AD causing presenilin mutations could also affect neuronal plasticity by interfering with the putative plasticity functions of PS1 and shifting APP processing toward AβP (1-42) (Mesulam, 1999). In transgenic mice, the expression of presenilin 1 and APP induces an

increase in AβP (1-42) production compared to the expression of either protein alone, suggesting that synergism may be operating (Epstein, 1999).

The cytosolically released intracellular C-terminal domain of the APP, which is released into the cytosol following gamma secretase cleavage, may be important in cell signaling related to the AD pathology. The C-terminal fragment may activate 3, 3-diaminobenzidine tetrahydrochloride (Dab), an intracellular adaptor protein, which leads to tau phosphorylation through the activation of cyclin dependent kinases (Cdk5). Another hypothesized route is through translocation of the C-terminal fragment of APP to the nucleus. This process, which is thought to occur simultaneously with the release of AβP, may be the pathological process, whereas the release of AβP may be pathologically irrelevant (Bothwell and Giniger, 2000).

Congophilic dense cores and neuritic plaques are not dominant in those suffering from AD and expressing the PS1δ exon 9 mutation. Researchers then hypothesized that classic amyloid may not be the culprit in AD, but membrane bound AβP, soluble AβP oligomers or intracellular AβP may be responsible. An experiment demonstrated that overexpression of the PS1 mutation in the brains of transgenic mice increased neuro-degeneration in the absence of classical amyloid deposition as assessed by anti- AβP immunohistochemistry. Increased intracellular AβP (1-42) levels were detected by a polyclonal antibody. Therefore, increased AβP deposition in classical congophillic deposits may not be necessary for neurodegeneration (Golde et al, 2000).

Presenilin involvement in AD could be determined through homogeneous purification and subsequent determination of in vitro catalytic activity toward the APP. It would purportedly be very difficult to purify intact presenilins and isolate a pure extract (Checler, 2001).

Apolipoprotein E (APOE)

Within the blood stream, neutral lipids circulate as lipoproteins. Lipoproteins are composed of a phospholipid and cholesterol shell and a triglyceride and cholesteryl ester interior with stabilization via surficial apoproteins. Apoproteins also act as cofactors for enzymatic reactions and ligands that bind to lipoprotein receptors (Ladu and Reardon, 2000). Lipoproteins containing APOE bind to cell surface receptors and are internalized by the process of endocytosis.

Recently, APOE, a 35kDa glycoprotein was implicated in AD. Apolipoprotein E plays an important role in triglyceride-rich lipoprotein metabolism and cholesterol homeostasis throughout the body, and early work assumed a similar role in the CNS (Roses, 1995). In the CNS, APOE is expressed predominantly by astrocytes and microglia. In addition, APOE manufactured in the liver travels via the blood stream to the brain, crossing the BBB. A variety of receptors in the low-density lipoprotein receptor (LDLR) family are expressed in the brain, with neurons expressing Low Density Lipoprotein Receptor Related Protein (LRP), APOE receptor 2 and the very low-density lipoprotein receptor (VLDLR) (Ladu and Reardon, 2000). Following injury in the peripheral or CNS, APOE may play a role in growth, repair and maintenance of myelin and axonal membranes (Roses, 1995).

APOE is polymorphic, with three common alleles, APOE epsilon 2 (ε2), APOE epsilon 3 (ε3) and APOE epsilon 4 (ε4) (Swartz et al, 1999). The ε3 allele appears to be the most common (Saunders et al, 2000). In those suffering from AD the ε4 allelic frequency is

approximately 40% higher than the general population. The risk of developing AD and developing it at a younger age appears to increase with number of ε4 alleles. On average, people homozygous for ε4 develop AD younger than those heterozygous for the allele, who in turn develop AD younger than those without the ε4 allele. It has also been hypothesized that those carrying the ε2 allele may be less likely to develop the disease (Swartz et al, 1999).

Apolipoprotein E binds to AβP in an isoform specific manner. Deposition of AβP into cytotoxic beta pleated sheet formations appears to differ with APOE allele, with ε4 promoting the most, ε3 intermediate amounts and ε2 the least. The ε4 allele appears to inhibit AβP polymerization the least, possibly a result of its inefficiency at forming soluble complexes with AβP. Epsilon 2 and ε3 allele products bind more readily to AβP and prevent AβP aggregate formation, thus slowing or preventing the formation of senile plaques (Swartz et al, 1999). Amyloid beta peptide induced neurotoxicity in neuronal cell cultures and primary rat astrocytes was prevented with the addition of exogenous APOE. With primary rat hippocampal neurons, it was determined that ε3 and not ε4 protects against AβP induced neurotoxicity and receptor associated protein, an APOE antagonist, inhibited the process (Ladu and Reardon, 2000).

Apolipoprotein E binds to tau, both in the neurite and neuron. This binding may slow down the formation of paired helical filaments (Swartz et al, 1999). For binding to occur, APOE has to enter the cytoplasm. It is proposed that APOE ε2, APOE ε3 and APOE ε4 all bind to the β tubulin binding site on tau, but APOE ε4 is more loosely bound; so over time tau can bind

additional tau molecules, leading to the formation of PHF and neurofibrillary tangles (Roses, 1995).

It is purported that APOE is important for neuronal plasticity. This was shown in APOE deficient mice, which displayed impairments in reactive synaptogenesis. It has been determined that the presence of the ε 3 allele promotes plasticity, whereas that of the ε4 allele inhibits neurite growth and dendritic plasticity (Mesulam, 1999).

Endocytosis and APOE

Neural endocytic pathways may also be influenced by APOE allele. The endocytic pathway (EP) internalizes and processes extracellular nutrients and trophic factors, recycles, modifies and degrades receptors and other integral membrane proteins after neurotransmitter release and directs information to intracellular biosynthetic pathways (Cataldo et al, 2000). Some acid proteases, like cathepsin D, which possesses beta/gamma secretase activity, are found in early endosomes, the site of internalization and modification of proteins relevant to AD, like APP and APOE. Alzheimer's disease patients expressing ε4 appear to manifest an earlier appearance of enlarged endosomes. It appears that these endosomal abnormalities are accelerated at the earliest stages by the ε4 allele, but not at moderate or late pathological stages, where no detectable difference in neuronal endosome volume was detected between individuals with ε4 and those with ε2 or ε3. Therefore, it appears that the ε4 allele influences clinical disease onset, but not progression. This EP activation is an AD specific response of susceptible

neuronal populations. Endocytic pathway activation has not been detected in other neurodegenerative diseases and is not a generalized response to neuronal injury or degeneration. These EP abnormalities associated with AD are consistent with APOE binding to its neuronal receptor and activating endocytosis and APOE interacting with APP in the endosomal compartment (Cataldo et al, 2000).

Enhanced endocytotic activation could result in sequestration and inappropriate degradation of plasma membrane proteins, growth factors or receptors by the early endosomes, ultimately reducing cellular viability. The EP also allows entry of AβP and APOE, which could selectively damage lysosomal integrity, impairing its function. Transgenic cell lines that mimic the increased hydrolase trafficking to endosomes seen in AD secrete enhanced levels of AβP (1-40) and AβP (1- 42), suggesting that APOE could be responsible for the increased release of AβP seen with AD (Cataldo et al, 2000).

APOE as a Free Radical Scavenger

APOE acts as a free radical scavenger in an isoform dependent way. In contrast, APOE is susceptible to free radical damage and this damage has been correlated with AD (Christen, 2000). The low free radical scavenging activity of the ε4 allele may contribute to its association with AD (Tamaoka et al, 2000). Apolipoprotein ε4 may not be toxic, but just produces a less favorable antioxidant effect (Christen, 2000). Apolipoprotein E is a strong chelator of copper and iron, important redox-active transition metals (Smith et al 2000). Isoform specific chelating ability has not been investigated.

Tamaoka et al assayed postmortem human brain and found that the highest quantity of thiobarbituric acid-reactive substances (TBARS), resulting from lipid peroxidation occurred in ε4 homozygous brains. Successively less generation of TBARS was seen with ε3/ε4, ε3/ε3 and ε2/ε3 brains, with statistically significant differences between AD brains with APOE ε4/ε4 and ε3/ε3 (Tamaoka et al, 2000). Compatible with these results are the findings from a rat neuronal cell line, where the cells were protected from oxidative cell death by APOE in an allele specific fashion, with ε2 offering the most protection, ε3 offering moderate protection and ε4 offering the least protection (Behl and Holsboer, 2000; Christen, 2000).

The level of lipid peroxidation in AD brains was found to be inversely proportional to the concentration of APOE found within the brain. This finding adds weight to the hypothesis that APOE is beneficial against lipoperoxidation. The beneficial effect is more pronounced when the patient has the ε2 or ε3 allele (Behl and Holsboer, 2000).

EGB 761

When ginkgo biloba extract 761 (EGB 761), an antioxidant, comprised of flavonoids, terpenoids and organic acids, was added in vitro, to AD brain tissue, lipoperoxidation levels were reduced (LeBars et al, 1997; Christen, 2000). EGB 761 could be important in protecting neurons from AβP induced lipoperoxidation whether the less potent and more susceptible ε4 isoform is present or not, with the major antioxidant benefit accruing when the ε4 isoform predominates.

EGB 761 has been studied in clinical investigations in the United States and Germany with a modest positive effect on cognitive indexes with questionable clinical significance (Le Bars et al, 1997; Chandler, 2000; Christen, 2000). A review of 30 articles found a small but significant effect of this drug on cognition in AD. Another well-studied antioxidant, vitamin E (alpha tocopherol) did not improve cognition among AD patients. It is not known whether EGB 761 improves cognition. In addition to acting as an antioxidant EGB 761 may provide a protective effect on neuroreceptors in aging subjects and act as a monoamine oxidase (MAO) inhibitor (Christen, 2000).

Alternately, it was shown that APOE is sensitive to attack by free radicals, with isoform specific responses. The ε4 isoform is more sensitive to free radical attack than ε3, which is more susceptible than ε2. Ginkgo biloba EGB 761 extract was found to protect APOE from oxidation in an isoform dependent way, with ε4 protected the most and ε2 the least (Christen, 2000).

Estrogen

Prevalence of AD is higher among women than men, even after adjusting for differences in age distribution (Henderson, 1997). The risk for AD conferred by possession of the ε4 allele appears to be greater for women than for men, although this has been disputed (Henderson, 1997; Swartz et al, 1999). Another risk factor for women is postmenopausal estrogen deprivation. Therefore it is believed that estrogen replacement therapy may reduce the risk of AD among women. Estrogens readily cross the BBB and therefore can interact with nuclear and

membrane bound neuronal estrogen receptors or through receptor independent interactions (Henderson, 1997; Behl and Holsboer, 2000). Estrogen administration was found to reduce plasma levels of APOE and its isoform specificity is presently unknown (Henderson, 1997).

Estrogen exhibits intrinsic antioxidant activities. 17β-Estradiol, 17α-estradiol and ethinyl estradiol protect cultured neurons against oxidative cell death characteristic of AD. This neuroprotective activity was found to be independent of estrogen receptor activation and requires an intact phenolic group (Behl and Holsboer, 2000). 17β-Estradiol protected neuroblastoma cells from oxidation and suppressed AβP induced membrane oxidation in hippocampal neurons (Christen, 2000). Because various isoforms of APOE possibly induce oxidative stress on neurons indirectly through their proposed inability to prevent AβP beta pleated sheet formation, it is possible that antioxidants like estrogen and EGB 761 could increase cognitive functioning in those suffering from AD.

Estrogens can apparently regulate APP metabolism in vitro. Investigators have demonstrated that estradiol diminishes AβP generation (Gandy and Petanceska, 2000). A multicentre, population-based, cohort study of Italian elderly women supports the hypothesis that estrogen replacement therapy is associated with a reduced prevalence of AD in post-menopausal women (Baldereschi et al, 1998). It is hypothesized that regulated cleavage of APP is occurring. Protein kinase C (PKC) could be the signal that activates alpha secretase, which in turn results in increased alpha secretase activity and a decrease in beta secretase activity. The negative correlation between hormone replacement therapy in post-menopausal women and AD development may be in part due to the AβP lowering effect of estrogen (Gandy and Petanceska, 2000).

A randomized, placebo-controlled double blind, parallel group design clinical study, although small, with ten women receiving 0.10 mg/day 17β-estradiol by skin patch and ten women receiving a placebo skin patch, determined that 17β-estradiol administration may enhance attention and memory for post-menopausal women afflicted with AD. It is thought that 17β-estradiol is the most active form of estrogen and Premarin, used in other studies, is not as effective, due to containing higher levels of estrone and lower levels of estradiol. It is postulated by the authors that estrogen may modulate cholinergic neurotransmission and increase blood flow, both of which may account for the cognitive benefits observed in the study subjects (Asthana et al, 2001).

Hirano Bodies

Hirano bodies are intraneuronal eosinophilic rod-like inclusions 15 to 30 micrometers in length, localized primarily in the hippocampus and which increase in frequency with age and AD progression. The complete composition of Hirano bodies is unknown, but actin, alpha-actinin, vinculin and tropomyosin are distributed throughout the structure. The known structure has led scientists to hypothesize that microfilament system breakdown is involved in its formation. Hirano bodies also appear to increase with normal aging (Smith, 1998).

Rationale for Experiments

Phospholipase Activations

Our research builds on experimental evidence relating to the documented decrease in phospholipids and corresponding increase in metabolites found in AD brain tissue. Phospholipases are enzymes that catalyze the hydrolysis of membrane phospholipids and are classified by the phospholipid bond cleaved. Phospholipase A_2 (PLA_2) hydrolyzes the fatty acid from the *sn*-2 position; phospholipase C (PLC) hydrolyzes the bond between glycerol and phosphate and phospholipase D (PLD) hydrolyzes the amino alcohol moiety from a phospholipid (Kaiser et al, 1990). Phospholipase A_2, PLC and PLD activations are hypothesized to be early events following the interaction of AβP with the neuronal plasma membrane (Kanfer et al, 1998). Activations of PLA_2, PLC and PLD are equivalent for AβP (25-35), AβP (1-40) and AβP (1-42) (Kanfer et al, 1999).

Amyloid beta peptide (25-35) stimulates PLA_2 and PLD in LA-N-2 cells, a human neuroblastoma cholinergic cell line, PLA_2 in PC12 cells, PLC in differentiated mouse brain cells and PLD of hippocampal cells. These interactions are potentially mediated, via receptor(s) interaction(s) or membrane perturbations, through a pertussis toxin (PTX) sensitive G protein (Kanfer et al, 1998; Rymer and Good, 2001). In rat ventral hippocampal and frontal cortex membrane preparations, AβP (25-35), 100 μM, stimulated G protein activities 104 % over control. Activation of Gi/Go proteins by AβP (25-35) was prevented following a PTX pre-treatment, suggesting that phospholipase activation via AβP is mediated through PTX sensitive

Gi/Go proteins (Soomets et al, 1999). Pertussis toxin also inhibited Gi/Go protein activation by AβP (1-40) and AβP (1-42) in PC12 membranes. Additionally, cell surface receptors were purportedly not required for AβP activation of Gi/Go (Rymer and Good, 2001).

G-proteins, characterized by seven membrane spanning domains, an extracellular N terminus and a cytoplasmic C terminus, are composed of α β and γ components encoded by distinct genes (Gether and Kobilka, 1998; Lopez-Ilasaca, 1998). Each gene type represents a family exhibiting varying degrees of complexity, with the most diverse encoding the α-subunits. There are at least 17 Gα genes, divided into four sub-families: G_s, G_i, G_q and G_{12}. There are also multiple genes encoding at least four β and six γ subunits. Under basal conditions the G-proteins exist as heterotrimers with GDP bound to the α chain. Activation promotes GDP release and subsequent binding of GTP and dissociation of GTP-Gα from the Gβγ complex. GTP-Gα and Gβγ then interact with effectors, resulting in signal transduction. Endogenous GTPase activity then hydrolyzes GTP-Gα to GDP-Gα and promotes the re-association of GDP-Gα and Gβγ (Lopez-Ilasaca, 1998).

PLA_2

Phospholipase A_2s, a ubiquitous superfamily of esterases, are important phospholipid degrading enzymes that are extremely heat and acid stable and highly cross-linked with disulphide bonds (Cummings et al, 2000). There are two main PLA_2 enzyme types, the secretory ($sPLA_2$) and the intracellular ($cPLA_2$, $iPLA_2$). The $sPLA_2$ requires millimolar concentrations of calcium, exhibits no phospholipid fatty acyl chain preferance and is prominent in pathological

processes. Intracellular $cPLA_2$ possesses specificity for arachidonic acid (AA) at the phospholipid *sn*-2 position and is regulated by micromolar calcium concentrations. The $iPLA_2$ is calcium independent, exhibits no phospholipid fatty acyl chain preferance and may be involved in "housekeeping", maintenance of membrane phospholipid composition and phagocytosis (Yedgar et al, 2000).

Membrane phospholipid hydrolysis by PLA_2 results in free fatty acid and lysophospholipid release. Released fatty acids may act as energy stores and more importantly, AA is metabolized by the cyclooxygenase (COX) and lipoxygenase (LOX) pathways generating eicosanoids like prostaglandins and leukotrienes, potent mediators of inflammation and signal transduction (Cummings et al, 2000; Yedger et al, 2000). Eicosanoids are involved in a wide range of physiological activities and excess production is involved in the pathophysiology of diseases like inflammation, allergy, brain injury, cancer development and metastasis and cardiovascular disorders (Yedgar et al, 2000). In addition, lysophospholipids are important in cell signaling, phospholipid remodeling, and membrane perturbation (Cummings et al, 2000; Six and Dennis, 2000).

In response to PLA_2 activity, membrane integrity may decrease. In addition, the free fatty acids and lysophospholipids may decrease membrane integrity by acting as detergents. In contrast, PLA_2 may decrease free-radical induced membrane phospholipid damage by hydrolyzing oxidized phospholipids from the membrane. Subsequently, the damaged lipids are removed from the cell, decreasing toxicity. Reacylation of the phospholipids by CoA-dependent acyltransferase and CoA-independent transacylase results in a return to normal functioning (Cummings et al, 2000).

Apoptosis involving PLA_2 has been reported and purportedly involves caspase 3 activation of cytosolic PLA_2, followed by the activation of downstream caspases. Phospholipase A_2 inhibition has been shown in some studies to decrease apoptosis, but other studies demonstrated that PLA_2 inhibition increases apoptosis. For example, pre-incubation of human umbilical vein endothelial cells with the PLA_2 inhibitors manoalide, 3-(4-octadecyl) benzoylacrylic acid and oleyloxethylphosphorylcholine resulted in an increase in apoptosis (Cummings et al, 2000).

Oxidative stress may produce membrane phospholipid alterations resulting in *sn*-2 fatty acid exposure, subsequently increasing PLA_2 activity and resulting in the release of free fatty acids and lysophospholipids. In addition, oxidation may result in release of intact membrane phospholipids and subsequent exposure of the *sn*-2 bond to PLA_2. The above two processes may be blocked by PLA_2 inhibitors. In addition, increases in intracellular calcium may result from cellular injury, which in turn activates protein kinase C (PKC) followed by PLA_2 (Cummings et al, 2000).

Phospholipase A_2 stimulation appears to be dependent on calcium release from a ryanodine sensitive intracellular endoplasmic reticulum storage site (Singh et al, 1996; Kanfer et al, 1998). It is postulated that since extracellular calcium is not required for intracellular calcium release, cyclic ADP-ribose generated by ADP-ribose-cyclase is responsible for the calcium release from the ryanodine sensitive store. Protein kinase A (PKA) and PKC activations and free radical formation have been eliminated as potential mediators of AA release (Singh et al, 1996).

Phospholipase A_2 inhibition is hypothesized to control the potential for cellular destruction. A major projected difficulty with this approach is that cells express various PLA_2

isoforms, which participate in normal phospholipid metabolism and non-selective inhibition of PLA_2 activity would impair vital phospholipid metabolism and reduce cell viability. Presently, the PLA_2 isoforms involved in AD have not been determined. It has been hypothesized that $sPLA_2$ enzymes should be targeted in the inflammation process, due to being the major contributors to the production of AA under pathological conditions and their ability to activate $cPLA_2$. Extracellular PLA_2 inhibitors include natural macromolecules like hyaluronic acid, heparin and chondroitin sulphates and polymers used in drug administration and clinical treatment like hydroxyethyl starch and polygeline (Yedgar et al, 2000).

PLC

Hydrolysis of phosphatidylinositol 3, 4, 5-triphosphate (PIP_3) by PLC generates the second messengers: inositol-1, 4, 5-trisphosphate (IP_3) and diacylglycerol (DAG), with IP_3 promoting the release of calcium ions from intracellular stores and DAG activating PKC, resulting in the phosphorylation of intracellular proteins. Activated PKC phosphorylates the lipocortin moiety of the lipoprotein - PLA_2 complex, liberating PLA_2. In the presence of elevated cytosolic calcium ions, PLA_2 is activated, hydrolyzing phospholipids into AA and lysophospholipid (Kaiser et al, 1990).

PLD

Phospholipase D was first discovered in plants and found to hydrolyze phosphatidylcholine (PC) to phosphatidic acid (PA) and choline. In addition to hydrolysis, PLD catalyzes a transesterification reaction (transphosphatidylation), using short-chain primary alcohols like methanol, ethanol, propranol or butanol as phosphatidyl group acceptors (Laychock and Rubin, 1999; Liscovitch et al, 2000) Phosphatidylalcohols are produced only by PLDs and are not normally found in biological membranes. Because of their unique origin, low basal levels and relative metabolic stability, phosphatidylalcohols serve as convenient and sensitive markers for PLD activation in cultured cells. When an alcohol is present, the transphosphatidylation reaction occurs at the expense of the hydrolytic reaction, generating greater amounts of the phosphatidylalcohol and less PA (Liscovitch et al, 2000). This observation has been supported by in-vitro research using synaptosomally bound PLD and determined that there was increased production of phosphatidylethanol (PtdEtOH) at the expense of PA (Singh et al, 1993).

Phosphatidylalcohols are also a more sensitive marker for PLD activation in cultured cells, because PA can be produced, in addition to phospholipid hydrolysis, by diacylglycerol kinase and by acylation of glycerol 3-phosphate, potentially confounding any measurements of PA levels (Liscovitch et al, 2000).

Receptor mediated PLD stimulation by AβP and quisqualate, a metabotropic receptor agonist, was postulated to occur. The PLD stimulations by AβP and quisqualate were prevented by 2, 3-Dihydroxy-6-Nitro-7-Sulfanoyl Benzo (f) Quinoxaline (NBQX), a known quisqualate

antagonist, but not by other excitatory amino acid antagonists. In addition, desensitization to subsequent challenges by AβP and quisqualate occurred with quisqualate or AβP treated LA-N-2 cells. Phospholipase D stimulation by AβP and quisqualate resumed following resensitization. The observations of antagonist selectivity, desensitization and resensitization appear consistent with a receptor-mediated event. It was further postulated that AβP intercalates within the plasma membrane, resulting in a general membrane perturbation. The perturbation was thought to result in conformational changes similar to those established by the binding of an agonist to its receptor and ultimately resulting in the activation of phospholipases (Singh et al, 1998a).

The Phospholipase Induced Pathology

Early phospholipid destruction likely results in the cell replenishing the phospholipids. As AD progresses the amyloid load increases, potentially resulting in the rate of phospholipid breakdown exceeding the rate of phospholipid synthesis. The increased energy expenditure on phospholipid synthesis compromises other cellular functions including the Na^+/K^+ ATPase, ion pumps and axoplasmic transport. It is predicted that the cell would eventually die from the increased channeling of energy into phospholipid biosynthesis and away from other important cellular functions. In addition, increased phospholipase activity results in increased levels of second messengers like DAG, IP_3, AA, PA and lysophospholipid which may produce unnecessary and exaggerated responses (Kanfer et al, 1998).

Nicotine

Following a review of 19 case control epidemiological studies, it was suggested that tobacco smoking, and by inference nicotine, is protective against the development of AD (Lee, 1994). Nicotine is one of about 4000 chemicals found in tobacco smoke and accounts for the acute pharmacological effects of smoking and dependence on cigarettes. Absorption of nicotine can occur through any site in or on the body. Nicotine stimulates nicotinic acetylcholine receptors in the CNS, producing increases in psychomotor activity, cognitive function, sensorimotor performance, attention and memory consolidation. The adverse, long term cardiovascular, pulmonary and carcinogenic effects of cigarettes are related to other compounds in tobacco (Julien, 1998)

Nicotinic Acetylcholine Receptors (nAChRs)

Nicotine may exert its protective effect through nAChRs, which are mainly found in various cortical areas, periacqueductal grey matter, basal ganglia, thalamus, hippocampus, cerebellum and retina. Non-neuronal distribution of nAChRs has been found in keratinocytes, muscle cells, lymphoid tissues and neurosecretory cells (Clementi et al, 2000). The most common classes of these receptors in the CNS are the $(\alpha 4)_2$ $(\beta 2)_3$ and the $(\alpha 7)_5$ receptors (Garrido et al, 2000). Each receptor consists of five subunits arranged around an aqueous channel in the plasma membrane. Heteromeric neuronal nAChRs have acetylcholine binding sites located in the large extracellular N-terminal domain, at the interface between the α and non-

α subunit. Homomeric α7 receptors have five identical acetylcholine binding sites per receptor molecule, one on each subunit (Clementi et al, 2000).

nAChRs Functions

Nicotinic acetylcholine receptor functions in the brain are presently unclear, but are known to be involved in complex cognitive functions like attention, learning, memory consolidation, arousal, sensory perception, control of locomotor activity, pain perception and body temperature regulation. The majority of these effects are thought to be due to presynaptic nAChRs that modulate the release of neurotransmitters. Postsynaptic nAChRs are also thought to play important roles. In addition, nAChRs are thought to be involved in neuronal survival. Their involvement in axon guidance and directional growth suggests that they could also be involved in shaping and maintaining neuronal circuitry (Clementi et al, 2000).

nAChRs and AD

A decrease in neuronal nAChRs is associated with AD. The α4β2 receptor type is reduced the most, while the observation of an α7 receptor reduction is presently being debated (Clementi et al, 2000). The results of one study suggest that α7 receptors are significantly reduced in the hippocampus (Guan et al, 2000). Deficits in nAChR expression may be due to disturbances in subunit transcription, protein synthesis and modification or receptor transport and turnover. Changes at the genomic level are unlikely (Wevers et al, 2000). Tau protein

hyperphosphorylation, oxidative stress and cell membrane modification during the development of AD might be related to decreased nAChRs (Guan et al, 2000).

Nicotine Inhibition of AβP Induced Cytotoxicity

Nicotine was found to significantly reduce AβP (25-35) cytotoxicity in cultured rat cortical neurons in a concentration-dependent manner and the protective effect was significantly antagonized by the nonselective nicotinic receptor antagonists hexamethonium and mecamylamine. In addition, it is suggested that the protective effect of nicotine is mediated, at least in part, through the α7 receptor due to its abundant presence in the hippocampus, neocortex and basal ganglia and also because the protective effect of nicotine was blocked by the selective α7 receptor antagonist alpha bungarotoxin (α-BTX) (Kihara et al, 1997; Dajas-Bailador et al, 2000; Garrido et al, 2000). An additional study determined that nicotine significantly reduced AβP (25-35) induced cytotoxicity in hippocampal neurons in a dose dependent fashion, with maximum inhibition at 10 μM/L. The degree of protection was found to be significantly larger when samples were exposed to nicotine for 5 days as apposed to 15 minutes. This inhibition was also found to be receptor mediated (Zamani and Allen, 2001). A six-month AD clinical trial using the full nicotinic α4β2 agonist ABT-418, an isoxazole bioisostere of nicotine, as a transdermal patch, failed to show a differentiation from placebo (Lloyd and Williams, 2000).

The protective mechanism, if operating through α7 nicotinic acetylcholine receptors, may be calcium dependent, due to the receptor being highly permeable to calcium (Dajas-Bailador et al, 2000). An additional mechanism of nicotine inhibition of AβP cytotoxicity may be through

nicotine binding to histidine residues of AβP and subsequent inhibition of aggregation and resulting cytotoxicity (Garrido et al, 2000). The theory of nicotine inhibiting AβP aggregation has recently been questioned due, to the finding that nicotine failed to affect the AβP-induced elevation in PLC activity (Zamani and Allen, 2001).

Additional mechanisms of nicotine inhibition of AβP cytotoxicity could include production of trophic factors and the stimulation of neurotransmitter release. Nicotine in the CNS may substitute for the downregulated cholinergic system of AD patients, resulting in increased neurotransmitter release at cholinergic sites. This action is predicted to result in improved cognitive functioning. Recently, data suggest that nicotine protects neurons against AβP induced cytotoxicity by inducing an increase in the secreted products of α secretase enzymatic activity, resulting in neurotrophic effects and the regulation of calcium levels (Zamani and Allen, 2001). In addition, tobacco smoking has been found to elevate nicotine binding sites in brain regions such as the neocortex. Nicotine treatment up-regulates both nicotine binding sites and subunit protein levels in cultured cells (Guan et al, 2000).

Due to the finding that nicotine protected rat neuronal and hippocampal cell cultures from AβP induced neurotoxicity, it was hypothesized that nicotine would prevent the activation of phospholipases by AβP. It was discovered that (-)-nicotine blunted the AβP activations of PLA_2 and PLD, but not PLC in LA-N-2 cells, a human cholinergic neuroblastoma cell line. These nicotine inhibitions likely did not occur through traditional nicotinic receptors, due to the inability of hexamethonium and D-tubocurarine, established antagonists, to block the inhibitions. If the nicotine inhibition of AβP activations is receptor occupancy mediated then it operates through an atypical receptor type (Singh et al, 1998b). The research we conducted attempted to

determine the location(s) and mechanism(s) of nicotine inhibition of AβP induced neurotoxicity, under the assumption that inhibition is occurring through a PTX sensitive G-protein.

Activation of PLA_2 and PLD of a Human Neuroblastoma Cell Line (LA-N-2) by N-dodecyl-L-lysine amide (Compound 24), a Putative G Protein Activator: Characteristics of Inhibition by (-)-Nicotine

Introduction

Although the mechanism remains elusive, AßP is implicated in the pathophysiology of neuronal degeneration found in Alzheimer's disease (Iverson et al, 1995; Price et al, 1995; Selkoe, 1994; Cummings et al, 1996). We have documented the AßP-induced activation of PLA_2 (Singh et al, 1996), PLC (Singh et al, 1997) and PLD (Singh et al, 1998a) in a human neuroblastoma cell line, LA-N-2. These phospholipase activations are reportedly prevented by PTX pre-treatment, suggesting the requirement for a PTX-sensitive G protein (Katada and Ui, 1982). We have previously reported that (-)-nicotine prevented the activation of PLA_2 and PLD by AßP, kainate and quisqualate, but were unable to rationalize its mechanism of action (Singh et al, 1998b). One possible site of action could be at the level of the PTX-sensitive G protein(s) required for mediating the phospholipase activations (Kanfer et al, 1998).

To test this hypothesis, the direct stimulation of PTX-sensitive G proteins was required. A series of alkyl-substituted amino acid amides had been synthesized and shown to activate the PTX-sensitive G proteins G_i / G_o (Leschke et al, 1997). We demonstrated that one of the amides, N-dodecyl-L-lysine amide (compound 24), stimulates both PLA_2 and PLD activities in LA-N-2 cells. These activations by compound 24 were not inhibited by pre-treatment with (-)-nicotine.

We then demonstrated that a PLD stimulation or a trend toward PLA_2 stimulation occurred following pre-treatment of LA-N-2 cells with PTX, an irreversible ribosylator of Gi/Go, followed by addition of compound 24. Nicotine inhibited the increase in compound 24 induced PLD activity and showed a trend toward inhibition of PLA_2 activity.

Materials

[9,10-^{3}H] Myristic acid (specific activity 51 Ci/mmol) and [5,8,9,11,13,14,15-^{3}H]arachidonic acid (specific activity 209 Ci/mmol) were purchased from Amersham Life Sciences (Oakville, ON, Canada). Silica gel G 60 thin-layer chromatography (TLC) plates were purchased from Merck (Darmstadt, Germany). Phosphatidylethanol was purchased from Avanti Polar Lipids Inc. (Alabaster, AL, USA). Phosphatidic acid was purchased from Serdary Research Labs (Englewood Cliffs, NJ, USA). Leibovitz L-15 medium, oligo $(dT)_{12-18}$ primer, 5X first strand buffer, dithiothreitol (0.1 M), dNTPs (100 mM), Moloney murine leukemia virus (MMLV) reverse transcriptase, nicotinic receptor α4 and α7 subunit forward and reverse primers, agarose, 100 bp and 1 Kb DNA ladders, EcoR I and React 3 buffer were purchased from Gibco BRL Life Technologies (Burlington, ON, Canada). Heat-inactivated (collect Gold) fetal bovine serum (FBS) was obtained from ICN Biomedicals, Inc. (Costa Mesa, CA, USA). (-)-Nicotine was obtained from Research Biochemicals International (Natick, MA, USA). Melittin, oleylamine, fatty acid-free bovine serum albumin (BSA), pertussis toxin and cholera toxin were obtained from Sigma Chemical Co. (St. Louis, MO, USA). The QIAquick gel extraction kit was obtained from Qiagen (Mississauga, ON, Canada). The SNAP total RNA isolation kit was purchased from Invitrogen (Carlsbad, CA, USA). Ready To Go™ PCR beads were purchased from Amersham Pharmacia Biotech (Psicataway, NJ, USA). N-Dodecyl-L-lysine amide x 2.3 HCl (compound 24, MW=397.4) was synthesized at the Freie Universität Berlin. LA-N-2 cells, a human neuroblastoma cell line, were obtained from Dr. Robert Seeger,

University of California (Los Angeles, CA, USA), and the cultures were maintained as described previously (Singh et al, 1990).

Experimental Procedures

Phospholipase Stimulation Measurements

Measurement of PLA_2 activation was conducted with LA-N-2 cells incubated at 37 °C with 1 μCi [^{3}H] AA in L-15 medium, containing 15% FBS, for 48 h. The cells were rinsed 5 times with FBS-free L-15 medium containing 1 mg /ml fatty acid-free BSA. The pre-labeled cells were incubated with agents as indicated for 1 h at 37 °C. Cells were then harvested mechanically and a lipid extraction was performed using the Folch method. The [^{3}H] AA produced was quantitated by TLC, as described previously (Singh et al, 1995). The quantity of radioactivity present as AA is expressed as a percentage of the total radioactivity recovered from the TLC plate for each sample.

Measurement of PLD activation was conducted with LA-N-2 cells incubated at 37 °C with 5 μCi of [^{3}H] myristic acid in L-15 medium containing 15% FBS for 48 h. The cells were rinsed 3 times with FBS-free L-15 medium containing 1mg/ml fatty acid-free BSA. Cells were incubated in L-15 medium with the agents in the presence of 0.5% v/v (85 mM) ethanol for 1 h. at 37 °C. Cells were then harvested mechanically and a lipid extraction was performed using the Folch method. The transphosphatidylation product of PLD activity, [^{3}H] phosphatidylethanol (PE), was quantitated by TLC as described previously (Singh et al, 1998a). The quantity of radioactivity present as PE is expressed as a percentage of the total radioactivity recovered from the TLC plate for each sample

Phospholipase Stimulations in CTX and PTX Pre-treated Cells

[^{3}H] AA or[^{3}H] myristic acid labeled LA-N-2 cells were prepared as described above. The cells were incubated with the indicated pre-treatment agents for 18 h at 37 °C. Following toxin removal, the cells were incubated with the indicated treatment agents for 2 h at 37 °C. Cells assessed for PLD activity were incubated with 0.5% v/v (85 mM) ethanol in addition to the indicated treatment agents. The treated cells were then incubated for 2 h at 37 °C. Cells were harvested mechanically and a lipid extraction was performed using the Folch method. The [^{3}H] AA produced, a measure of PLA_2 activation and [^{3}H] PE, a measure of PLD activity, were quantitated by TLC, as described previously and expressed as described above (Singh et al, 1995; Singh et al, 1998a).

Experiments Involving Quantification of the Phosphatidate Phosphohydrolase Catalyzed Conversion of Phosphatidic Acid to Diglycerides

[^{3}H] Myristic acid labeled LA-N-2 cells were prepared as described above. The cells were incubated with the indicated pre-treatment agents for 18 h at 37 °C. Following toxin removal, the cells were incubated with the indicated treatment agents, in the presence of 0.5 % v/v (85 mM) ethanol, for 2 h at 37 °C. Cells were harvested mechanically and a lipid extraction was performed using the Folch method. The lipid extracts were agitated and divided in half. One half of the lipid extract from each sample was subjected to thin layer chromatography (TLC) with an appropriate solvent system that separates [^{3}H] PA, a product of PLD metabolism (Singh

et al, 1998a). The remaining half lipid extract from each sample, containing [^{3}H] diglycerides, was separated by TLC using a solvent system composed of diethyl ether (45 mL), benzene (50 mL), absolute ethanol (2 mL) and glacial acetic acid (0.2 mL). The liberated [^{3}H] PA and [^{3}H] DG was quantitated by TLC, as described previously (Singh et al, 1998a). The quantity of radioactivity present in [^{3}H] PA or [^{3}H] DG is expressed as a percentage of the total radioactivity recovered from the TLC plate for each sample.

Molecular Biological Determination of α 4 and α 7 nAChR Subunit Expression in LA-N-2 Cells

Ribonucleic acids (RNA) were extracted from confluent LA-N-2 cells with the SNAP Total RNA Isolation Kit and treated with DNase I. Reverse transcriptase polymerase chain reaction (RT-PCR) was conducted to amplify the cDNA of interest using specially designed primers to detect the α 4 and α 7 subunits. The RT step used 5 μg total RNA and three μL oligo $(dT)_{12\text{-}18}$ primer (100 ng/μL) placed in a total volume of 40 μL, with the difference comprised of diethyl pyrocarbonate (DEPC) treated water. The mixture was incubated at 65 ° C for 10 minutes and allowed to cool at room temperature for 10 minutes, allowing adherence of the oligo (dT) primers to the poly-A tails of the respective RNAs. Following incubation, 12 μL of 5X first strand buffer, 2 μL of dithiothreitol DTT (0.1M), 4 μL of deoxy-nucleotide triphosphates (dNTPs) (100 mM) and 2 μL of reverse transcriptase (RT) (200 U/μL) were added producing a total volume of 60 μL. The mixture was incubated at 37 ° C for 60 minutes. The above incubations were conducted under controlled conditions in a Minicycler PCR machine.

Polymerase chain reaction (PCR) was then conducted in a total reaction volume of 25 μL per tube. Four tubes were prepared for PCR, one to determine the existence of the α 7 nicotinic acetylcholine receptor subunit (nACHR) cDNA, another to determine the existence of an α 4 nACHR subunit cDNA, one to assess the presence of glyceraldehyde-3'-phosphate dehydrogenase (GAPDH), an ubiquitous housekeeping gene, as a loading control and another to assess the presence of DNA contamination in the original RNA extract as a negative control.

To each tube containing Ready To Go™ PCR beads was added 2 μL of the α 7 primers, 5' primer 5'-GACTCAACATGCGCTGCTC-3' and the 3' primer 5'-CTGACCGATGGTACGGATGT-3' to produce a 1755-bp product, α 4 primers, 5' primer 5'-CCCACAGGAGAAGACGAACC-3' and the 3' primer 5'-CCGGTCCCTTCCTAGATCAT-3' to produce a 2035-bp product or GAPDH primers, 5' primer 5'-GCTGGGGCTCACCTGAAGGG-3' and the 3' primer 5'-GGATGACCTTGCCCACAGCC-3' to amplify a 384-bp product. In addition, 20 μL distilled water and 3 μL of cDNA from the RT step or 3 μL of RNA for the negative control were added to the tubes. The above was mixed gently and placed in the Minicycler for thirty cycles with the first step being denaturation of the cDNA at 94 ° C for one minute followed by annealing in the presence of excess primers at 55 ° C for one minute and thirty seconds and elongation at 72 ° C for two minutes. Following the final cycle the cDNA was incubated for ten minutes at 72 ° C and held at -9°C. The amplified cDNA from the above round of PCR was then subjected to a second round of PCR, further amplifying the cDNA. The above protocol was utilized for the second round of PCR with all four tubes receiving 3 μL of cDNA from the preliminary PCR round.

An electrophoresis gel and running buffer working solution was created using 0.04 M Tris-acetate, 0.001 M ethylenediaminetetraacetic acid (EDTA) and 14.4 μL ethidium bromide. A 1% agarose gel was created with a portion of the above working solution. Samples were prepared for running on the gel by mixing 2 μL loading buffer plus 10 μL sample cDNA for each of the cDNAs amplified. 2 μL of a 1000 or 100 base pair DNA ladder plus 1 μL of loading buffer were used as standards. These samples were then pipetted into wells on the solidified agarose gel, with the gel placed in the running buffer and electrophoresed for two hours at 104 v. Band sizes were roughly determined by using an ultraviolet light table and comparing ethidium bromide bands on the gel, corresponding to the cDNA bands of interest, with expected positions on the respective DNA ladder.

A restriction endonuclease assay was conducted with α 7 nAChR subunit cDNA bands purified from the electrophoresis gel. The bands were removed from the gel and the cDNA extracted with the QIAquick gel extraction kit according to the manufacturers protocol. The cDNA was used as a substrate for the restriction endonuclease EcoR I. Twenty-four μL of cDNA, 3μL EcoR I, (10 U/μL) and 3 μL React 3 buffer were mixed together in a tube and incubated for 24 hours at 37 °C. The gel preparation and electrophoresis system described earlier were utilized to separate the components of the EcoR I digestion. Band sizes were roughly determined by using an ultraviolet light table and comparing ethidium bromide bands on the gel, corresponding to the cDNA bands of interest, with expected positions on the respective DNA ladder.

All phospholipase stimulation experiments were conducted in triplicates and each experiment repeated on at least 3 separate occasions. Raw data from the phospholipase

stimulation experiments using a toxin pre-treatment and experiments assessing phosphotidate phosphohydrolase catalysis were log transformed prior to statistical analysis. Data are presented as mean $\pm$ SD. The results were evaluated statistically by ANOVA and the post hoc Tukey HSD multiple comparison tests using Prism and SYSTAT computer programs (Wilkinson, 1990; Motulsky, 1995). A significant difference is accepted when $P < 0.05$ by a two-tailed test. Molecular biological experiments were also repeated on at least 3 separate occasions.

Results

The Stimulation of PLA_2 and PLD Activities by Compound 24 in the Presence or Absence of (-) - Nicotine

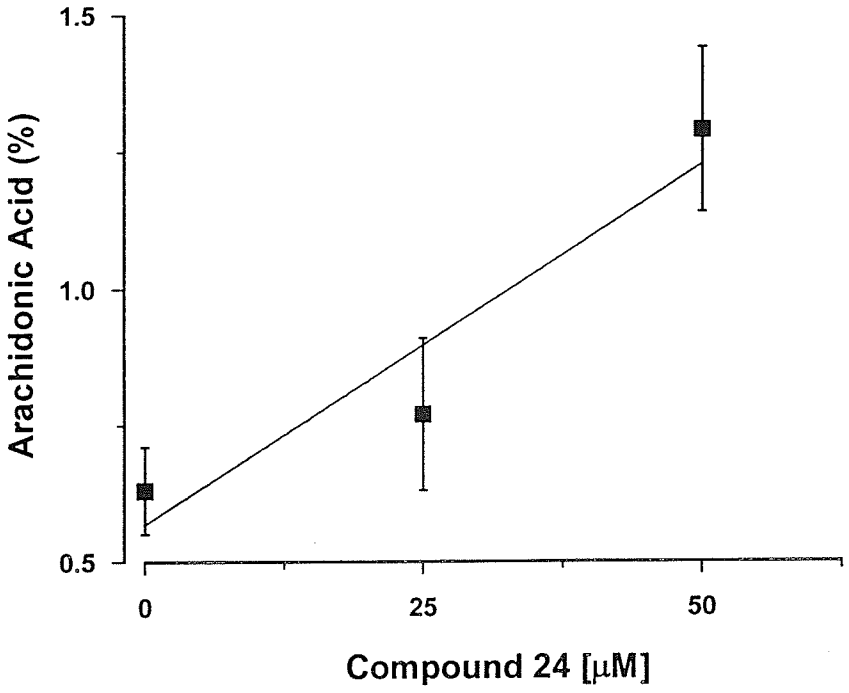
As a measure of PLA_2 stimulation, there was a progressive increase of [^{3}H] AA release with increasing concentrations of compound 24 (Figure 1). The activity at 100 μM compound 24 was 1.32 ± 0.15%, and equivalent to the stimulation observed with 50 μM compound 24 (1.29 ± 0.18%). This maximal stimulation was followed by a gradual decline at higher concentrations (0.84 ± 0.16% for 500 μM compound 24) (Sorrentino et al, 1999). The EC_{50} value for compound 24 stimulation of PLA_2 was calculated to be 32 μM (Sorrentino et al, 1999).

We were interested in determining if (-)-nicotine could inhibit the activation of PLA_2 by compound 24. Melittin, a reported general PLA_2 activator (Singh et al, 1995), was also employed for these studies. Melittin and compound 24 both stimulated PLA_2 and (-)-nicotine did not inhibit these activations (Table 1).

As a measure of PLD activation, there was a progressive increase in PE production with increasing concentrations up to 100 μM of compound 24 (Figure 2). The formation of PE was 0.18 ± 0.05% (Sorrentino et al, 1999). The calculated EC_{50} of compound 24 for PE production was 56 μM (Sorrentino et al, 1999). There was no effect of (-)-nicotine on the compound 24 stimulated formation of PE (Table 2).

Figure 1

The activation of phospholipase A_2 by varying concentrations of compound 24 as represented by arachidonic acid liberation. Data are presented as means $\pm$ S.D. The regression line represents the best fit to the data ($r^2 = 0.90$)



(Sorrentino et al, 1999)

Table 1

The stimulation of phospholipase A_2 activity by compound 24 and melittin in the presence or absence of (-)-nicotine.

	Phospholipase A_2 activity[b]
	% Arachidonic Acid
Control	0.34 ± 0.11
Melittin (5 μg)	68.6 ± 6.5[c]
Melittin + (-)-nicotine (500 μM)[a]	65.7 ± 5.4[c]
Compound 24 (100 μM)	1.21 ± 0.33[c]
Compound 24 + (-)-nicotine[a]	1.22 ± 0.41[c]

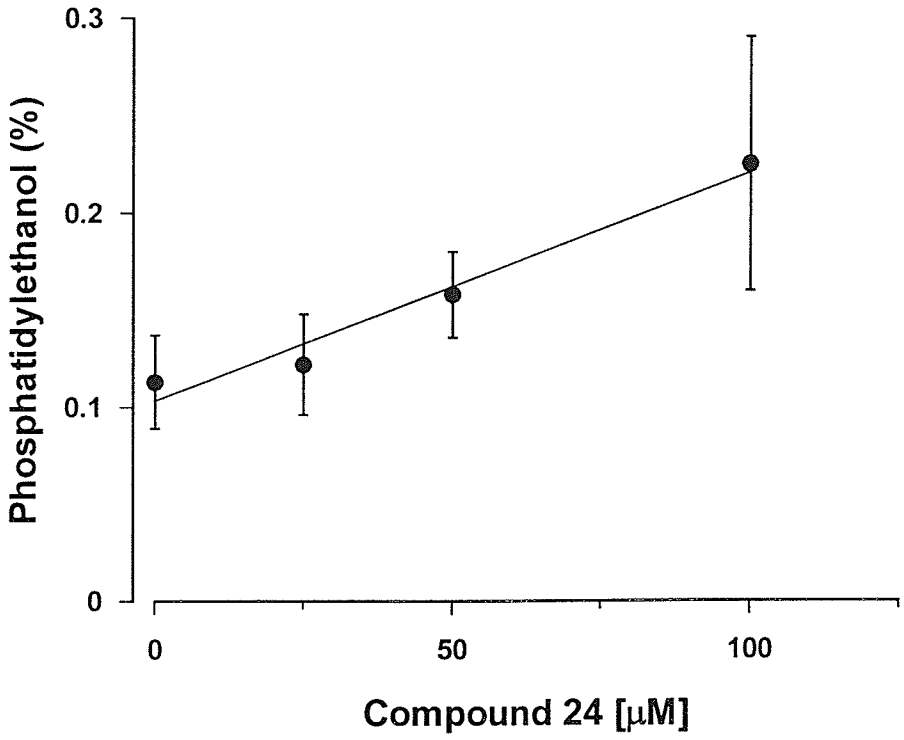
[a][^{3}H]Arachidonic acid prelabeled cells were exposed to 500 μM (-)-nicotine for 30 min. These solutions were removed and replaced with melittin or compound 24 in L-15 medium.

[b]Values are expressed as means ± S.D.

[c]$P<0.001$ compared to control data (ANOVA and Tukey's HSD tests).

Figure 2

The activation of phospholipase D by varying concentrations of compound 24 as represented by phosphatidylethanol liberation. Data are presented as means ± S.D. The regression line represents the best fit to the data ($r^2 = 0.97$ for phosphatidylethanol formation)



(Sorrentino et al, 1999)

Table 2

The stimulation of phospholipase D activity by compound 24 in the presence and absence of (-)-nicotine.

	Phospholipase D activity[b]
	% Phosphatidylethanol
Control	0.11 ± 0.02
Compound 24 (100 μM)	0.23 ± 0.07[c]
Compound 24 + (-)-nicotine (500 μM)[a]	0.22 ± 0.10[c]

[a][^{3}H]Myristic acid prelabeled cells were exposed to (-)-nicotine for 30 min. This solution was removed and replaced with compound 24 in L-15 medium.

[b]Values are expressed as means ± S.D.

[c]$P<0.001$ compared to respective control data (ANOVA and Tukey's HSD tests).

PLA_2 and PLD Activities in LA-N-2 Cells Pre-Treated with PTX + L-15, CTX + L-15 or L-15 Media Only, Followed by Compound 24 Treatment in the Presence or Absence of (-)-Nicotine

Addition of compound 24 to LA-N-2 cells pre-treated with L-15 or CTX resulted in increased PLA_2 hydrolysis activity and increased AA release compared to control (Table 3) (Figure 3, Panels A and C). Nicotine did not inhibit the compound 24 induced stimulation of PLA_2. A trend toward a compound 24 induced increase in PLA_2 hydrolysis activity and (-)-nicotine inhibition in PTX pre-treated cells is suggested from analyzing Table 3 and Figure 3, Panel B, though statistical significance was not reached ($P < .051$). Melittin stimulated PLA_2 and increased AA production over control in all three pre-treatment categories (Table 3).

Addition of compound 24 to LA-N-2 cells pre-treated with L-15, PTX or CTX resulted in increased PLD hydrolysis activity and PE production compared to control (Table 4) (Figure 4). Nicotine modestly inhibited the compound 24 stimulated production of PE when LA-N-2 cells were pre-treated with PTX (Table 4) (Figure 4, Panel B). Oleylamine stimulated PLD and increased PE production over control in all three pre-treatment categories (Table 4).

Table 3

Percentage [^{3}H] arachidonic acid produced by PLA2 hydrolysis of plasma membrane phospholipids for three pre-treatment categories and four treatment categories.

	% Arachidonic Acid [b]		
Pre-Treatments	L-15	Pertussis Toxin (200 ng/ml)	Cholera Toxin (200 ng/ml)
Treatments			
Control	0.58 ± 0.26	0.78 ± 0.66	0.60 ± 0.33
Compound 24 (100 μM)	1.16 ± 0.68**	1.93 ± 2.18	1.27 ± 0.81*
Compound 24 + (-)-Nicotine (500 M) [a]	1.12 ± 0.35**	1.22 ± 0.48	1.19 ± 0.56*
Melittin (5 μg)	25.8 ± 11.0***	20.9 ± 5.48***	22.5 ± 4.92***

[a][^{3}H] Arachidonic acid prelabeled cells were exposed to (-)-nicotine in L-15 media 30 min prior to compound 24 administration.

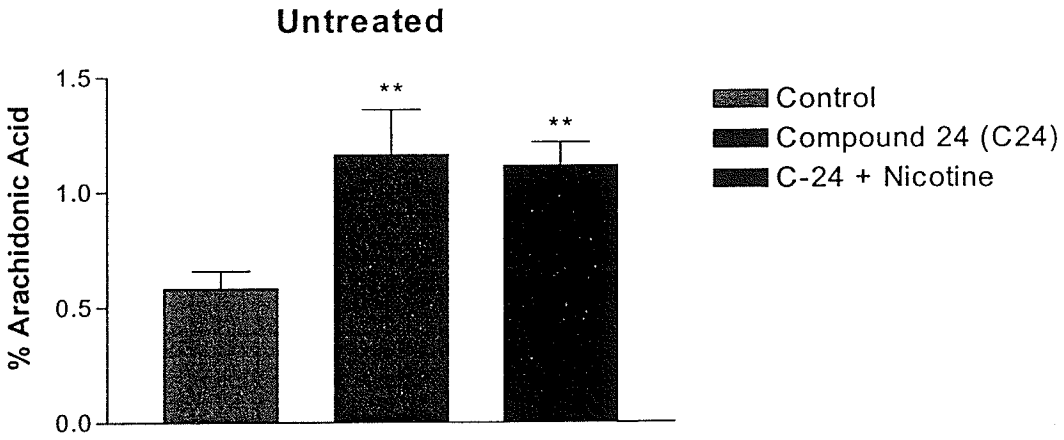
[b]Values are expressed as means ± S.D.

* $P < 0.05$ compared to control data
** $P < 0.01$ compared to control data
*** $P < 0.001$ compared to control data
(ANOVA and Tukey's HSD tests)

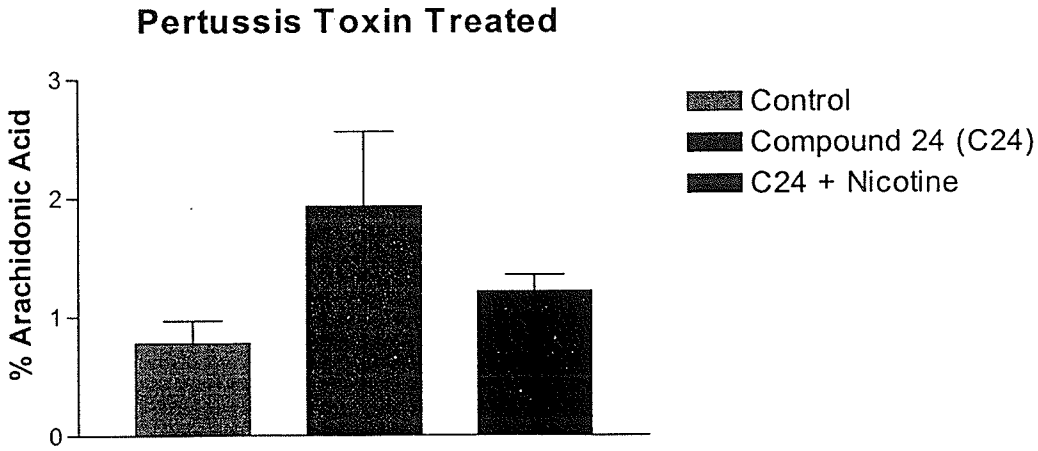
Figure 3

Percent arachidonic acid liberation following pre-treatments with L-15 media (Panel A), pertussis toxin in L-15 media (Panel B) or cholera toxin in L-15 media (Panel C). The pre-treatments were followed by treatments with L-15 media, compound 24 in L-15 media or compound 24 plus nicotine in L-15 media. Cells in the compound 24 plus nicotine in L-15 treatment category received nicotine 30 min prior to being administered compound 24.

Panel A



Panel B



Panel C

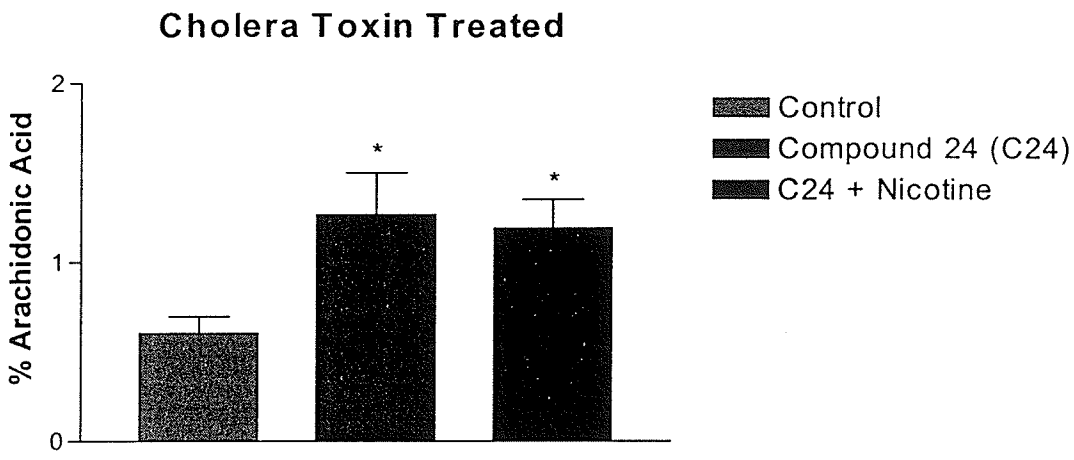

Cholera Toxin Treated
% Arachidonic Acid
2
1
0
*
*
Control
Compound 24 (C24)
C24 + Nicotine

Table 4

Percentage [^{3}H] phosphatidylethanol produced by PLD hydrolysis of plasma membrane phospholipids for three pre-treatment categories and four treatment categories.

	% Phosphatidylethanol [(b)]		
Pre-Treatments	L-15	Pertussis Toxin (200 ng/ml)	Cholera Toxin (200 ng/ml)
Treatments			
Control	0.34 ± 0.14	0.36 ± 0.14	0.43 ± 0.17
Compound 24 (100 μM)	0.79 ± 0.53***	0.76 ± 0.27***	0.82 ± 0.29***
C24 + (-)-Nicotine (500 μM) [(a)]	0.73 ± 0.42***	0.53 ± 0.20**#	0.70 ± 0.19**
Oleylamine (10 mM)	0.90 ± 0.40***	0.85 ± 0.27***	0.87 ± 0.30***

[(a)][^{3}H] Myristic acid pre-labeled cells were exposed to (-)-nicotine in L-15 media 30 min prior to compound 24 administration.

[(b)]Values are expressed as means ± S.D.

* $P < 0.05$ compared to control data

** $P < 0.01$ compared to control data

*** $P < 0.001$ compared to control data

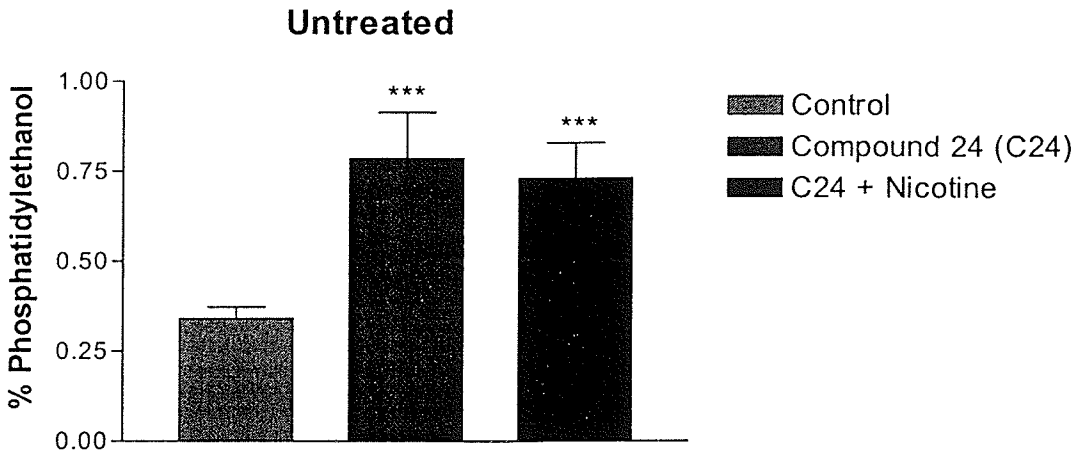
\# $P < 0.05$ compared to compound 24 data

(ANOVA and Tukey's HSD tests)

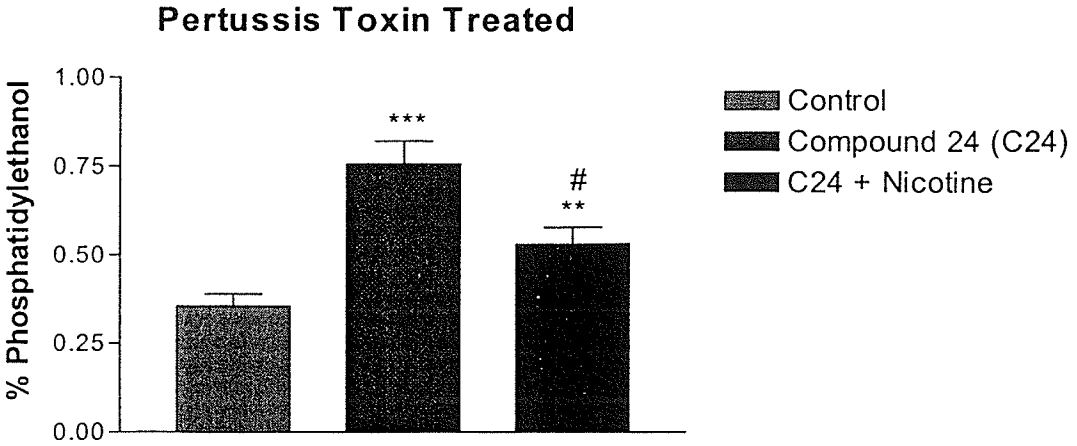
Figure 4

Percent phosphatidylethanol liberation following pre-treatments with L-15 media (Panel A), pertussis toxin in L-15 media (Panel B) or cholera toxin in L-15 media (Panel C). The pre-treatments were followed by treatments with L-15 media, compound 24 in L-15 media or compound 24 plus nicotine in L-15 media. Cells in the compound 24 plus nicotine in L-15 treatment category received nicotine 30 min prior to being administered compound 24.

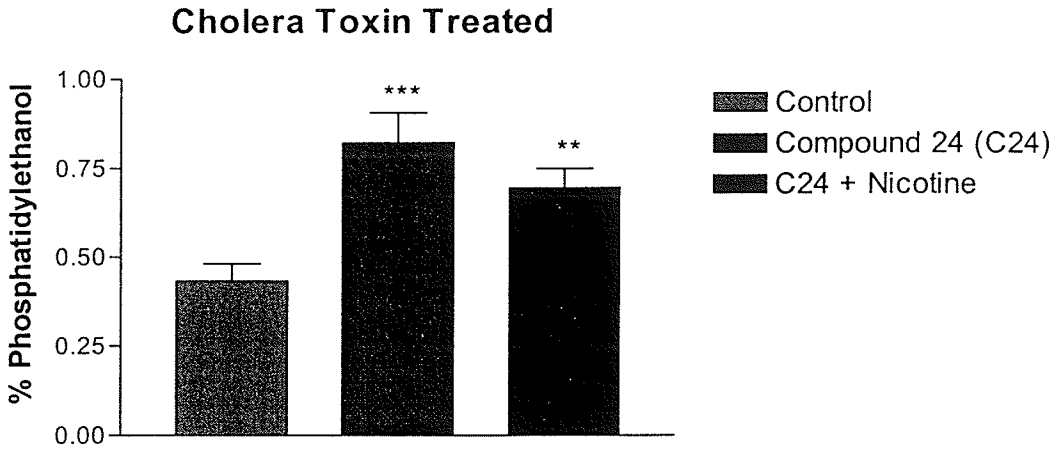
Panel A



Panel B



Panel C


Cholera Toxin Treated
% Phosphatidylethanol
1.00
0.75
0.50
0.25
0.00
***
**
Control
Compound 24 (C24)
C24 + Nicotine

PLD and Phosphatidate phosphohydrolase Activities in LA-N-2 Cells Pre-Treated with PTX + L-15, CTX + L-15 or L-15 Media Only, Followed by Compound 24 Treatment in the Presence or Absence of (-)-Nicotine

Addition of compound 24 to LA-N-2 cells pre-treated with L-15 resulted in increased phosphatidate phosphohydrolase activity and increased DG formation compared to control. Compound 24 addition to LA-N-2 cells pre-treated with PTX resulted in a trend towards increased phosphatidate phosphohydrolase activity and resulting DG production, though statistical significance was not reached ($P > 0.05$). Nicotine did not inhibit the C24 induced increase in DG production. Oleylamine stimulated an increase in DG production compared to control when pre-treated with L-15, PTX or CTX (Table 5).

Determination of α 4 or α 7 nAChR Subunit Expression in LA-N-2 Cells

The expression of α4 and α7 nAChR subunit mRNA was not detectable by RT-PCR. In contrast, the housekeeping gene GAPDH was amplified, indicating that the RNA isolated from LA-N-2 cells was of good quality. In some experiments, a second PCR amplification was performed using the preliminary PCR products as templates. This approach resulted in detectable levels of α7 but not α4 nAChR subunit cDNA (Figure 5). The size of the PCR product was consistent with the expected size of 1755 BP. Restriction digestion with EcoR I resulted in two bands consistent with the expected sizes of 1416 and 339 BP respectively (Figure 6).

Table 5

Percentage [^{3}H] phosphatidic acid produced by PLD hydrolysis of plasma membrane phospholipids and percentage [^{3}H] Diglycerides produced by phosphatidate phosphohydrolase, for three pre-treatment categories and four treatment categories.

		Percentage Phosphatidic Acid	
Pre-Treatments	L-15	Pertussis Toxin (200 ng/ml)	Cholera Toxin (200 ng/ml)
Treatments			
Control	3.36 ± 1.50	3.48 ± 1.22	4.60 ± 1.61
C24 (100 μM)	4.10 ± 1.78	3.91 ± 1.58	4.40 ± 2.01
C24 + (-)-Nicotine (500 μM)	5.29 ± 2.75	6.00 ± 2.60*	5.87 ± 2.40
Oleylamine (10 mM)	4.00 ± 1.93	4.52 ± 1.95	5.11 ± 1.72
		Percentage Diglycerides	
Pre-Treatments	L-15	Pertussis Toxin (200 ng/ml)	Cholera Toxin (200 ng/ml)
Treatments			
Control	2.26 ± 0.37	3.54 ± 2.40	4.51 ± 0.75
C24 (100 μM)	3.87 ± 0.57**	6.02 ± 3.06	6.16 ± 2.30
C24 + (-)-Nicotine (500 μM)	5.18 ± 2.58***	5.68 ± 2.40	4.84 ± 3.46
Oleylamine (10 mM)	11.0 ± 4.82***	17.1 ± 5.45***	11.9 ± 6.08*

Values are expressed as means ± S.D.

* $P < 0.05$ compared to control data
** $P < 0.01$ compared to control data
*** $P < 0.001$ compared to control data
(ANOVA and Tukey's HSD tests)

Figure 5

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) Electrophoresis Gel showing α 7 cDNA (1755 bp), corresponding to α 7 mRNA expression in LA-N-2 cells. The cells were treated for 2 hours with L-15 media, L-15 media+FBS, L-15 media+nicotine or L-15 media+FBS+nicotine. The positive control used was GAPDH. The data were obtained through a second PCR amplification performed on the preliminary PCR products.

Treatment Categories

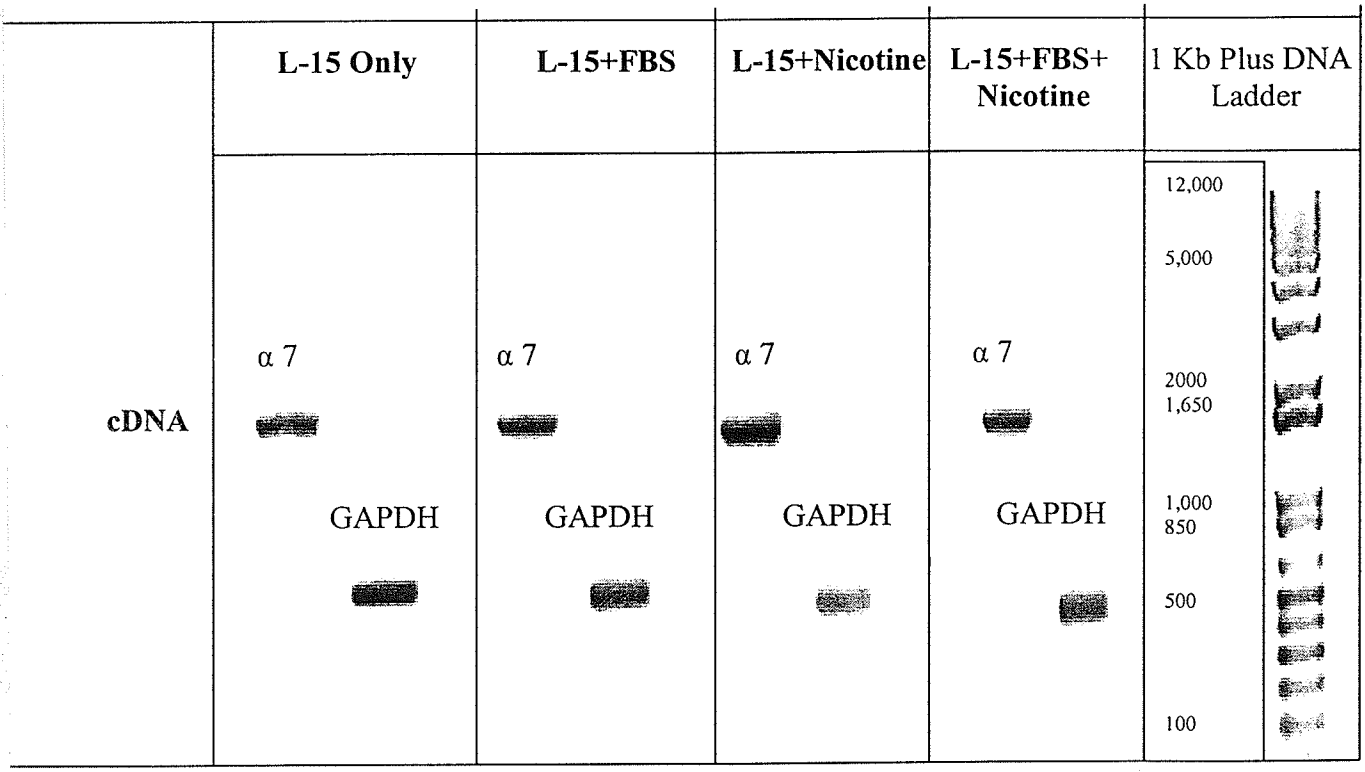
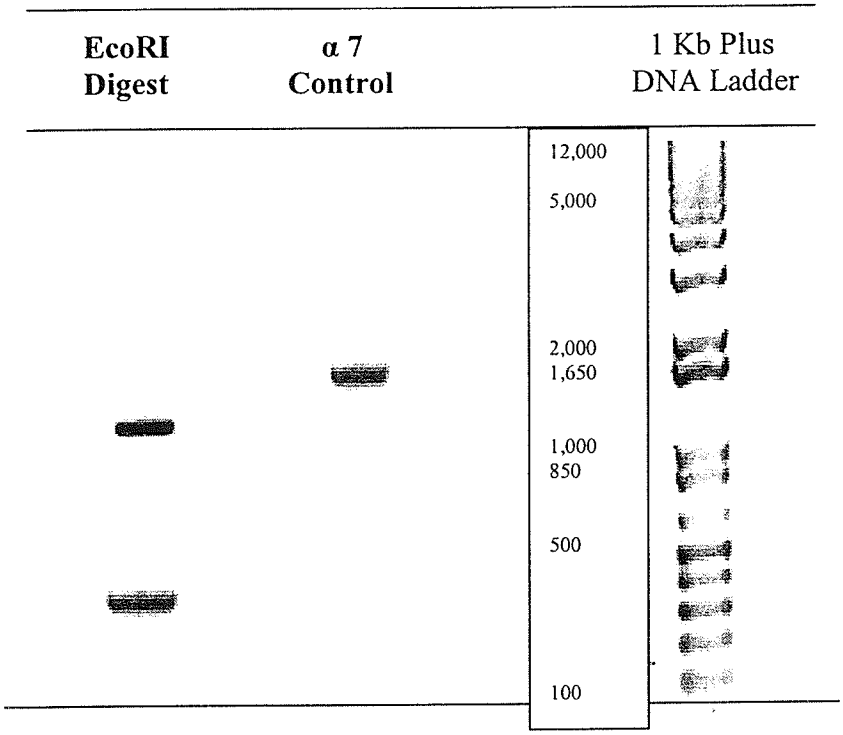


Figure 6

An electrophoresis gel showing the results of a restriction endonuclease assay, which used the EcoRI restriction enzyme to cleave the 1755 bp α 7 cDNA into 1416 bp and 339 bp fragments.



Discussion

Compound 24 was previously shown to directly stimulate PTX-sensitive G proteins in cell free systems (Leschke et al, 1997). We have shown that compound 24 is capable of stimulating the activities of PLA_2 and PLD in intact LA-N-2 cells, with resulting increases in AA liberation and PE production, respectively. In contrast to the ability of (-)-nicotine to prevent the activation of PLA_2 by AβP or kainite and PLD by AβP or quisqualate, there was no (-)-nicotine inhibition of these phospholipase activations by C24 unless cells were pre-treated with PTX. The failure of (-)-nicotine to inhibit PLA_2 and PLD activations by C24 suggests that the major inhibitory effect of nicotine is occurring proximal to the PTX sensitive Gi/Go proteins. In addition, the occurrence of PLA_2 and PLD stimulations following PTX pre-treatment suggests that multiple phospholipase stimulation mechanisms may be operating in the presence of C24.

Arachidonic acid, cleaved from the phospholipid *sn*-2 position by PLA_2, is reported to be a good indicator of PLA_2 catalytic activity (Cummings et al, 2000; Yedger et al, 2000). The only perceived difficulty with using AA as an indicator of PLA_2 stimulation is that AA may be further metabolized to leukotrienes and prostaglandins, hence, the data may underestimate AA release. Phosphatidylethanol, the transphoshatidylation product of PLD activity at the *sn*-4 position, is purportedly a good indicator of PLD catalytic activity (Laychock and Rubin, 1999; Liscovitch et al, 2000). Through conducting experiments, we determined that, PA, another reputed indicator of PLD activity, is converted rapidly by phosphatidate phosphohydrolase to DG, limiting its usefulness as an indicator. Diglycerides, on the other hand, may be a useful indicator of PLD activity if phosphatidate phosphohydrolase is expressed in the utilized cell model.

When PTX is present, it is postulated that plasma membrane channels are opened, enabling nicotine to flow down its concentration gradient into the cytosol. Pertussis toxin may be increasing cell membrane permeability to nicotine indirectly through irreversible ADP ribosylation of G_i/G_o, resulting in increased adenylyl cyclase activity and increased levels of cAMP, a cellular second messenger. Cyclic AMP then activates protein kinase A (PKA), which phosphorylates channel proteins on serine, threonine or tyrosine residues resulting in activation or inhibition (Smith, 1996). Changes in membrane permeability directly related to PTX have been ruled out due to its mechanism of direct entry through membrane insertion (Todar, 1997).

Once inside the cell, in adequate concentrations, (-)-nicotine may prevent the AßP, kainate and quisqualate activations by interference with couplings between a PTX-sensitive G protein and PLD. Although mean data appear to indicate that (-)-nicotine may also inhibit PLA_2 stimulation by C24 in LA-N-2 cells pretreated with PTX, the variability of the C24 effect precludes such a conclusion at present (Table 3) (Figure 3, Panel B). Another possibility could be direct inhibition of phospholipases, as observed in a study documenting nicotine inhibition of PLA_2 following joint stimulation of AMPA and metabotrophic glutamate receptors in primary Swiss mouse embryo neuronal cultures (Marin et al, 1997). We were unsuccessful in demonstrating inhibition of PLA_2 activity of LA-N-2 cell homogenates by (-)-nicotine (Singh et al, 1998b). This result is supported by the observation that (-)-nicotine did not prevent PLA_2 stimulation by melittin.

Intracellular α 7 receptors are expressed in SH-SY5Y cells, a human neuroblastoma cell line, leading us to postulate their presence in LA-N-2 cells and to speculate that (-)-nicotine inhibition may be mediated, at least partially, through these receptors following PTX treatment

and resulting intracellular accumulation of (-)-nicotine (Lukas, 1992; Puchacz et al, 1994; Ke et al, 1998). Binding of (-)-nicotine to a site on an intracellular subunit in an incompletely assembled nAChR could promote sub-unit assembly, leading to an increase in numbers of nAChR radioligand binding sites (Ke et al, 1998). Therefore (-)-nicotine appears to possess the potential to exert inhibitory effects at multiple levels, depending on the composition of the environment bathing the cell.

Our findings are consistent with the interpretation that (-)-nicotine inhibition occurs primarily at the level where AßP interacts with the cell membrane, possibly through nAChRs, as an in-vitro study using fetal rat primary cerebral cortex cultures concluded, or partially through nAChRs as eluded to by an in vitro study using cultured spinal cord neurons (Kihara et al, 1997; Garrido et al, 2000). An additional study determined that incubation of α7SK-N-MC cells with nicotine prior to administration of AβP 1-42, promoted cell survival, likely through an interaction with the α7 nAChR (Wang et al, 2000). However, we previously determined that acetylcholine does not inhibit the AßP stimulations of phospholipases, suggesting the participation of a non-cholinergic nicotinic receptor (Singh et al, 1998b). In addition, we determined that the α 4 receptor is not expressed and the α 7 receptor is expressed in very small quantities in LA-N-2 cells, suggesting that α 7 receptors play an insignificant role in our cell culture model of (-)-nicotine inhibition of AßP peptide induced stimulation of PLA_2 and PLD. These observations support the hypothesis that (-)-nicotine inhibition of AβP-induced activation of PLA_2 and PLD operates through a non-cholinergic nicotinic receptor.

When inhibition of AβP neurotoxicity is mediated through nAChRs, as found with other cell lines, it occurs in a time dependent manner, with greater protection resulting from multiple

day exposures (Ke et al, 1998; Zamani and Allen, 2001). This suggests that up regulation of nAChRs is not occurring within the time course of our experiments, which supports our argument that (-)-nicotine's inhibitory effect is not being mediated through traditional nAChRs. In conclusion, we believe that the protective effect of nicotine is consistent with interactions, primarily at a non-cholinergic cell membrane receptor site(s), unless cellular permeability to (-)-nicotine is enhanced by conditions duplicated by PTX in vitro.

Summary and Concluding Statements

Presently, research is being conducted globally in an attempt to document, understand and explain the AD pathology and to develop effective treatments for this presently incurable and debilitating disease. Our work attempted to determine the mechanism(s) of nicotine inhibition of AβP induced neurotoxicity in a LA-N-2 cell culture model. Previous research conducted in this laboratory suggested that AβP may be activating PLA_2 and PLD through interpolations or receptor interactions at the cellular plasma membrane. Phospholipase activations are not postulated to occur at nAchRs, due to an inability of acetylcholine to block them. (-)-Nicotine inhibited the PLA_2 and PLD activations, but its mechanism of action was undetermined. Through experiments using research tools such as compound 24, mellitin, oleylamine, PTX and CTX, we attempted to determine the location(s) where (-)-nicotine inhibited the AβP induced stimulations of PLA_2 and PLD.

We were successful in determining that compound 24 stimulated PLA_2 and PLD and that these stimulations were not inhibited by (-)-nicotine unless PTX was present. We are presently unable to definitively explain why the inhibition occurred, although a number of theories were proposed. These theories were supported by research conducted in other laboratories. Ultimately, we believe that the protective effect of (-)-nicotine against AβP induced PLA_2 and PLD activations is consistent with interactions, primarily at a nicotinic non-cholinergic receptor, located proximal to Gi/Go, unless cellular permeability to (-)-nicotine is enhanced by conditions duplicated by PTX in vitro. If PTX, or conditions duplicated by PTX, are present, (-)-nicotine inhibition may occur at multiple sites.

It is purported that α4 and α7 nAChRs are involved in the AD pathology. We determined the existence of the α 7 nAChR in LA-N-2 cells, albeit in very small quantities. The relative quantities did not change despite cellular exposure to (-)-nicotine, which is thought to upregulate nAChRs. Experiments consisting of multi-day (-)-nicotine exposures could be conducted to better determine the extent of α 4 and α 7 nAChR involvement in (-)-nicotine inhibition.

Future work could attempt to address some of the questions/concerns that arose while the experiments were being conducted, including, the extent of Gi/Go ribosylation with PTX and weather compound 24 activates Gi/Go exclusively. Additional studies could attempt to definitively determine if DG are effective indicators of PLD activity. Additional/alternate direct Gi/Go activators should be sought and used in an attempt to determine a definitive mechanism for the (-)-nicotine inhibition of the AβP induced stimulation of PLA_2 and PLD. If a definitive neuroprotective mechanism is determined for (-)-nicotine, then steps should be taken to develop analogs, which could be used in an attempt to block the postulated neurotoxicity resulting from AβP induced PLA_2 and PLD stimulations in the brains of AD patients.

References

Asthana, S., Baker, L., D., Craft, S., Stanczyk, F., Z., Veith, R., C., Raskind, M., A., Plymate, S., R.: High-Dose Estradiol Improves Cognition for Women With Alzheimer's Disease. Neurology. 57: 605-612, 2001.

Baldereschi, M., Di Carlo, A., Lepore, V., Bracco, L., Maggi, S., Grigoletto, F., Scarlato, G., Amaducci, L.: Estrogen-replacement Therapy and Alzheimer's Disease in the Italian Longitudinal Study on Aging. Neurology. 50: 996-1002, 1998.

Behl, C., Holsboer, F.: The Female Sex Hormone Oestrogen as a Neuroprotectant. Trends. Pharmacol. Sci. 20 (11): 441-444, 2000.

Bodles, A., M., Guthrie, D., J., S., Harriott, P., Campbell, P., Irvine, B.: Toxicity of non-Aβ component of Alzheimer's disease amyloid, and N-terminal fragments thereof, correlates to formation of β-sheet structure and fibrils. Eur. J. Biochem. 267: 2186-2194, 2000.

Bothwell, M., Giniger, E.: Alzheimer's disease: Neurodevelopment converges with neurodegeneration. Cell, 102: 271-273, 2000.

Cataldo, A.M., Peterhoff, C.M., Troncoso, J.C., et al.: Endocytic Pathway Abnormalities Precede Amyloid Beta Deposition in Sporadic Alzheimer's Disease and Down Syndrome: Differential Effects of APOE Genotype and Presenilin Mutations. Am. J. Path. 157 (1): 277-286, 2000.

Chandler, F.: Herbs, Everyday Reference for Health Professionals, Canadian Pharmacists Association and Canadian Medical Association. Ottawa. 2000.

Chauhan, A., Indrani, R., Chauhan, V.P.S.: Interaction of Amyloid Beta-Protein with Anionic Phospholipids: Possible Involvement of Lys^{28} and C-Terminus Aliphatic Amino Acids. Neurochem. Res. 25: 423-429, 2000.

Checler, F.: Processing of the β-amyloid precursor protein and its regulation in Alzheimer's disease. J. Neurochem. 65: 1431-1444, 1995.

Checler, F.: The Multiple Paradoxes of Presenilins. J. Neurochem. 76: 1621-1627, 2001.

Christen, Y.: Oxidative Stress and Alzheimer's Disease. Am. J. Clin. Nutr. 71 (suppl): 621S-629S, 2000.

Chyan, Y. J., Poeggeler, B., Omar, R. A., Chain, D., G., Frangione, B., Ghiso, J., Pappolla, M., A.: Potent neuroprotective properties against the Alzheimer β-amyloid by an endogenous melatonin-related indole structure, indole-3-propionic acid. J. Biol. Chem. 274: 21937-21942, 1999.

Clementi, F., Fornasari, D., Gotti, C.: Neuronal nicotinic receptors, important new players in brain function. Eur. J. Pharmacol. 393: 3-10, 2000.

Copani, A., Condorelli, A., Caruso, A., Vancheri, C., Sala, A., Giuffrida, A., M., Canonico, P., L., Nicoletti, F., Sortino, M., A.: Mitotic signaling by β-amyloid causes neuronal death. FASEB J. 13: 2225-2234, 1999.

Cummings, B.J., Pike, C.J., Shankle, R., Cotman, C.W.: Beta-amyloid deposition and other measures of neuropathology predict cognitive status in Alzheimer's disease. Neurobiol. Aging. 17: 921-933, 1996.

Cummings, B. S., McHowat, J., Schnellmann, R.: Phospholipase A_2s in Cell Injury and Death. J. Pharmacol Exp. Ther. 294: 793-799, 2000.

Cummings, J. L.: Alzheimer's disease management. J. Clin. Psychiatry. 59: 4-5, 1998.

Dajas-Bailador, F. A., Lima, P. A., Wonnacott S.: The alpha 7 nicotinic receptor subtype mediates nicotine protection against NMDA excitotoxicity in primary hippocampal cultures through a Ca (2+) dependent mechanism. Neuropharmacology. 13: 2799-2807, 2000.

Eckert, G. P., Cairns, N. J., Maras, A., Gattaz, W., F., Muller, W., E.: Cholesterol modulates the membrane-disordering effects of beta-amyloid peptides in the hippocampus: specific changes in Alzheimer's disease. Dement Geriatr Cogn Disord. 11: 181-186, 2000.

Emmerling, M. R., Watson, M. D., Raby, C. A., Spiegel, K.: The role of complement in Alzheimer's disease pathology. Biochim Biophys Acta 1502: 158-171, 2000.

Epstein, F., H.: Molecular basis of the neurodegenerative disorders. N. Engl. J. Med. 340: 1970-1980, 1999.

Gandy, S., Petanceska, S.: Regulation of Alzheimer β-amyloid precursor trafficking and metabolism. Biochim Biophys Acta. 1502: 44-52, 2000.

Garrido, R., Malecki, A., Hennig, B., Toberek, M.: Nicotine attenuates arachidonic acid-induced neurotoxicity in cultured spinal cord neurons. Brain Res. 861: 59-68, 2000.

Gauthier, S.: Advances in the Pharmacotherapy of Alzheimer's Disease. JAMC. 166 (5): 616-623, 2002.

Gether, U., Kobilka, B. K.: G protein-coupled receptors. J. Biol. Chem. 273: 17979-17982,1998.

Golde, T., E., Eckman, C., B., Younkin, S., G.: Biochemical detection of Aβ isoforms: implications for pathogenesis, diagnosis and treatment of Alzheimer's disease. Biochim. Biophys Acta. 1502: 172-187, 2000.

Guan, Z., Z., Zhang, X., Ravid, R., Nordberg, A.: Decreased protein levels of nicotinic receptor subunits in the hippocampus and temporal cortex of patients with Alzheimer's disease. J. Neurochem. 74: 237-243, 2000.

Halliday, G., Robinson, S. R., Shepherd, C., Kril, J.: Alzheimer's disease and inflammation: a review of cellular and therapeutic mechanisms. Clin. Exp. Pharmacol. Physiol., 27: 1-8, 2000.

Harkany, T., Abraham, I., Konya, C., Nyakas, C., Zarandi, M., Penke, B., Luiten, P.: Mechanisms of β-amyloid neurotoxicity: perspectives in pharmacotherapy, Rev. Neurosci. 11: 329-382, 2000.

Haugabook, S. J., Yager, L. D., Zenk, B., Healy, E., A., Eckman, C., Prada, L., Younkin, P., Murphy, I., Pinnix, L., Onstead, K., Sambamurti, T., E., Golde, Dickson, D., Younkin, S., G., Eckman, C., B.: Reduction of Aβ accumulation in the Tg2576 animal model of Alzheimer's disease after oral administration of the phosphatidylinositol kinase inhibitor wortmannin. FASEB J. Express article 10.1096/fj.00-0528fje: 1-12, 2000.

Henderson, V.W.: The Epidemiology of Estrogen Replacement Therapy and Alzheimer's Disease. Neurology. 48 (supple 7): S27-S35, 1997.

Hirakura, Y., Satoh, Y., Hirashima, N., Toshiharu, S., Kagen, B., L., Kirino, Y.: Membrane perturbation by the neurotoxic Alzheimer amyloid fragment β25-35 requires aggregation and β-sheet formation. Biochem. Mol. Biol. Int. 46: 787-794, 1998.

Iverson, L.L., Mortshire-Smith, R.J., Pollack, S.J., Shearman, M.S.: The toxicity in vitro of ß-amyloid protein. Biochem. J. 311: 1-16, 1995.

Iwai, A.: Properties of NACP/α-synuclein and its role in Alzheimer's disease. Biochim. Biophys. Acta. 1502: 95-109, 2000.

Jorm, A. F.: Alzheimer's disease; risk and protection. Med. J. Aust. 167: 443-446, 1997.

Julien, R., M.: A primer of drug action: A concise nontechnical guide to the actions, uses and side effects of psychoactive drugs, W. H. Freeman and Company, New York, 1998, 165-180.

Kaiser, E., Chiba, P., Zaky, K.: Phospholipases in biology and medicine. Clin. Biochem. 23: 349-370, 1990.

Kanfer, J.N., Sorrentino, G., Sitar, D.S.: Phospholipases as mediators of amyloid beta peptide neurotoxicity: an early event contributing to neurodegeneration characteristic of Alzheimer's disease. Neurosci. Lett. 257: 1-4, 1998.

Kanfer, J. N., Sorrentino, G., Sitar, D. S.: Amyloid beta peptide membrane perturbation is the basis for its biological effects. Neurochem. Res. 24: 1621-1630, 1999.

Katada,T., Ui, M.: Direct modification of the membrane adenylate cyclase system by islet-activating protein due to ADP-ribosylation of a membrane protein. Proc. Natl. Acad. Sci. USA. 79: 3129-3133, 1982.

Ke, L., Eisenhour, C.M., Bencherif, M., Lukas, R.J.: Effects of chronic nicotine treatment on expression of diverse nicotinic acetylcholine receptor subtypes, dose and time dependent effects of nicotine treatment. The J. Pharm. Exp. Thera. 286: 825-840, 1998.

Kihara, T., Shimohama, S., Sawada, H., Kimura, J., Kume, T., Kochiyama, H., Maeda, T. and Akaike, A.: Nicotinic receptor stimulation protects neurons against beta-amyloid toxicity. Ann. Neurol. 42: 159-163, 1997.

Kumar-Singh, S., De Jonghe, C., Cruts, M., Kleinert, R., Wang, R., Mercken, M., De Strooper, B., Vanderstichele, H., Lofgren, A., Vanderhoeven, I., Backhovens, H., Vanmechelin, E., Kroisel, P., M., Van Broeckhoven, C.: Nonfibrillar diffuse amyloid deposition due to a γ42-secretase site mutation points to an essential role for N-truncated $A\beta_{42}$ in Alzheimer's disease. Hum. Mol. Genet. 9: 2589-2598, 2000.

Kuo, Y. M., Emmerling, M. R., Lampert, H. C., Hempelman, S., R., Kokjohn, T., A., Woods, S., A., Cotter, R., J., Roher, A., E.: High levels of circulating Aβ42 are sequestered by plasma proteins in Alzheimer's disease. Biochem. and Biophys. Res. Commun. 257: 787-791, 1999.

Laychock, S., G., Rubin, R., P., Lipid Second Messengers. CRC Press, New York, 1999, 1-15.

Ladu, J.L., Reardon L.: Lipoproteins in the Central Nervous System. Ann. N. Y. Acad. Sci. 903: 167-175, 2000.

Lansbury, P., T.: Evolution of amyloid: what normal protein folding may tell us about fibrillogenesis and disease. Proc. Natl. Acad. Sci. 96: 3342-3344, 1999.

Le bars, P., L., Katz, M., M.,Berman, N., Itil, T., M., Freedman, A., M., Schatzberg, A., F.: A Placebo-controlled, Double-blind, Randomized Trial of an Extract of Ginkgo Biloba for Dementia. JAMA. 278: 1327-1332, 1997.

Leschke, C., Storm, R., Breitweg-Lehmann, E., Exner, T., Nürnberg, B. and Schunack, W.: Alkyl-substituted amino acid amides and analogous di- and triamines: new non-peptide G protein activators. J. Med. Chem. 40: 3130-3139, 1997.

Liscovitch, M., Czarny, M., Fiucci, G., Tang, X.: Phospholipase D: molecular and cell biology of a novel gene family. Biochem. J. 345: 401-415, 2000.

Lloyd, K., L., Williams, M.: Neuronal nicotinic acetylcholine receptors as novel drug targets. JPET. 292: 461-467, 2000.

Lopez-Ilasaca.: Signaling from G-protein-coupled receptors to mitogen-activated protein (MAP)-kinase cascades. Biochem. Pharmacol. 56: 269-277, 1998.

Lukas, R.J.: Expression of ganglia-type nicotinic acetylcholine receptors and nicotinic ligand binding sites by cells of the IMR-32 human neuroblastoma clonal line. J. Pharmacol. Exp. Ther. 265: 294-302, 1992.

Marin, P., Hamon, B., Glowinski, J., Premont, J.: Nicotine-induced inhibition of neuronal phospholipase A_2. J. Pharmacol. Exp. Ther. 280: 1277-1283, 1997.

Martinez-Senec, M., Villalain, J., Gomez-Fernandez, J., C.: Structure of the Alzheimer β Amyloid peptide (25-35) and its interation with negatively charged phospholipid vesicles. Eur. J. Biochem. 265: 744-753, 1999.

Mattson, M. P., Partin, J. and Begley, J. G.: Amyloid β-peptide induces apoptosis-related events in synapses and dendrites. Brain Res. 807: 167-176, 1998.

Mesulam, M. M.: Neuroplasticity failure in Alzheimer's disease: bridging the gap between plaques and tangles. Neuron. 24: 521-529, 1999.

Motulsky, H., Prism, GraphPad Software Inc., San Diego, CA. 1995.

Muller, W., E., Koch, S., Eckert, A., Hartmann, H., Scheuer, K.: β-amyloid peptide decreases membrane fluidity. Brain Res. 674: 133-136, 1995.

Pillot, T., Drouet, B., Queille, S., Labeur, C., Vandekerckhove, J., Rosseneu, M., Pincon-Raymond, M., Chambaz, J.: The nonfibrillar amyloid β-peptide induces apoptotic neuronal cell death: involvement of its c-terminal fusogenic domain. J. Neurochem. 73: 1626-1634, 1999.

Price, D.L., Sisodia, S.S., Gandy, S.E.: Amyloid beta amyloidosis in Alzheimer's Disease. Curr. Opin. Neurol. 8: 268-274, 1995.

Puchacz, E., Buisson, B., Bertrand, D., Lukas, R.J.: Functional expression of nicotinic acetylcholine receptors containing rat alpha 7 subunits in human SH-SY5Y neuroblastoma cells. FEBS Lett. 354: 155-159, 1994.

Roses, A.D.: Apolipoprotein E and Alzheimer's Disease. Sci. Am. Sept/Oct: 16-25, 1995.

Roth, G. S., Joseph, J. A., Mason, R. P.: Membrane alterations as causes of impaired signal transduction in Alzheimer's disease and aging. Trends Neurosci. 18: 203-206, 1995.

Rymer, D. L., Good, T. A.: The role of G protein activation in the toxicity of amyloidogenic Aβ (1-40), Aβ-(25-35), and Bovine Calcitonin. J. Biol. Chem. 276: 2523-2530, 2001.

Saunders, A.M., Trowers, M.K., Shimkets, R.A.: The Role of Apolipoprotein E in Alzheimer's Disease: Pharmacogenomic target selection. Biochim Biophys. Acta. 1502: 85-94, 2000.

Seilheimer, B., Bohrmann, B., Bondolfi, L., Muller, F., Stuber, D., Dobeli, H.: The toxicity of the Alzheimer's β-amyloid peptide correlates with a distinct fiber morphology. J. Struct. Biol. 119: 59-71, 1997.

Selkoe, D.J.: Alzheimer's disease: a central role for amyloid. J. Neuropathol. Exp. Neurol. 53: 438-447, 1994.

Selkoe, D. J.: Translating cell biology into therapeutic advances in Alzheimer's disease. Nature. 399 (supp): 23-31, 1999.

Serpell, L. C.: Alzheimer's amyloid fibrils: structure and assembly. Biochim Biophys Acta. 1502: 16-30, 2000.

Sigurdsson, M., E., Permanne, B., Soto, C., Wisniewski, T., Frangione, B.: In vivo reversal of amyloid-β lesions in rat brain. J Neuropathol. Exp. Neurol. 59: 11-17, 2000.

Singh, I. N., Massarelli, R., Kanfer, J. N.: Activation of phospholipases D and A by amphiphilic cations of cultured LA-N-2 cells is G protein and protein kinase C independent. J. Lipid Mediat. 7: 85-96, 1993.

Singh, I.N., McCartney, D.G., Kanfer, J.N.: Amyloid beta protein (25-35) stimulation of phospholipases A, C and D activities of LA-N-2 cells. FEBS Lett. 365: 125-128, 1995.

Singh, I.N., Sorrentino, G., Kanfer, J.N.: Amyloid beta protein (25-35) activation of phospholipase A_2 in LA-N-2 cells. Alzheimer's Res. 2: 121-128, 1996.

Singh, I.N., Sorrentino, G., Kanfer, J.N.: Amyloid ß protein (25-35) stimulation of phospholipase C in LA-N-2 cells. J. Neurochem. 69: 252-258, 1997.

Singh, I.N., Sorrentino, G., Kanfer, J.N.: Activation of LA-N-2 cell phospholipase D by amyloid beta protein (25-35). Neurochem. Res. 23: 1225-1232, 1998a.

Singh, I.N., Sorrentino, G., McCartney, D.G., Massarelli, R., Kanfer, J.N.: Enzymatic activities during differentiation of the human neuroblastoma cells, LA-N-1 and LA-N-2. J. Neurosci. Res. 25: 476-485, 1990.

Singh, I.N., Sorrentino, G., Sitar, D.S., Kanfer, J.N.: (-)-Nicotine inhibits the activations of phospholipases A2 and D by amyloid ß peptide. Brain Res. 800: 275-281, 1998b.

Six, D. A. Dennis, E. A.: The expanding superfamily of phospholipase A2 enzymes: classification and characterization. Biochim. Biophys. Acta. 1488: 1-19, 2000.

Skoog, I.: Detection of Preclinical Alzheimer's Disease. N. Engl. J. Med. 343 (7): 502-503, 2000.

Smith, C.U.M., Elements of Molecular Neurobiology, John Wiley and Sons, Toronto, 1996, 154-156.

Smith, M.A.: Alzheimer Disease. International Review of Neurobiology. 42: 1-53, 1998.

Smith, M.A., Rottkamp, C.A., Nunomura, A.: Oxidative Stress in Alzheimer's Disease. Biochim. Biophys. Acta. 1502: 139-144, 2000.

Snowdon, D. A., Kemper, S. J., Mortimer, J. A., Greiner, L., H., Wekstein, D., R., Markesbery, W., R.: Linguistic ability in early life and cognitive function and Alzheimer's disease in late life. JAMA. 275: 528-532, 1996.

Soomets, U., Mahlapuu, R., Tehranian, R., Jarvet, J., Karelson, E., Zilmer, M., Iverfeldt, K., Zorko, M., Graslund, A., Langel, U.: Regulation of GTPase and adenylate cyclase activity by amyloid β-peptide and its fragments in rat brain tissue. Brain Res. 850: 179-188, 1999.

Sorrentino, G., Fitzpatrick-Wong, S., Schunack, W., Nurnberg, B., Sitar, D. S., Kanfer, J, N.: (-)-Nicotine does not block G protein activation of phospholipases A_2 and D of LA-N-2 cells. J. Neurochem. 72S: S71A, 1999.

Subramaniam, R., Koppal, T., Green, M., Yatin, S., Jordan, B., Drake, J., Butterfield, D., A.: The free radical antioxidant vitamin E protects cortical synaptosomal membranes from amyloid β-peptide (25-35) toxicity but not from hydroxynonenal toxicity: relevance to the free radical hypothesis of Alzheimer's disease. Neurochem. Res. 23: 1403-1410, 1998.

Swartz, R.H., Black, S.E., St. George-Hyslop, P.: Apolipoprotein E and Alzheimer's Disease: a Genetic, Molecular and Neuroimaging Review. Can. J. Neurol. Sci. 26: 77-88, 1999.

Tamaoka, A., Miyatake, F., Matsuno, S.: Apolipoprotein E Allele-Dependent Antioxidant Activity in Brains with Alzheimer's Disease. Neurology. 54 (12): 2319-2321, 2000.

Todar, K.: Bacteriology 330 Lecture Topics: Bordetella pertussis and Whooping Cough. The University of Wisconsin Department of Bacteriology. Madison,1-6, 1997.

Veld, B., A., Ruitenberg, A., Hofman, A., Launer, L., J., van Duijn, C., M., Stijnen, T., Breteler, M., Stricker, B.: Nonsteroidal Antiinflammatory Drugs and the Risk of Alzheimer's Disease. N. Engl. J. Med. 345: 1515-1521, 2001.

Versteeg, H., H., Henegouwen, P., van Deventer, S., Peppelenbosch, M., P.: Cyclooxygenase-dependent Signaling: molecular events and consequences. FEBS Lett. 445: 1-5, 1999.

Wang, H. Y., Lee, D., D'Andrea, M. R., Peterson, P. A., Shank, R. P., Reitz, A. B.: β-amyloid 1-42 binds to nicotinic acetylcholine receptor with high affinity. J. Biol. Chem. 275: 5626-5632, 2000.

Wevers, A., Burghaus, L., Moser, N., Witter, B., Steinlein, O., T., Schutz, U., Achnitz, B., Krempel, U., Nowacki, S., Pilz, K., Stoodt, J., Lindstrom, J., De Vos, R., Steur, E., Schroder, H.: Expression of nicotinic acetylcholine receptors in Alzheimer's disease: postmortem investigations and experimental approaches. Behav. Brain Res. 113: 207 215, 2000.

Wilkinson, L., SYSTAT: the system for statistics, Systat Inc., Evanston, IL. 1990.

Yan, S. D., Roher, A., Chaney, M.: Cellular cofactors potentiating induction of stress and cytotoxicity by amyloid β-peptide. Biochim. Biophys. Acta. 1502: 145-157. 2000.

Yedgar, S., Lichtenberg, D., Schnitzer, E.: Inhibition of phospholipase A_2 as a therapeutic target. Biochim Biophys Acta. 1488: 182-187, 2000.

Yuan, J. and Yankner, B., A.: Apoptosis in the nervous system. Nature. 407: 802-809, 2000.

Zamani, M.R., Allen, Y.S.: Nicotine and its interaction with beta amyloid Protein: a short review. Biol. Psychiatry. 49: 221-232, 2001.

Zhu, Y. J., Lin, H., Lal, R.: Fresh and nonfibrillar amyloid β protein (1-40) induces rapid cellular degeneration in aged human fibroblasts: evidence for AβP-channel-mediated cellular toxicity. FASEB J. 14: 1244-1254, 2000.