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Role of Calcium in Inflammation: Relevance to Alzheimer's Disease

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FLORIDA INTERNATIONAL UNIVERSITY

Miami, Florida

ROLE OF CALCIUM IN INFLAMMATION: RELEVANCE TO
ALZHEIMER'S DISEASE

A dissertation submitted in partial fulfillment of the
requirements for the degree of
DOCTOR OF PHILOSOPHY

in

BIOLOGY

by

Amita Quadros

2007

To: Interim Dean Mark D. Szuchman
College of Arts and Sciences

This dissertation, written by Amita Quadros, and entitled Role of Calcium in Inflammation: Relevance to Alzheimer's Disease, having been approved in respect to style and intellectual content, is referred to you for judgment.

We have read this dissertation and recommend that it be approved.

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The dissertation of Amita Quadros is approved.

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Dean George Walker
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Florida International University, 2007

DEDICATION

I would like to dedicate my thesis to my husband Naveen Quadros and my daughter Nicole Quadros who have sacrificed a lot these past 6 years so that I could get my most sought after doctoral degree. They have encouraged and supported me throughout these years and helped me achieve this momentous task. I also would like to dedicate this thesis to my brother Savio D'Souza and all my friends and family who have been with me all through this long and arduous journey.

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ABSTRACT OF THE DISSERTATION
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ALZHEIMER'S DISEASE

by

Amita Quadros

Florida International University, 2007

Miami, Florida

Professor Ophelia Weeks, Major Professor

Alzheimer's disease (AD) is neuropathologically characterized by excessive beta-amyloid ($A\beta$) plaques and neurofibrillary tangles composed of hyperphosphorylated tau in the brain. Although the etiology of genetic cases of AD has been attributed to mutations in presenilin and amyloid precursor protein (APP) genes, in most sporadic cases of AD, the etiology is still unknown and various predisposing factors could contribute to the pathology of AD. Predominant among these possible predisposing factors that have been implicated in AD are age, hypertension, traumatic brain injury, diabetes, chronic neuroinflammation, alteration in calcium levels and oxidative stress. Since both inflammation and altered calcium levels are implicated in the pathogenesis of AD, we wanted to study the effect of altered levels of calcium on inflammation and the subsequent effect of selective calcium channel blockers on the production of pro-inflammatory cytokines and chemokines. Our hypothesis is that $A\beta$, depending on its conformation, may contribute to altered levels of intracellular calcium in neurons and glial cells. We wanted to determine which conformation of $A\beta$ was most pathogenic in terms of increasing inflammation and calcium influx and further elucidate the possibility

of a link between altered calcium levels and inflammation. In addition, we wanted to test whether calcium channel blockers could inhibit the inflammation mediated by the most pathogenic form of A β , by antagonizing the calcium influx triggered by A β .

Our results in human glial and neuronal cells demonstrate that the high molecular weight oligomers are the most potent at stimulating the release of pro-inflammatory cytokines IL-6 and IL-8 as well as increasing intracellular levels of calcium compared to other conformations of A β . Further, L-type calcium channel blockers and calmodulin kinase inhibitors are able to significantly reduce the levels of IL-6 and IL-8. These results suggest that A β -induced alteration of intracellular calcium levels contributes to its pro-inflammatory effect.

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CHAPTER ONE

INTRODUCTION:

1.1. NEUROPATHOLOGICAL FEATURES OF ALZHEIMER'S DISEASE

Alzheimer's disease (AD) a protein misfolding disease named after Alois Alzheimer, is the major cause of dementia in the elderly in Western countries. The neuropathology of AD brains is characterized by the progressive accumulation of intracellular neurofibrillary tangles (NFT) and extracellular parenchymal senile plaques (Wilcock and Esiri 1982). The tangles consist of hyperphosphorylated tau while the plaques comprise of beta-amyloid (A β) peptides (Jellinger and Baner 1998). Biological markers like the presence of A β and tau in the cerebrospinal fluid (CSF) of AD patients provide an important tool in the diagnosis of the disease but they are not foolproof as other neurodegenerative diseases also show the presence of A β and tau in the CSF (Hampel et al. 2003). Reports have shown that compared to normal control patients, brains of AD patients exhibit significant cortical synaptic loss, A β deposition, NFT formation, and inflammation (Lue et al. 1996). Although senile plaques and NFTs are the neuropathological hallmarks of AD, significant microvascular pathology including reduced vascular density, atrophic vessels, and vascular amyloid deposits as cerebral amyloid angiopathy (CAA) have also been well described as part of AD pathology (Hashimura et al. 1991, Buee et al. 1994, Kalaria and Hedera 1996, Jeynes and Provias 2006). For over a decade it has been thought that A β may play an important role in mediating the initial pathogenic events in AD since all the mutations associated with familial forms of AD affect processing of A β from its substrate amyloid precursor protein

(APP) (Selkoe 1996). The familial cases of AD are mainly due to genetic mutations in the presenilin genes (PS1 and PS2) and in the APP gene. The APOE $\epsilon 4$ allele is also reported as a genetic risk factor for AD (Wilhelmus et al. 2005). The two leading hypotheses for AD etiology are the ‘amyloid cascade hypothesis’ implicating $A\beta$ as the central mediator while the ‘neuronal cytoskeletal degeneration hypothesis’ implicates tau as the prime suspect in causing AD. Other reports also implicate α -synuclein a synaptic protein implicated in Lewy bodies to also be responsible for AD pathology (Wirhth and Bayer 2003).

1.1.1. AMYLOID CASCADE HYPOTHESIS:

According to the ‘amyloid cascade’ hypothesis that was initiated in the 1980’s, $A\beta$, the major protein present in plaques is produced as a result of the endoproteolytic cleavage of APP by beta (β) and gamma (γ) secretases, and $A\beta$ aggregates deposited around neurons as senile plaques (Russo et al. 2002, Hardy and Higgins 1992, Glenner and Wong 1984). The processing of APP results in the formation of $A\beta$ that under normal circumstances is degraded by neprilysin or other proteases and then eliminated. In neurons of AD patients as compared to controls there may be a decrease in alpha (α) secretase activity over β -secretase activity resulting in increased $A\beta$ production or lack of neprilysin/clearance mechanisms to degrade $A\beta$ and clear it from the body (Buee et al. 1994, Rojas-Fernandez et al. 2002, Iwata et al. 2001). The two major enzymes, which enable the generation of $A\beta$ from APP, are the β and γ secretases. β -secretase cleaves APP at the N-terminus to release sAPP β , a 100-kD soluble N-terminal fragment

and C99, a 12-kD C-terminal fragment which remains membrane bound (Benjannet et al. 2001). Cleavage by α -secretase produces sAPP α and C83, a 10-kD membrane-bound C-terminal fragment. Both C-terminal fragments, C99 and C83, then become the substrate for one or more γ -secretases that cleave the fragments within their transmembrane domains, leading to the release and secretion of A β and the nonpathogenic p3 peptide, respectively (Neve et al. 2000). Hence, preventing the β -secretase cleavage and/or enhancing α -secretase activity would decrease the formation of A β (Citron 2002, Vassar 2002, Tyler et al. 2002). Many studies are being carried out in vitro and in vivo to determine if blocking β -secretase activity may reduce the pathology associated with AD. The relative amount of β versus α secretase cleavage is higher in AD compared to controls and the major β -secretase is BACE 1 over BACE 2 (Zhao et al. 2007). Studies have shown that the subcellular localization and phosphorylation of BACE affects its cleavage specificity for APP (Walter et al. 2001).

Elucidation of the proteolytic processing of APP resulting in the release of A β revealed that γ -secretase that cleaves intramembranous APP may most likely be presenilins (Xu et al. 2002) since mutations in PS resulted in early-onset Alzheimer's disease. Also the fact that PS could specifically influence the C-terminal cleavage of A β , in particular the A β 1–40/1–42 ratio was another reason for researchers to believe that PS could be the elusive γ -secretase enzyme. Other researchers disagreed with this theory because PS and APP are located in different secretory compartments, and nicastrin also plays an important role in γ -secretase activity (Li et al. 2003). Researchers have been trying to elucidate a possible mechanism of APP cleavage by presenilin (Annaert et al.

1999). There is an ongoing debate if PS1 is the actual γ -secretase due to the difference in subcellular localization of PS1 and APP and also because γ -secretase is composed of other proteins like nicastrin, PEN-2, Aph-1 (Cupers et al. 2001).

A β peptide in AD brains is composed of 39-43 amino acids. Data suggest that soluble forms of A β correlate better with the severity of dementia observed in AD patients than aggregated forms (McLean et al. 1999, Kim et al. 2003). Several isoforms of A β exist, however A β 1-40 is the more soluble isoform compared to A β 1-42 which aggregates more rapidly. However in AD brains, the amount of A β soluble oligomers found is elevated compared to control brains. Soluble oligomers are also referred to as amyloid-beta derived diffusible ligands (ADDLs). There are two pools of A β in AD brains, one pool with soluble forms include monomers/dimers and soluble oligomers and the other pool of aggregated insoluble forms of A β form the senile plaques and give rise to cerebral amyloid angiopathy (CAA). Several studies indicate that A β undergoes conformational changes when it interacts with the cell membrane resulting in A β assuming functional properties of an ion channel (Bhatia et al. 2000). It has been well established that the different forms of A β induce neurotoxicity. However, the exact mechanism of action for this neurotoxicity has not been established and is still under investigation by various research groups. One of the mechanisms by which A β mediates toxicity is by forming ion channels (Lin et al. 2001). Researchers speculate that this neurotoxicity mediated by A β due to its ion-channel forming abilities is because A β undergoes conformational changes when it interacts with the lipid in the cell membrane (Arispe et al. 1993, Kaye et al. 2004).

1.1.2. CYTOSKELETON HYPOTHESIS:

According to the ‘cytoskeleton hypothesis’ tau is implicated as the prime suspect in causing AD. Weingarten and colleagues first discovered a microtubule-associated protein tau (MAPT) in 1975 (Weingarten et al. 1975). The major function of tau in the CNS is in the stabilization of microtubules in neurons and tau might be involved in the establishment and maintenance of neuronal polarity. The tau gene located on human chromosome 17q21 in the human genome, contains 16 exons with the major tau isoform being encoded by 11 exons (Goedert et al. 1989, Spillantini and Goedert 1998). Alternative pre-mRNA splicing of exons 2, 3 and 10 in the single tau gene results in the formation of six different isoforms in the adult human brain (Buee et al. 2000). These isoforms ranging from 352-441 amino acids are responsible for the modulation of tau function and are characterized by the presence of three (3R tau) or four (4R tau) tandem repeats of 31-32 amino acids located in the carboxy terminal end which is also the microtubule binding domain of tau (Goedert and Jakes 2005). In adult human brain the ratio of 3R tau to 4R tau is approximately 1 and this balance is disrupted in the case of tau mutations associated with exon 10 (D’Souza and Schellenberg 2005). Various kinases and phosphatases are involved in the regulation of tau phosphorylation that occurs at a number of serine, threonine and proline residues (Butler and Shelanski 1986, Ferrer et al. 2005).

Tau protein has been used in various configurations as a diagnostic marker of degenerative changes in the CNS. Tau hyperphosphorylation is at the crux of most tauopathies since hyperphosphorylation dissociates tau from microtubules, destabilizes them and forms paired helical filaments (PHF) in vitro (Lindwall and Cole 1984, Alonso

et al. 1994). Tau phosphorylation is regulated by an exquisite equilibrium between kinase and phosphatase activities. An imbalance of these two enzymatic processes can result in abnormal hyperphosphorylation of tau and generation of PHF. Mutations in the tau gene and tau hyperphosphorylation have been observed in many neurodegenerative diseases as well as in senescent brains. Neurodegenerative diseases of note include Pick's disease, AD, Frontotemporal dementia with Parkinsonism linked to chromosome 17 (FTDP-17), progressive supranuclear palsy (PSP), corticobasal degeneration, Niemann Pick's disease, etc. These diseases are all referred to as tauopathies since they all share a common pathology which is aggregated tau.

Tau is a major constituent of microtubules, and since microtubules offer stabilization to neurons, tau hyperphosphorylation and subsequent aggregation may be central in the neurodegeneration observed in AD (Alonso et al. 2001). Studies suggest that cognitive deterioration occurs only after the development of neurofibrillary tangles with hyperphosphorylated tau. Tau hyperphosphorylation in AD results in the reduced ability of tau to bind to microtubules. In AD, tau is hyperphosphorylated at 30 specific amino acid sequences throughout its 441 amino acids (Gong et al. 2005). In vitro studies suggest that increases in A β production may potentiate tau phosphorylation by activation of kinases such as glycogen synthase kinase-3 (GSK-3) (Phiel et al. 2003). Phosphorylated tau protein at Threonine 231 is seen in postmortem brain tissue of patients with AD and can be detected in CSF (Buerger et al. 2002). In vitro studies with cultured cells have shown that A β induced tau phosphorylation is neurotoxic to cultured cells (Busciglio et al. 1995). Other supportive evidence for the tau hypothesis in AD can be found from studies with transgenic mouse models of AD that overexpress mutant APP

or PS. These animals develop senile plaques with age, but they fail to develop NFTs and exhibit little neuronal loss (Holcomb et al. 1998). Researchers supporting the tau hypothesis claim that tau alone is sufficient to cause dementia and neurodegeneration since mutations in tau cause frontotemporal dementia (FTD). In addition, studies with transgenic mice having the 'Swedish' mutation (TgAPPsw), that causes early onset AD in humans, reveal the presence of abundant A β deposits that are visible beginning at 9 months of age (Duff et al. 1996). However, these mice do not show substantial neurodegeneration despite the presence of A β plaques. Although amyloid and tau are often present in the same brain areas, namely hippocampus and entorhinal cortex, a mechanistic link between them has yet to be established. Recent data suggest that A β is upstream of tau and that A β deposition induces accumulation of tau resulting in memory impairment (Gotz et al. 2004, Ribe et al. 2005). Hence there could be a possible interaction between amyloid and tau which is responsible for the pathogenesis associated in AD. However, there is no evidence to date demonstrating that abnormal tau phosphorylation or accumulation causes amyloid deposition.

1.1.3. NEUROVASCULAR HYPOTHESIS:

Another recent hypothesis that is gaining momentum with regards to the pathogenesis of AD is the neurovascular hypothesis. Due to the link between AD and atherosclerosis (Kalback et al. 2004) neurovascular dysfunction is believed to be responsible for the cognitive decline and neurodegeneration observed in AD. The neurovascular unit in the brain consists of astrocytes, neurons, pericytes in close association with the vascular endothelium to maintain cerebral homeostasis and serve as

the first line of defense against pathogenic factors (Frontczak-Baniewicz and Walski 2006). According to the neurovascular hypothesis for AD faulty clearance of A β across the blood-brain barrier (BBB) together with aberrations in angiogenesis results in brain hypoperfusion and neurovascular inflammation (Zlokovic 2005, Zlokovic et al. 2005). The BBB regulates A β transport via two main receptors, the low density lipoprotein receptor related protein 1 (LRP1) and the receptor for advanced glycation end products (RAGE) (Deane and Zlokovic 2007). The RAGE receptor is thought to be a primary transporter of A β across the BBB into the brain from the systemic circulation, while the LRP-1 mediates transport of A β out of the brain (Donahue et al. 2006). The RAGE receptor is a member of the immunoglobulin superfamily and exists in three major isoforms in the brain. They are expressed in neurons, microglia, astrocytes, pericytes, smooth muscle cells and cerebral endothelial cells. The adverse consequences of RAGE interaction with A β include perturbation of neuronal properties and functions, amplification of glial inflammatory responses, elevation of oxidative stress and amyloidosis, increased A β influx at the BBB and vascular dysfunction (Lue et al. 2005). Previous studies on microglia demonstrate that binding of A β to RAGE receptor results in the production of macrophage-colony stimulating factor (M-CSF) via the NF κ B dependent pathway thereby suggesting that inflammation is one of the mechanisms involved in AD neurotoxicity (Du et al. 1997). The neurovascular hypothesis in AD suggests that A β causes a reduction in cerebral blood flow (CBF) due to vasoconstriction of cerebrovessels. A β also causes regression of cerebrovessels due to faulty mechanisms involving angiogenesis and subsequent activation of glial cells resulting in

neuroinflammation. Hence therapies aimed at clearance of A β from the brain, enhancing CBF and preventing regression of cerebrovessels are new therapeutic targets for AD according to the neurovascular hypothesis.

The differentiation of AD from vascular dementia (VD) is hampered by clinical diagnostic criteria with lowered sensitivity and specificity. The most frequently examined biomarkers in the diagnosis of dementia are cerebrospinal fluid (CSF) tau, phospho-tau which is phosphorylated at threonine 181, and A β 1-42 (de Jong et al. 2006). Studies indicate that total tau and IL-6 levels were not significantly different between AD patients and VD patients. However, AD patients had higher level of phospho-tau as compared to VD patients (Jia et al. 2005). This is also supported by other studies that show higher levels of CSF phospho-tau in AD patients as compared to VD patients thereby suggesting that phospho-tau is a valuable diagnostic tool to differentiate between AD and VD cases (Hu et al. 2002).

1.2. ROLE OF BRAIN INFLAMMATION IN ALZHEIMER'S DISEASE:

The vast majority of AD cases are sporadic with no history of associated mutations. In addition to plaques and NFT's, gliosis is another important feature of AD pathology (McGeer and McGeer 2001, McGeer et al. 1989). Microglia the resident macrophages of the brain may identify A β oligomers or A β aggregates as foreign material and this recognition may prompt their activation. Activated microglia found in and around amyloid plaques are morphologically characterized by short tortuous ramified processes (Meda et al. 1995). Astrocytes that become activated in AD brain are characterized by morphologically long sinuous processes that reach in and around the

amyloid plaque. These cells upregulate glial fibrillary acidic protein (GFAP) the most specific marker of astrocytosis. The release of pro-inflammatory molecules accounts for the activated astrocytes which express GFAP (Little et al. 2001). Reactive astrocytes result in the production of other acute phase reactants such as α -1-antichymotrypsin which are found co-localized with fibrillar cores of mature A β plaques thereby promoting A β aggregation (Abraham and Potter 1989). These activated microglia and astrocytes are considered to be signs of an inflammatory response in AD brain. The cyclooxygenase (COX) enzymes COX-1 and COX-2 responsible for the production of prostaglandins (PGs) from the substrate arachidonic acid are also upregulated in regions of the AD brain undergoing degeneration supporting the inflammation hypothesis (Masferrer et al. 1995, Zhang et al. 1997, Hoozemans et al. 2001). Also, pro-inflammatory cytokines have shown to be neurotoxic in vitro at high doses (Meda et al. 1995, Downen et al. 1999). Studies reveal that A β is responsible for the stimulation and production of pro-inflammatory eicosanoids and cytokines such as TNF α in various in vitro models (Paris et al. 2000, Lue et al. 1999). Pro-inflammatory cytokines such as IL-1 β , TNF α , IL-6 and IL-8 are typically found to be elevated in AD brain compared to non-AD subjects (McGeer and McGeer 1999, Lue et al. 2001). IL-6 stimulation in AD brains increases tau phosphorylation which further causes neurodegeneration (Quintanilla et al. 2004). IL-6 cytokine is produced within the CNS by astrocytes and microglia and under normal physiological conditions have a neuroprotectant and neurotrophic role. However, chronic exposure of IL-6 causes neurodegeneration (Nelson et al. 2002). Patients with traumatic brain injury revealed higher levels of IL-6 and IL-8 (Kushi et al. 2003) suggesting that

these cytokines and other chemokines could also serve as potential biomarkers for neuroinflammatory diseases such as AD, multiple sclerosis and Parkinson's disease (Tan et al. 2007, Nagatsu et al. 2000). Converging lines of evidence from epidemiological studies, post mortem evaluations and animal models of AD all support the hypothesis that chronic inflammation plays a deleterious role in the pathophysiology of AD. Numerous epidemiological studies have demonstrated that NSAIDs such as ibuprofen and indomethacin significantly reduce the risk for the incidence of AD (McGeer and McGeer 1999, Imbimbo 2004).

TgAPP^{sw} mice develop age dependent gliosis with increasing amyloid levels. AD-like pathology is also observed in double transgenic mice with the PS1 M146L mutation together with the TgAPP^{sw} mutation (Holcomb et al. 1998, Duff et al. 1996). These mice develop gliosis as detected by thioflavin S staining and anti-A β antibody (Holcomb et al. 1998). Studies with 10 month old TgAPP^{sw} mice treated with ibuprofen showed reduced amyloid pathology and decreased microglial activation (Lim et al. 2000). Other studies demonstrate that this amyloid lowering property of ibuprofen in transgenic mice was due to its ability to reduce A β 1-42 production by affecting APP processing (Yan et al. 2003). Thus animal models of AD develop not only A β deposit pathology but also manifest gliosis comparable to that found in AD patient brains. Studies in transgenic mouse models of AD particularly suggest that severity of microgliosis is directly proportional to A β burden. One study showed that high levels of A β 1-42 rather than A β 1-40 were able to activate microglia more potently as measured by the release of TNF α and nitric oxide secretion (Meda et al. 1995).

Although a plethora of research has been carried out on AD, there is to date no known cure for this disease and the neurodegeneration is without remission. All treatments available on the market today, namely, acetylcholine esterase inhibitors, NMDA receptor blockers, anti-inflammatory drugs, antioxidants offer only symptomatic relief and slow progression of the disease to some extent (Prasad et al. 2002).

1.3. ROLE OF CALCIUM IN INFLAMMATION AND AD:

Altered calcium homeostasis is considered to play an important role in neurodegeneration associated with normal aging, AD and related disorders (Mattson 1992). In vitro studies on cultured neurons with the calcium ionophore A23187 indicated that calcium influx resulted in microtubule disruption due to altered tau phosphorylation (Mattson et al. 1991). Studies of brain tissue from AD patients reveal increased activation of calcium-dependent proteases in neurofibrillary tangles. Abnormalities in calcium regulation in astrocytes, oligodendrocytes, and microglia have also been documented in experimental models of AD (Mattson and Chan 2003, Nusslein et al. 1996).

Calcium ion is vital for neuronal signaling and is tightly regulated. Receptor mediated changes in intracellular calcium is one of the major pathways involved in signal transduction. Calcium mediated signals are generated by four main processes namely calcium influx from extracellular spaces across the plasma membrane into the cytoplasm, calcium release from internal stores into the cytoplasm, calcium extrusion from cytoplasm across the plasma membrane and calcium sequestration into intracellular calcium stores (Moller 2002). Calcium influx across the plasma membrane into the cytoplasm occurs via receptor operated channels (ROCs) triggered by ligand binding,

voltage gated calcium channels (VGCCs) and calcium permeable store-operated channels (SOCs) which open up when intracellular stores of calcium are depleted. VGCCs are a group of voltage-gated ion channels found in neurons, glial cells, muscle cells, etc. involved in the release of neurotransmitters and hormones, muscular contraction, excitability of neurons and gene expression (Dolphin 2006). VGCCs include the neural N-type channel blocked by ω -conotoxins, the residual R-type channel involved in processes in the brain and muscle, the closely related P/Q-type channel blocked by ω -agatoxins, and the dihydropyridine-sensitive L-type channels responsible for excitation-contraction coupling of skeletal, smooth, and cardiac muscle and hormone secretion in endocrine cells. The reason I evaluated the effect of L-type calcium channel blockers over other type of calcium channel blockers on reducing inflammation was due to studies in aging hippocampal or cortical neurons of rodents and rabbits that reveal an increase in L-VGCC activity. Their hypothesis is that increased L-VGCC activity drives many of the other calcium related biomarkers of hippocampal aging together with ryanodine receptors (RyR) (Thiebault et al. 2007). Other studies have demonstrated that the age-related learning impairment in rabbits is reversed by the specific L-type calcium channel blocker nimodipine thereby suggesting that elevation of L-type channel activity causes neuronal dysfunction during aging (Davare and Hell 2003).

Studies reveal that pro-inflammatory cytokines like $\text{IFN}\gamma$ and $\text{IL-1}\beta$ induce calcium response and cause subsequent activation of microglia (Franciosi et al. 2002, Goghari et al. 2000). Influx of calcium also results in the release of PGs from the substrate arachidonic acid, the mediators of inflammation. Our previous data further support the hypothesis that $\text{A}\beta$ can activate calcium dependent secretory phospholipase

A2 (PLA2) resulting in the production of arachidonic acid which is metabolized into proinflammatory eicosanoids (Paris et al. 2000). PGE2 induces increases in intracellular calcium both in astrocytes and in neurons. The intracellular calcium increase in neurons may be mediated by glutamate released from astrocytes (Bezzi et al. 1998). Studies on rat hippocampal neurons treated with IL-6 resulted in an increased calcium influx via NMDA-receptor, since NMDA receptor antagonists blocked this IL-6 mediated calcium influx. Further this increase in cytosolic calcium induced tau protein hyperphosphorylation via the JAK/STAT pathway (Orellana et al. 2005). Previous studies with chronic exposure of Purkinje neurons to IL-6 reveal similar increases in intracellular calcium resulting in neurodegeneration (Nelson et al. 2002). Increases in intracellular calcium cause activation of microglia and release of cytokines and chemokines which are blocked when calcium is chelated (Hoffmann et al. 2003). Hence, my rationale was that perhaps calcium channel blockers (CCBs) or inhibitors of the calcium signaling pathway could help alleviate inflammation in AD. Several studies in patients with cardiovascular disease and hypertension indicate that these CCBs increase blood flow, dilate blood vessels, reduce nitric oxide release, which in turn prevents oxidative stress and reduces inflammation (Mason et al. 2003, Sudano et al. 2007). Clinical studies with Amlodipine, a calcium channel blocker showed reduced risk in the development and progression of coronary atherosclerosis on the basis of its anti-oxidant and anti-inflammatory properties. Amlodipine caused a reduction of the inflammatory chemokine monocyte chemoattractant protein-1 (MCP-1) (Kataoka et al. 2004).

1.3.1. RATIONALE FOR CALCIUM CHANNEL BLOCKERS BEHAVING AS ANTI-INFLAMMATORY AGENTS

Our previous studies in transgenic mouse models of AD have shown that A β causes vasoconstriction of cerebral blood vessels and is also responsible for stimulating Cox-2 activity resulting in the production of pro-inflammatory eicosanoids (Paris et al. 2003). This A β mediated vasoactivity can be alleviated by using calcium channel blockers both in vitro and in vivo (Paris et al. 2004). I also showed that organotypic brain slice cultures from TgAPP^{sw} mice over 9 months produced significant levels of TNF α and Cox-2 activity. These mice had significant amyloid pathology and when brain slices of these mice were treated with a selective Cox-2 inhibitor NS-398, levels of TNF α was significantly reduced (Quadros et al. 2003). A β has been shown to cause synaptic dysfunction and can render neurons vulnerable to excitotoxicity and apoptosis by a mechanism involving disruption of cellular calcium homeostasis (Mattson et al. 1992). Since it has been well established that inflammation and alteration in cellular calcium levels are some of the factors responsible for the pathogenesis in AD, I hypothesized that there could be a link between altered calcium levels and inflammation. My hypothesis was that A β could mediate inflammation by possibly altering cellular calcium levels. I also wanted to identify which conformation of A β was most potent in mediating inflammation in glial cells and correlate this pro-inflammatory effect to altered calcium levels. My other aim was to determine the effect of the various conformations of A β with respect to neurodegeneration and evaluate if this A β mediated neurotoxicity could be blocked by using calcium channel blockers or other inhibitors that are part of the

calcium signaling pathway. The other inhibitors of the calcium signaling pathway include nuclear factor-kappa B (NFkB) inhibitors, phospholipase C (PLC) inhibitors, protein kinase C (PKC) inhibitors and calmodulin kinase inhibitors. These inhibitors could possibly have anti-inflammatory effects with A β induced inflammation because of their property of reducing intracellular calcium. In vitro studies on neurons have shown that calcium is responsible for the stimulation of NFkB activity (Lilienbaum and Israel 2003). Other studies revealed that NFkB activity could be significantly reduced by using PKC or calmodulin kinase inhibitors (Bonizzi et al. 1999, Han and Logsdon 2000). Both PKC kinase and calmodulin kinase are activated by calcium entry into the cytosol which further activates NFkB activity in neurons (Lilienbaum and Israel 2003). Evidence from earlier studies reveals that NFkB activation causes inflammation (Hata et al. 2002, Granet et al. 2003). In addition, calcium channel blockers like amlodipine have shown to behave as anti-inflammatory agents in rat models of arteriosclerosis (Kataoka et al. 2004, Yoshii et al. 2006). Other studies on amlodipine reveal that it exerts protective vascular effects by probably suppressing pro-inflammatory cytokines and free radical generation (Chou et al. 2002, Richard 2005). Other calcium channel blockers more specifically the dihydropyridines such as nitrendipine, manidipine including amlodipine have also demonstrated anti-inflammatory properties with respect to other inflammatory diseases such as multiple sclerosis, experimental autoimmune encephalomyelitis (Toba et al. 2005, Brand-Schieber and Werner 2004). Hence my rationale that calcium channel blockers and other inhibitors of the calcium signaling pathway could have potential anti-inflammatory effects in human glial cells thereby protecting neurons from the pathogenic inflammatory insults mediated by A β in AD.

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CHAPTER TWO

Increased TNF α Production and Cox-2 Activity in Organotypic Brain Slice Cultures from APPsw Transgenic Mice

ABSTRACT

β -amyloid (A β) peptides are the principal component of senile plaques and vascular deposits in Alzheimer's disease (AD) and are derived from the proteolytic cleavage of the β -amyloid precursor protein (APP). I have previously shown that synthetic A β can stimulate cyclooxygenase-2 (Cox-2) activity in brain organotypic slice cultures. In the present study, I used brain slices from transgenic APP Swedish (TgAPPsw) mice and control littermates of different age groups to determine the effect of APP overexpression on the levels of prostaglandin and TNF α release. The production of eicosanoid and TNF α was increased as a function of age in organotypic brain slice culture from TgAPPsw mice compared to age matched control littermates. I also showed that the selective Cox-2 inhibitor NS-398 reduces the production of eicosanoid and TNF α in organotypic brain slice cultures of TgAPPsw mice. In conclusion, my data suggest that either activity or expression of Cox-2 is increased in TgAPPsw mice brains as a function of age, contributing to an increased production of pro-inflammatory eicosanoids and TNF α .

Keywords: Alzheimer's disease, inflammation, Cox-2, TgAPPsw mice, PGE₂, TNF α

INTRODUCTION:

Alzheimer's disease (AD) is a neurodegenerative disease characterized by extracellular A β deposits and intracellular neurofibrillary tangles (Wilcock and Esiri 1982). AD is a multifactorial disease, of which neuroinflammation is suggested to be one of the major contributing factors in the progression of the disease (Bamberger and Landreth 2002, Eikelenboom et al. 2002). This is evidenced by the upregulation of both cyclooxygenase (Cox) enzymes, Cox-1 and Cox-2 in AD brain (Ho et al. 2001) and by the fact that several epidemiological studies have revealed that non-steroidal anti-inflammatory drugs (NSAIDs) reduce the risk for developing AD (McGeer and McGeer 1998). NSAIDs block Cox-1 and Cox-2 that are responsible for the production of prostaglandins (Dannhardt and Kiefer 2001). Cerebral amyloid plaques in AD patients are co-localized with activated microglia and astrocytes. Several reports have revealed that activated microglia and astrocytes release eicosanoids and cytokines that may contribute to the inflammatory response observed in AD brains (Vehmas et al. 2003). We have shown previously in organotypic rat brain slices, that synthetic A β peptide can stimulate the production of prostaglandin E₂ (PGE₂) and TNF α via a Cox-2 dependent manner (Paris et al. 2002). A β progressively accumulates in the brains of transgenic mice overexpressing APP with the Swedish mutation (TgAPP^{sw} line 2576). Before 9 months of age, no β -amyloid plaque can be observed in the brain of these animals, after 9 months the amount of A β deposits increase with age (Kuo et al. 2001).

METHODS:

1 mm thick brain slices were sectioned using a mouse brain slicer from TgAPP^{sw} and control animals at different ages (3 month, 10 month, 14 month, 17 month) and cultured in neurobasal media supplemented with 5% B27 (Gibco-Invitrogen, CA) and Penicillin-Streptomycin-Fungizone mixture. The levels of PGE₂ (Fig. 1) and TNF α (Fig.2) that accumulate in the culture medium surrounding the brain slices after 24 h incubation at 37°C was measured using ELISA kits (Biosource, CA).

RESULTS:

No difference in the accumulation of PGE₂ and TNF α were observed between 3 month-old TgAPP^{sw} and control organotypic brain slice cultures. An increased accumulation of both PGE₂ and TNF α was observed in TgAPP^{sw} brain slice cultures compared to control brain slices cultures isolated from 10, 14 and 17 month-old animals. To determine whether the increased production of PGE₂ were dependent on COX-2 activity, I treated the brain slices with 20 μ M of the selective COX-2 inhibitor NS-398. NS-398 completely inhibited the increased production of PGE₂ (Fig. 3) in 14 M TgAPP^{sw} brain slice culture compared to control cultures suggesting that the increased PGE₂ production observed is mediated by COX-2. Interestingly, NS-398 also partially alleviated the increased TNF α production observed in organotypic brain slice culture from TgAPP^{sw} mice (Fig. 4).

DISCUSSION:

Microgliosis and astrocytosis are known to be associated with β -amyloid deposits in the brain of TgAPPsw mice (Gasic-Milenkovic et al. 2003) and *in vitro*, activated microglia and astrocytes are known to produce eicosanoids and TNF α (Casal et al. 2002). My data suggest that the increased production of PGE₂ and TNF α by organotypic brain slice cultures from TgAPPsw mice older than 9 months may be related to the glial reaction occurring in the brain of TgAPPsw mice. The fact that the selective Cox-2 inhibitor NS-398 was able to reduce TNF α levels suggests that metabolites of the Cox-2 pathway are also important for regulating TNF α release. TNF α is a cytokine produced in response to inflammation and brain injury by astrocytes and microglia (Lee et al. 2000). Studies in transgenic and knockout mouse models of TNF α have revealed that this cytokine plays a dual role in the brain (Probert et al. 1997). TNF α can be both protective and damaging depending on the levels produced and the duration of action (Pober 1987). Increased levels of TNF α activate microglia resulting in the production of additional cytokines that may cause neurodegeneration (Neumann et al. 2002). Altogether my data show that the brains of TgAPPsw mice are subjected to inflammatory stress with aging leading to increased Cox-2 activity and TNF α production. We have previously shown *in vitro* using rat brain slices that synthetic A β is capable of inducing the production of pro-inflammatory cytokines and TNF α (Paris et al 2002). My results using organotypic brain slice cultures from TgAPPsw and control littermates reveal a progressive stimulation of Cox-2 activity and TNF- α production in function of age. Products of the Cox-2 enzyme are known to play a role in long-term potentiation (LTP) in hippocampal

neurons and postsynaptic membrane excitability (Chen et al. 2002) suggesting that a stimulation of Cox-2 activity by A β may impair neuronal functions and affect learning and memory. My data support the fact that there is an active inflammatory process occurring in the brain of TgAPPsw mice. The fact that young TgAPPsw mice do not show this inflammatory condition suggests that inflammation parallels the accumulation of A β in the brain and might be associated with gliosis. Moreover, my data demonstrate that the use of selective Cox-2 inhibitors can block the increased PGE₂ release observed in TgAPPsw brain slices suggesting that Cox-2 activity is upregulated in the brain of TgAPPsw mice. Metabolites of the arachidonic acid cascade are important mediators of LTP and neuronal plasticity; the abnormal stimulation of Cox-2 activity observed may therefore lead to impaired neuronal function. Besides, other studies with TNF α suggest that, through activation of the transcription factor NF-kappaB, TNF α may modulate neuronal excitability and vulnerability to excitotoxicity (Furukawa and Mattson 1998). My data demonstrate that the selective Cox-2 inhibitor NS-398 can also reduce TNF α levels significantly. Hence, blocking Cox-2 enzyme activity by using selective Cox-2 inhibitors may be efficient at reducing neuroinflammatory events occurring in AD brains.

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Figure 1:

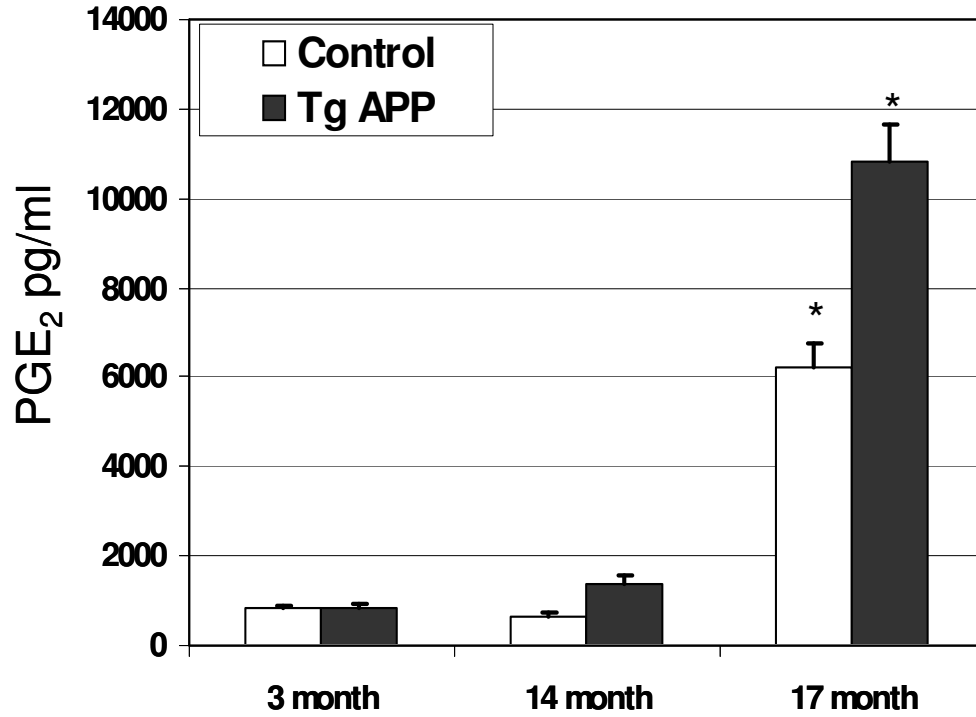


Fig. 1: PGE₂ release in culture media surrounding 3M, 14M, 17M old control and TgAPP sw mice brain slices. ANOVA revealed significant main effect of age ($p < 0.001$), of transgenicity ($p < 0.001$), interactivity between age and transgenicity ($p < 0.001$). Post-hoc analysis showed significant differences in PGE₂ levels between control and TgAPP sw 17M of age brain slice culture media.

Figure 2:

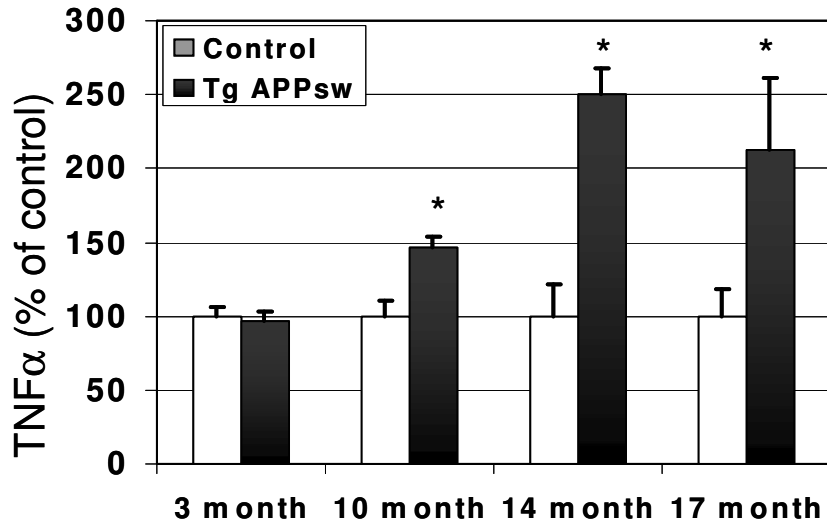


Fig. 2: TNF α levels in culture media surrounding 3M, 10M, 14M and 17M old control and TgAPP sw mice brain slices. ANOVA revealed significant main effect of age ($p < 0.009$), main effect of transgenicity ($p < 0.001$) and an interactivity between them ($p < 0.009$). Post-hoc analysis revealed significant differences in TNF α production between control 14M & TgAPP 14M ($p < 0.009$) as well as significance difference between control 17M & TgAPP 17M ($p < 0.05$).

Figure 3:

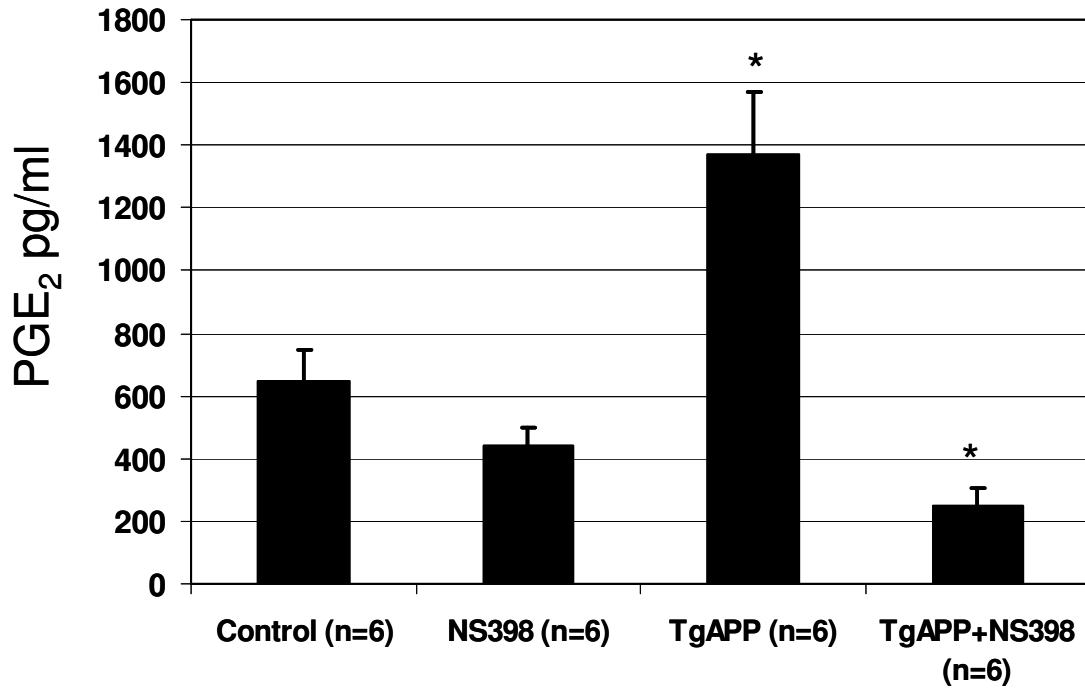


Fig. 3: PGE₂ release in culture media surrounding 14M old brain slices of control and TgAPP sw mice treated with and without 20µM NS398. ANOVA revealed significant main effect of transgenicity (p=0.038), a main effect of NS398 (p<0.001) as well as interactivity between NS398 and transgenicity (p<0.001). Post-hoc analysis showed significant differences between PGE₂ production in control cortex & TgAPP cortex but no significant differences between control cortex & TgAPP cortex & between NS398 and TgAPP cortex (p=0.4) showing that NS398 completely inhibits the production of PGE₂ in TgAPP brain slice.

Figure 4:

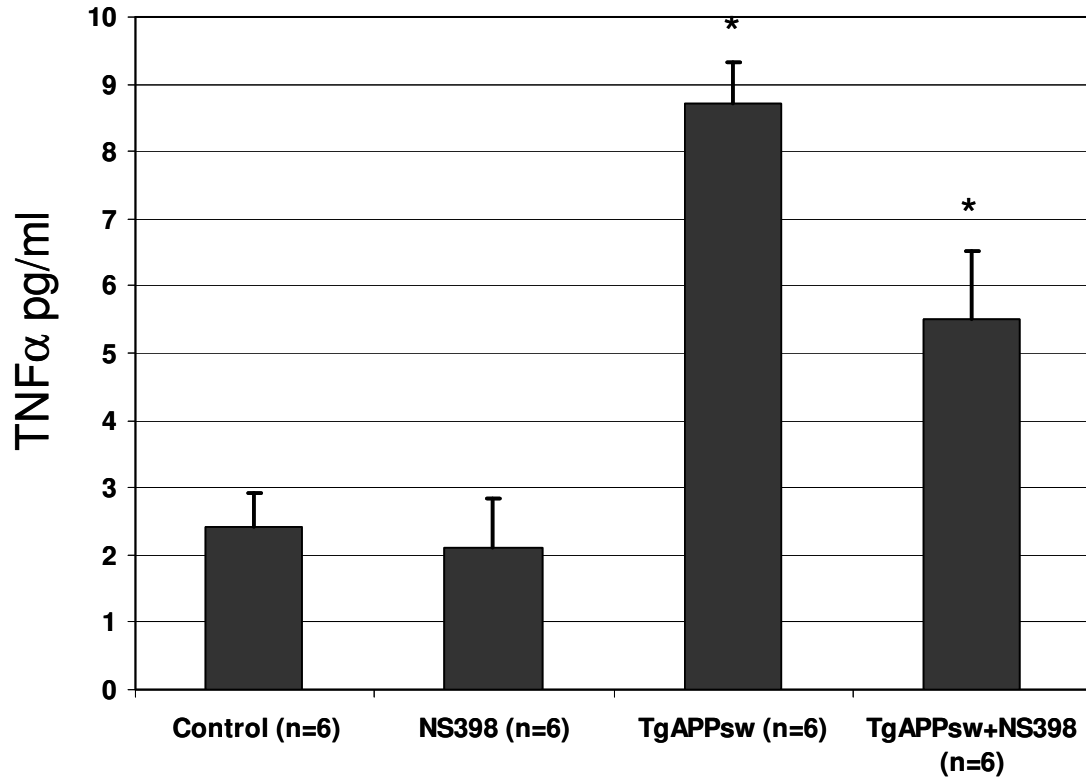


Fig. 4: TNF α levels in culture media surrounding 14M old brain slices of control and TgAPPsw mice treated with and without 20 μ M NS398. ANOVA revealed significant main effect of NS398 ($p < 0.04$), main effect of transgenicity ($p < 0.001$) as well as interactivity between the two ($p < 0.04$). Post-hoc analysis revealed significant difference in TNF α production between control and TgAPPsw cortex ($p < 0.001$). Hence, NS398 inhibits TNF α production by TgAPP sw brain slice.

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CHAPTER THREE

Increase of calcium entry by A β promotes inflammation in human astrocytes and microglia

ABSTRACT:

BACKGROUND: A β has been shown to cause synaptic dysfunction and can render neurons vulnerable to excitotoxicity and apoptosis by a mechanism involving disruption of cellular calcium homeostasis. Studies have shown that an increase in intracellular calcium plays an important role in the activation of glia and in the propagation of inflammatory reactions in the CNS. My aim was to identify whether A β could affect calcium entry in human astrocytes and microglia and whether a correlation between calcium dysregulation and the pro-inflammatory effect of A β could be established. My data show that high molecular weight oligomers (HMWO) of A β 1–42 stimulate calcium entry in microglia and astrocytes more potently than low molecular weight oligomers (LMWO) of A β 1–42 or preparations of freshly solubilized (FS) A β containing essentially dimeric forms of the peptide. In addition, I show that the HMWO forms of A β are more potent at stimulating inflammation in astrocytes and microglia than LMWO A β or FSA β .

METHODS: Primary culture of human astrocytes and microglia were used to determine the impact of the various forms of A β (FS, LMWO, HMWO) on calcium entry and on the levels of pro-inflammatory cytokines IL-6 and IL-8. The production of cytokines was quantified using Bio-plex cytokine profiling assay while calcium entry in the cells was monitored fluorimetrically.

RESULTS: My results indicate that HMWO A β are more potent than LMWO or FSA β at stimulating IL-6 and IL-8 levels in human astrocytes and microglia. The increase in pro-inflammatory cytokines induced by the oligomeric species of A β is inhibited by the intracellular chelator BAPTA-AM. The HMWO are more potent at stimulating IL-6 and IL-8 in astrocytes and microglia than LMWO A β or FSA β . In addition, HMWO are also more potent than FSA β at stimulating calcium entry in human astrocytes and microglia.

CONCLUSION: My data therefore suggests a link between the inflammatory effect of A β oligomeric species and their effect on calcium entry in human astrocytes and microglia.

BACKGROUND:

Several studies have revealed that cytokines serve as powerful regulators of glial cell function and contribute to CNS pathology. Studies have shown that an increase in intracellular calcium plays an important role in the production of cytokines by glia resulting in the propagation of inflammatory reactions in the CNS (Hoffmann et al. 2003). Although in principle, glial activation aims at CNS protection, excessive or sustained activation could significantly contribute to acute and chronic neuropathologies. Dysregulation of glial cytokine production could therefore disturb neural cell functions and result in neurotoxicity (Benveniste 1998, Benveniste et al. 2001). In AD, soluble A β comprises mainly of monomeric/dimeric and oligomeric species, whereas, insoluble A β consists of aggregated forms which are the major components of senile plaques. Studies in rodent hippocampus reveal that soluble A β mediates an inhibition of LTP, resulting in

memory loss (Rowan et al. 2005). My aims were to identify which form of A β 1–42 (FS, LMWO, HMWO) are most potent in activating microglia and astrocytes, as well as determine if this effect of A β is mediated in part by an alteration of intracellular calcium levels. Pro-inflammatory cytokines have been shown to induce calcium response and activate glial cells in experimental models of AD (Mattson et al. 1997, Goghari et al. 2000). In the present study I compared the effects of the various forms of A β 1-42 on calcium entry and their corresponding impact on the levels of pro-inflammatory cytokines.

METHODS AND MATERIALS:

Preparation of the different forms of A β 1-42:

Freshly solubilized: Lyophilized A β 1-42 peptide (Purity greater than 95%, Biosource, CA) was dissolved to 1 mg/ml in 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) (Sigma-Aldrich, MO) in order to minimize formation of β -sheet structures and promote α -helical secondary structure. A β 1-42 was allowed to air dry in a chemical fume hood for 1h, followed by further drying in a speed-vac for 30 minutes. The resulting clear film was re-suspended in 100% DMSO to a concentration of 1mM. Peptides were subsequently aliquoted and stored at -80°C. LMWO: An aliquot of the DMSO stock of A β 1-42 was diluted to 100 μ M in HBSS (Invitrogen, CA) not containing calcium and incubated overnight at 4°C. The peptide was then centrifuged at 16,000g for 30 mins at 4°C. The supernatant (containing LMW A β species) was collected and dissolved to appropriate concentration in HBSS. HMWO: HFIP treated A β 1-42 was diluted in DMSO to 100 μ M

in PBS and incubating for 5 days at 37°C. HMWO were pelleted by centrifugation at 15,000g for 15 mins and resuspended in HBSS. A β 1-42 concentrations in the different preparations were estimated by using A β 1-42 ELISA (Biosource, CA) after denaturing the different forms of A β with 80% formic acid. In addition, the different preparations of A β were evaluated on native and denaturing conditions using 4-20% gradient PAGE followed by electroblotting on Polyvinylidene fluoride (PVDF) membranes and immunodetection with the antibody 4G8 (Signet laboratories).

Cell culture: Primary cultures of human astrocytes and human microglia (ScienCell, MD) were cultured in astrocyte medium and microglia media respectively (ScienCell, MD) supplemented with 10% fetal bovine serum, astrocyte growth medium/microglia growth medium and 1X Penicillin-Streptomycin-Fungizone mixture (Cambrex Corp., NJ). The treatment groups included various forms (FS, LMWO, HMWO) of A β 1-42 at 1 μ M. Cells were also treated with 10 μ M of the calcium ionophore A23187 (Sigma-Aldrich, MO), 20 μ M of the intracellular calcium chelator BAPTA-AM (Sigma-Aldrich, MO), a PLC inhibitor, U73122 (Sigma-Aldrich, MO) at 10 μ M, a calmodulin kinase inhibitor KN62 (Sigma-Aldrich, MO) at 5 μ M and a L-type calcium channel blocker Nilvadipine (Fujisawa, Japan) at 5 μ M. Cell culture plates were incubated at 37°C and culture media collected 24h after treatment. IL-6 and IL-8 were quantified in the culture medium using the Luminex fluorescent-bead-based technology (de Jager et al. 2003, Patel et al. 2005). Human cytokine 2-plex panel kits (Bio-Rad, CA) were used according to the recommendation of the manufacturer.

Measurement of intracellular calcium levels: Intracellular calcium levels were evaluated by spectrofluorometry using Fluo-4 (Molecular Probes). Human astrocytes and microglia were grown on a black costar 96-well plates previously coated with collagen for 24 hrs. Confluent cells were treated with 5 μ M of Fluo-4 in DMEM for 30 minutes in a cell culture incubator. Cells were subsequently washed in DMEM followed by 3 washes in HBSS containing no calcium in order to remove any unincorporated Fluo-4. Intracellular calcium levels were quantified using a Bio-tek spectrofluorometer equipped with two plate microinjectors (Bio-tek Inc., VT) at specific excitation (485 nm) and emission (516 nm) wavelengths. Baseline intracellular calcium levels were continuously recorded for 1 minute. Immediately following treatment of the cells with 1 μ M FS, LMWO and HMWO A β 1–42, intracytoplasmic calcium was continuously recorded for one minute to determine the possible impact of the different forms of A β on calcium release by intracellular calcium stores. Finally, following one minute of treatment with different forms of A β , 2mM of CaCl₂ was added to the cells and intracellular calcium levels were continuously monitored for another minute in order to evaluate the impact of the different forms of A β on calcium entry.

Statistical analyses

For statistical analyses, ANOVA followed by post-hoc analysis using Bonferroni were performed using SPSS for Windows release 10.1. Results are represented as Mean +/- SEM.

RESULTS:

Analysis and evaluation of the different soluble forms of A β 1-42:

FS, LMWO and HMWO A β 1-42 loaded on a 4-20% gradient PAGE under non-denaturing conditions revealed that HFIP treated FS A β displayed tetramers in addition to dimers on immunodetection, LMWO A β prepared by aging HFIP A β for 24h at 4°C was detected as a band at 20kd and HMWO A β prepared by aging HFIP A β for 5 days at 37°C revealed a large band comprising of the different forms of A β , more specifically the large oligomers >90kd. Under denaturing conditions (Tris/glycine/SDS), immunodetection with the monoclonal antibody 4G8 showed that FSA β formed a band at around 4kd, LMWO bands were detected at 20kd while HMWO formed a large band at around 70kd indicating that oligomers are SDS resistant (Fig.1).

Effect of HMWO, LMWO and FS A β 1-42 on calcium influx in human astrocytes and microglia:

Data show that HMWO, LMWO and FSA β 1-42 tested do not have any major impact on the release of calcium by internal stores following one minute of treatment in human astrocytes and microglia in calcium-free HBSS (Fig. 2a, 2c). However, following addition of extracellular calcium in microglia and astrocytes, calcium entry was differentially stimulated by HMWO, LMWO and FS A β 1-42. Interestingly, HMWO and LMWO of A β 1-42 appear more potent than FSA β . at stimulating calcium entry in astrocytes and microglia. Mean peak amplitude of calcium entry was quantified for the three forms of A β 1-42 (Fig. 2b, 2d).

Effect of HMWO, LMWO and FS A β 1-42 on the production of IL-6 and IL-8 in human astrocytes and microglia:

Levels of IL-6 and IL-8 were quantified in media collected from human astrocytes and microglia following 24h treatment with HMWO, LMWO and FS A β 1-42. Pro-inflammatory cytokines IL-6 and IL-8 were elevated in both astrocytes and microglia following treatment with the HMWO as compared to control and FSA β (Fig.3a-3d). Levels of IL-6 and IL-8 in microglia were slightly increased by LMWO compared to control and FSA β (Fig. 3b, 3d). IL-8 levels in astrocytes were slightly increased by LMWO as compared to control and FSA β (Fig 3c).

Effect of A23187 and BAPTA-AM on the release of IL-6 and IL-8 by human astrocytes and microglia:

The calcium ionophore A23187 significantly increased IL-6 and IL-8 levels, whereas the intracellular calcium chelator BAPTA-AM reduced basal cytokine production of IL-6 and IL-8 in both astrocytes and microglia (Fig 4a-4d) showing that the production of IL-6 and IL-8 by microglia and astrocytes is dependent on intracellular calcium levels.

Effect of HMWO A β 1-42 on IL-6 and IL-8 production by human astrocytes and microglia after co-treatment with the other calcium signaling inhibitor and Nilvadipine, the L-type calcium channel blocker:

After establishing that the HMWO A β 1-42 is most potent in stimulating both IL-6 and IL-8 in astrocytes and microglia, we wanted to determine if Nilvadipine (the L-type

calcium channel blocker), U73122 (a PLC inhibitor) and KN62 (a calmodulin kinase inhibitor) could inhibit or reduce levels of these pro-inflammatory molecules stimulated by 1 μ M HMWO A β 1-42. My results demonstrated that 5 μ M Nilvadipine significantly reduces the production of both IL-6 and IL-8 mediated by 1 μ M HMWO A β in both astrocytes and microglia (Fig.5a-5d), suggesting that A β mediates the production of IL-6 and IL-8 in astrocytes and microglia by stimulating calcium entry via L-type calcium channels. My results also revealed a significant reduction in both IL-6 and IL-8 in astrocytes and microglia following treatment with 10 μ M U73122 and 5 μ M KN62 (Fig. 5a-5d), strongly suggesting that compounds affecting calcium signaling pathways can have anti-inflammatory properties.

DISCUSSION:

Activation of glia resulting in the production of pro-inflammatory cytokines is a feature observed in mouse models of AD and in pathological tissue samples from AD patients (Tarkowski et al. 2003, Patel et al. 2005). My previous studies with brain slices from TgAPPsw mice reveal that A β causes an increase in pro-inflammatory cytokines (Quadros 2003). Several studies have shown that dysregulation of calcium homeostasis and/or increases in intracellular levels of calcium are also responsible for the activation of glia and in the propagation of inflammatory reactions in the CNS (Hoffmann et al. 2003, Mattson and Chan 2003). Evidence from various studies reveal that intracellular calcium stimulates the release of pro-inflammatory cytokine IL-6 and chemokine IL-8 in human mast cells via activation of NF κ B (Kim et al. 2005, Mitsuyama et al. 2004, Kempuraj et

al. 2003). Studies in human microglia treated with IL-8 reveal that it enhances A β 1-42 to release other pro-inflammatory cytokines and increases the activity of COX-2 resulting in the production of eicosanoids (Franciosi et al. 2005). My aim was to determine which particular forms of A β 1-42 differentially affect calcium influx in glial cells and to determine to what extent this increase of calcium influx in glial cells mediates the production of the major proinflammatory cytokines IL-6 and IL-8. I observed that HMWO A β 1-42 is more potent than FS or LMWO at increasing calcium influx and cytokine production in both astrocytes and microglia. To further demonstrate that A β induced increases in pro-inflammatory interleukins IL-6 and IL-8 was dependent on increased intracellular levels of calcium, I used BAPTA-AM, an intracellular calcium chelator. My results demonstrated that the intracellular calcium chelator BAPTA-AM can oppose cytokine production triggered by HMWO and LMWO A β 1-42 suggesting that calcium increases induced by A β is necessary to promote the proinflammatory action of A β . Interestingly, I also observed that FSA β 1-42 was not a potent stimulus for increasing pro-inflammatory levels of IL-6 and IL-8 and was also not able to increase intracellular calcium levels.

In addition, both astrocytes and microglia produced elevated levels of both IL-6 and IL-8 in response to the calcium ionophore A23187. The elevated levels of IL-6 and IL-8 were reduced by treatment with Nilvadipine, U73122, and KN62. Studies on rat astrocytes have revealed that PLC stimulates the production of IL-6 in response to endothelins (Morga et al. 2000). Other studies on astrocytes have shown that a pro-inflammatory stimulus can induce the production of IL-6 via activation of the NF κ B by

calmodulin kinase enzyme (Schwaninger et al. 1999). Therefore, my data in glial cells treated with oligomeric forms of A β suggest that blocking calcium entry either by blocking L-type channels or by inhibiting PLC (which regulates intracellular calcium stores) or by inhibiting calmodulin kinase may be of interest for preventing glial activation in AD patients. Several reports indicate that calcium channel blockers used for the treatment of hypertension can lower the risk of developing dementia, including AD (Forette et al. 2002, Menne et al. 2006). Studies indicate that the brain loses its ability to downregulate intracellular levels of calcium with age (Toescu et al. 2004) and the reduced risk of dementia associated with calcium channel blockers is presumed to be related to their ability to prevent excess entry of calcium into neurons (Trompet et al. 2006). The relationship between glial activation and neurodegeneration remains unclear, although several cytokines and inflammatory mediators produced by activated microglia and astrocytes have the potential to initiate or exacerbate the progression of neuropathology in several CNS disorders. Hence, my data suggest that changes in intracellular levels of calcium mediated by oligomeric forms of A β 1-42 (mainly HMWO) may be responsible for the activation of glial cells in AD. Neuroinflammation is a prominent feature of AD pathology as evidenced by astrogliosis and microgliosis reported in various studies (Akiyama et al. 1999, Akiyama et al. 2000). Recent studies using primary cultures of human astrocytes demonstrate that A β mediated increases in NF κ B activity further stimulate inflammation, activating glial cells resulting in the production of more A β via activation of BACE 1 enzyme (Bourne et al. 2007). The reason HMWO A β are more potent than LMWO and FSA β in stimulating calcium influx

and cytokine release may be due to the fact that HMWO A β contains more β -sheet than the other two forms. Studies on neurons have indicated that neurotoxicity mediated by A β is related to the β -sheet content of the peptide (Watson et al. 2005). Our results indicate that the inflammation mediated by HMWO A β is caused by an increase in intracellular calcium levels, which can be blocked by using Nilvadipine. Calmodulin kinase inhibitors decrease the production of IL-6 and IL-8 in astrocytes and microglia by inhibiting intracellular calcium signaling. Our results strongly suggest that compounds that modulate calcium influx in glial cells decrease inflammation and therefore can be neuroprotective. Further studies on human neuronal cells need to be performed to determine the effect of these calcium channel blockers on HMWO A β 1-42 mediated neurotoxicity.

List of abbreviations:

CNS: Central Nervous system

AD: Alzheimer's disease

A β : beta amyloid

ELISA: Enzyme linked immunosorbent assay

IL-x: Interleukin-x

HBSS: Hank's balanced salt solution

NF κ B: Nuclear factor kappa B

CaCl₂: Calcium chloride

BAPTA: 1, 2-Bis (2-aminophenoxy) ethane-*N,N,N',N'*-tetraacetic acid

PIP₂: phosphatidylinositol 4, 5-biphosphate

IP3: inositol 1, 4, 5- triphosphate

Competing interests:

The authors declare that they have no competing interests.

Author's contributions:

AQ carried out the Bio-plex assay, performed the calcium entry experiments and drafted the manuscript. NP aided in calcium entry assays and treatment of the of the primary cell cultures. BB and ML aided in growing of the primary cells, collecting media for the Bioplex assays. MM aided in manuscript preparation and gave critical analysis of the manuscript. DP conceived and developed the methodology to perform calcium measurements and aided in manuscript preparation.

Acknowledgments:

The authors would like to thank Bob and Diane Roskamp for their generous support.

Figure 5:

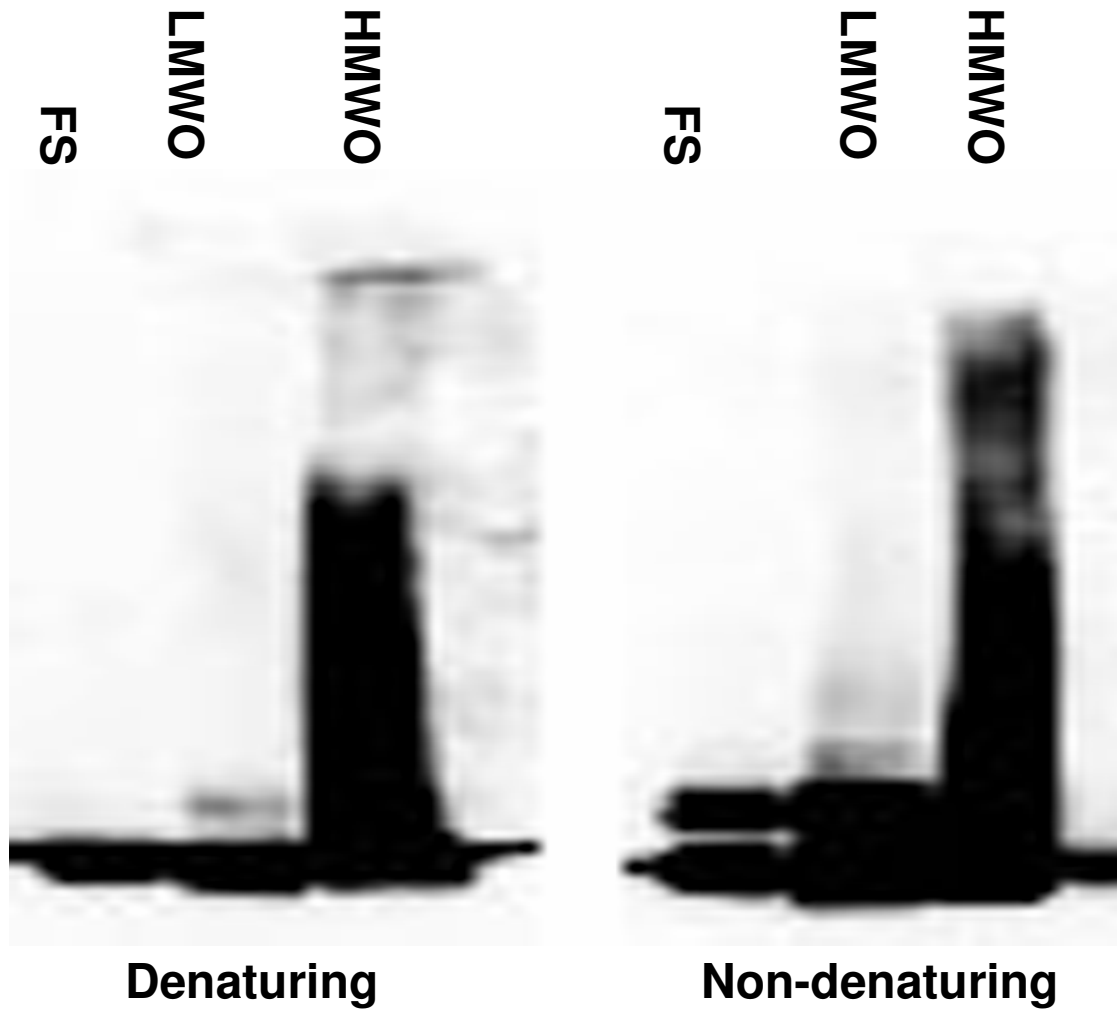


Fig. 5: Western blot of A β 1-42 using 4G8 monoclonal antibody under denaturing and non-denaturing conditions. FS, LMWO and HMWO A β 1-42 loaded on a 4-20% gradient PAGE under denaturing conditions (Tris/glycine/SDS) followed by electroblotting on PVDF and immunodetection with the monoclonal antibody 4G8 showed that FSA β formed a band at around 4kd, LMWO bands were detected at 20kd while HMWO formed a large band at around 70kd.

Figure 6a: Astrocytes

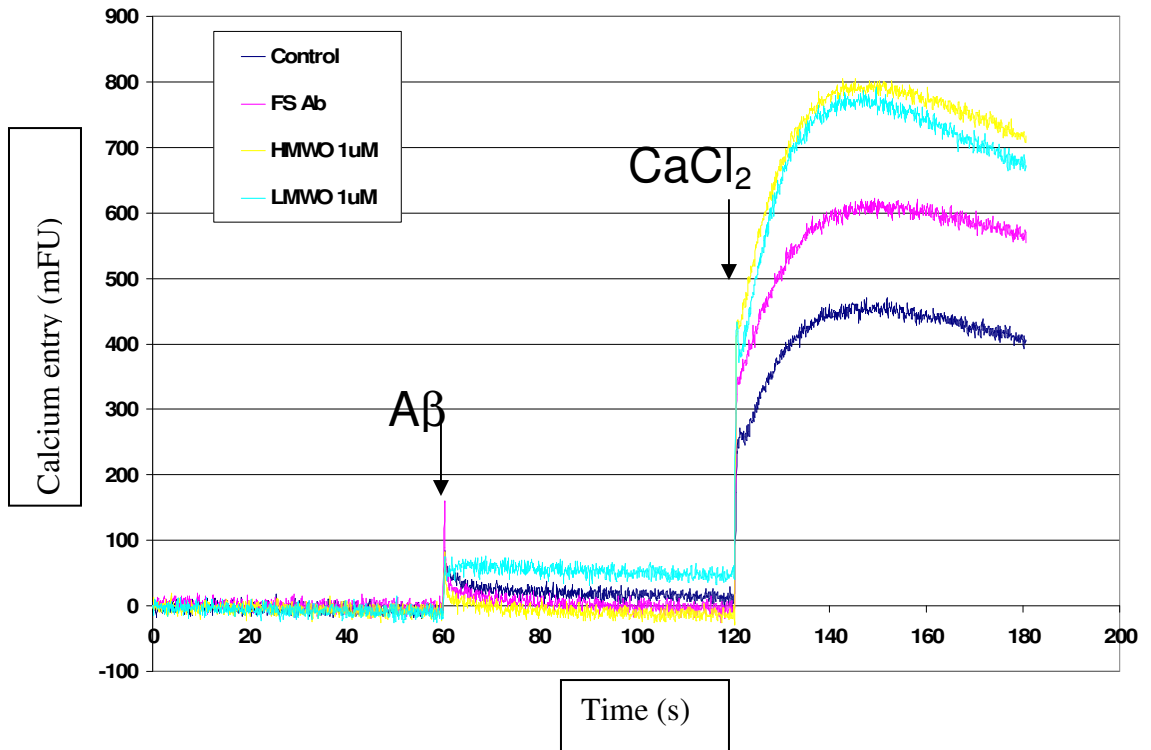


Figure 6b: Astrocytes

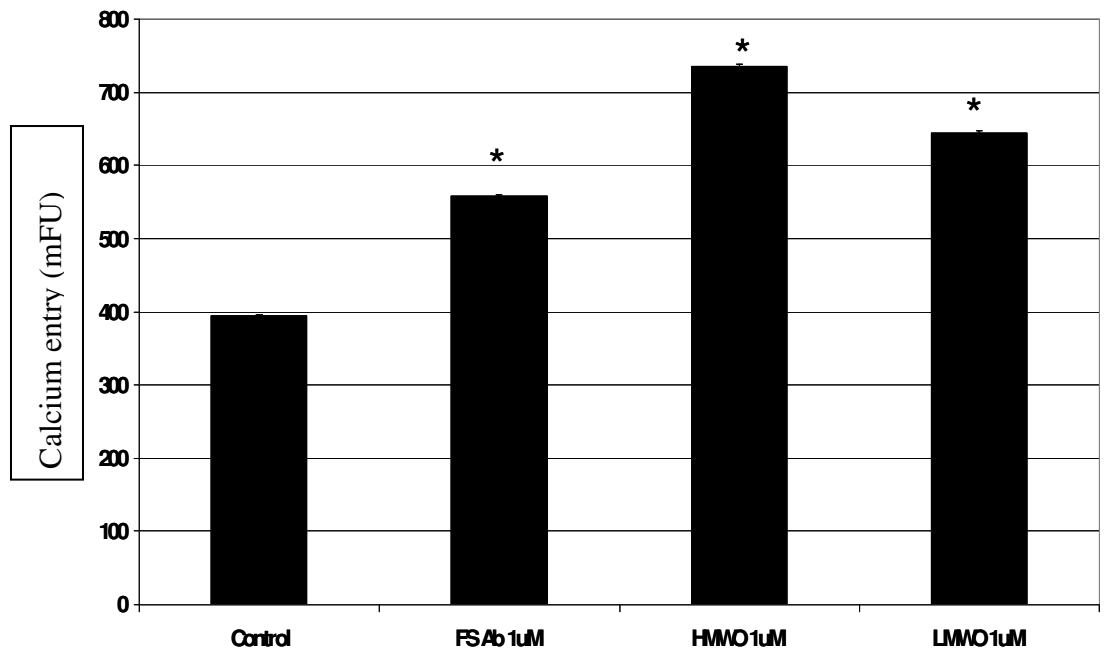


Figure 6c: Microglia

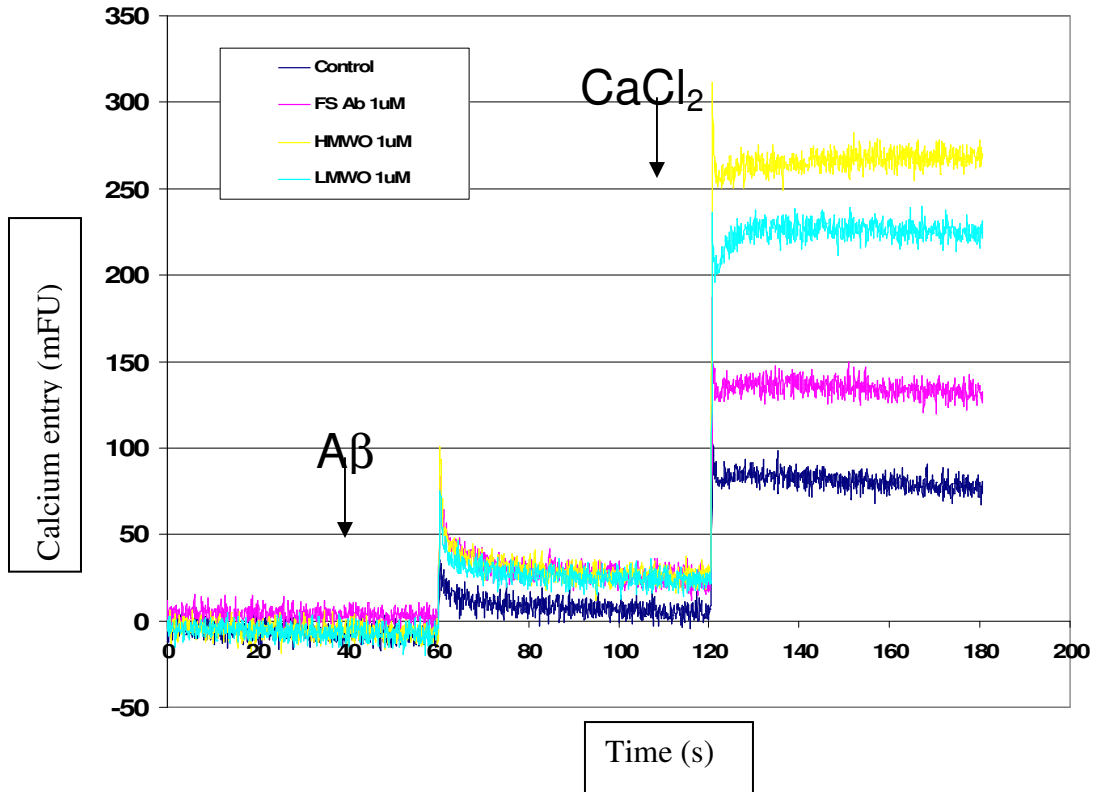


Figure 6d: Microglia

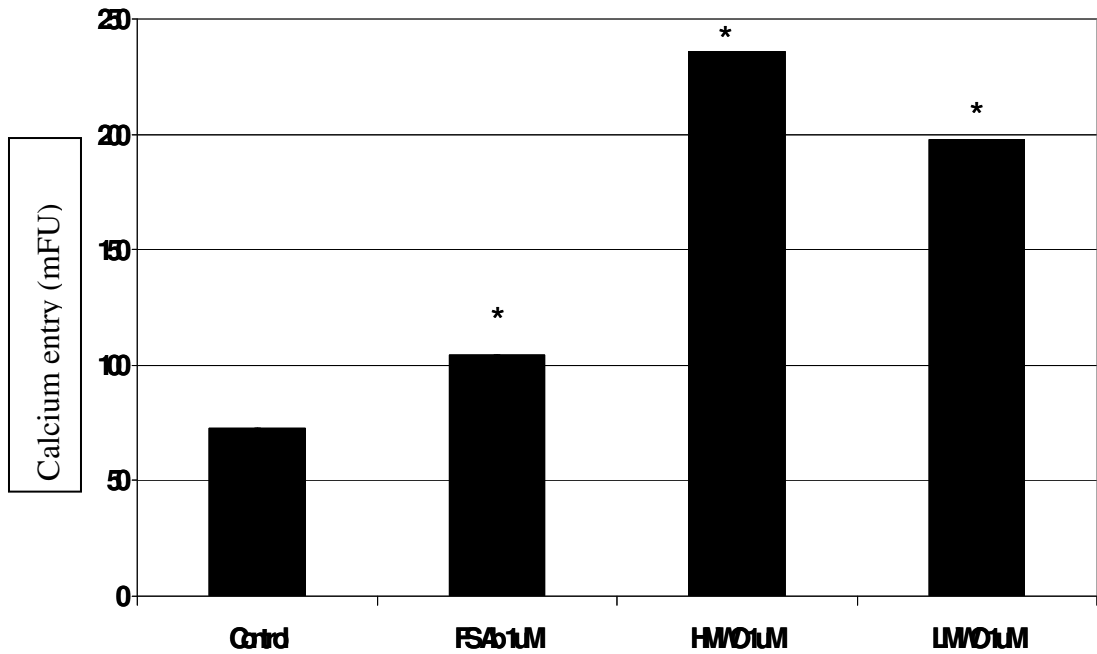


Fig. 6: a) Real time measurement of calcium entry in human astrocytes following treatment with FS, LMWO and HMWO A β 1-42 at 1 μ M dose (n=6 for each treatment group). b) Quantification of intracellular calcium entry in human astrocytes. ANOVA revealed significant main effects between control and HMWO, control and LMWO and control and FSA β in astrocytes. Post-hoc analysis by Bonferroni showed significant effect of all three treatment groups (p<0.05). c) Real time measurement of calcium entry in human microglia following treatment with FS, LMWO and HMWO A β 1-42 at 1 μ M dose (n=6 for each treatment group). d) Quantification of intracellular calcium entry in human microglia. ANOVA followed by post-hoc analysis showed significant main effects between control and HMWO, control and LMWO (p<0.05) but no significant difference between control and FSA β in microglia (p>0.05).

Figure 7a: Astrocytes

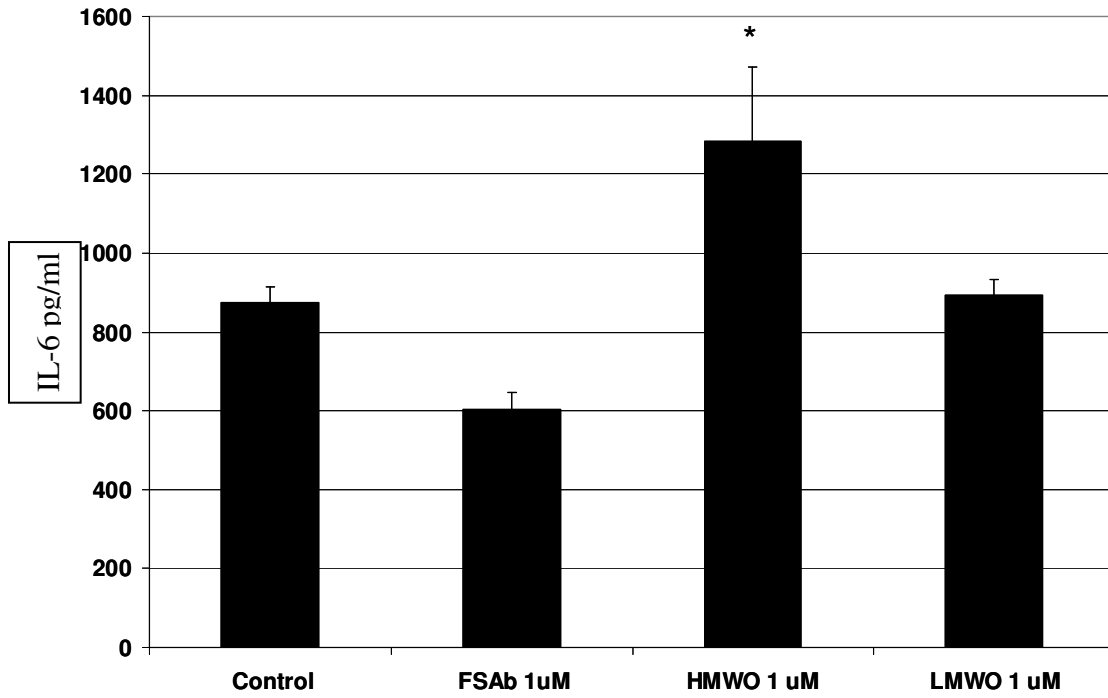


Figure 7b: Microglia

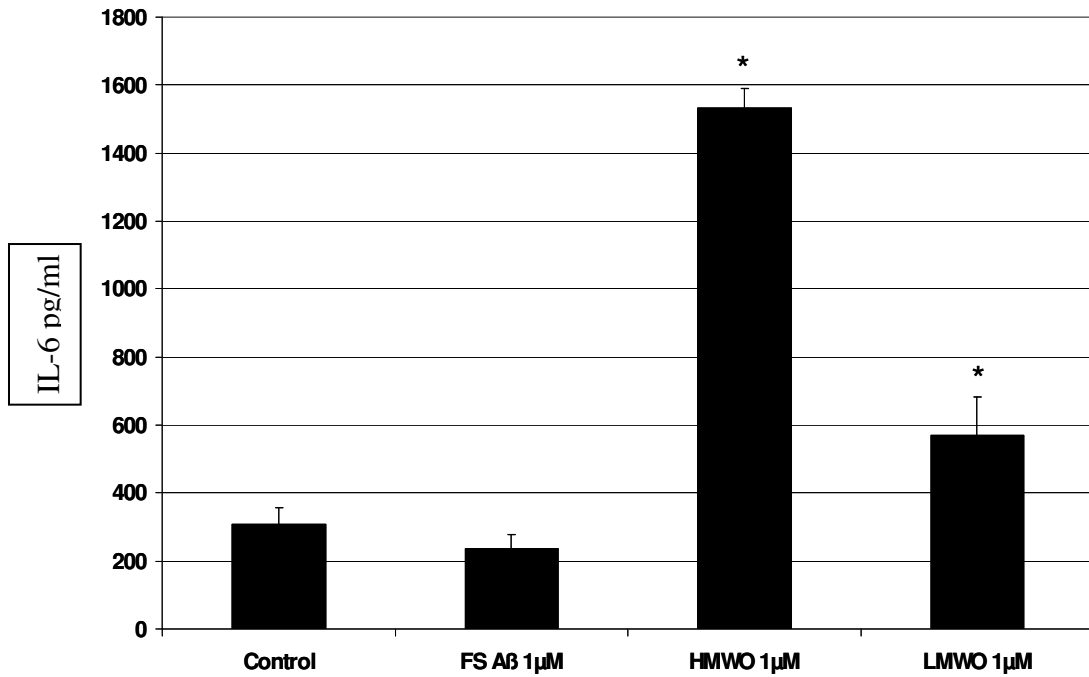


Figure 7c: Astrocytes

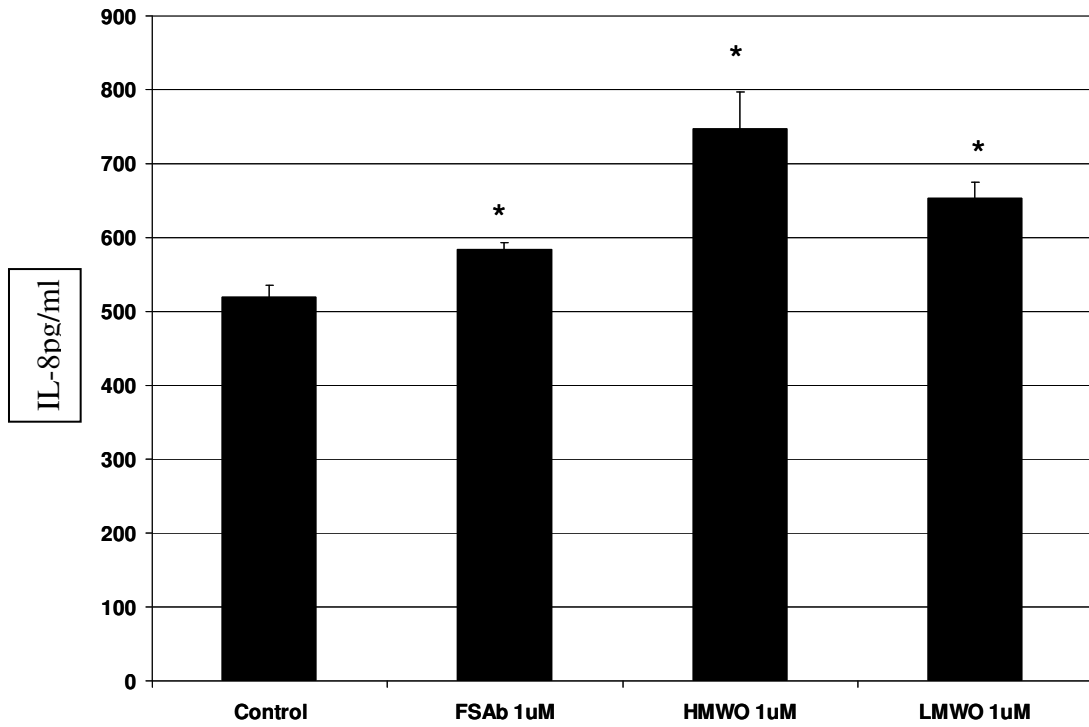


Figure 7d: Microglia

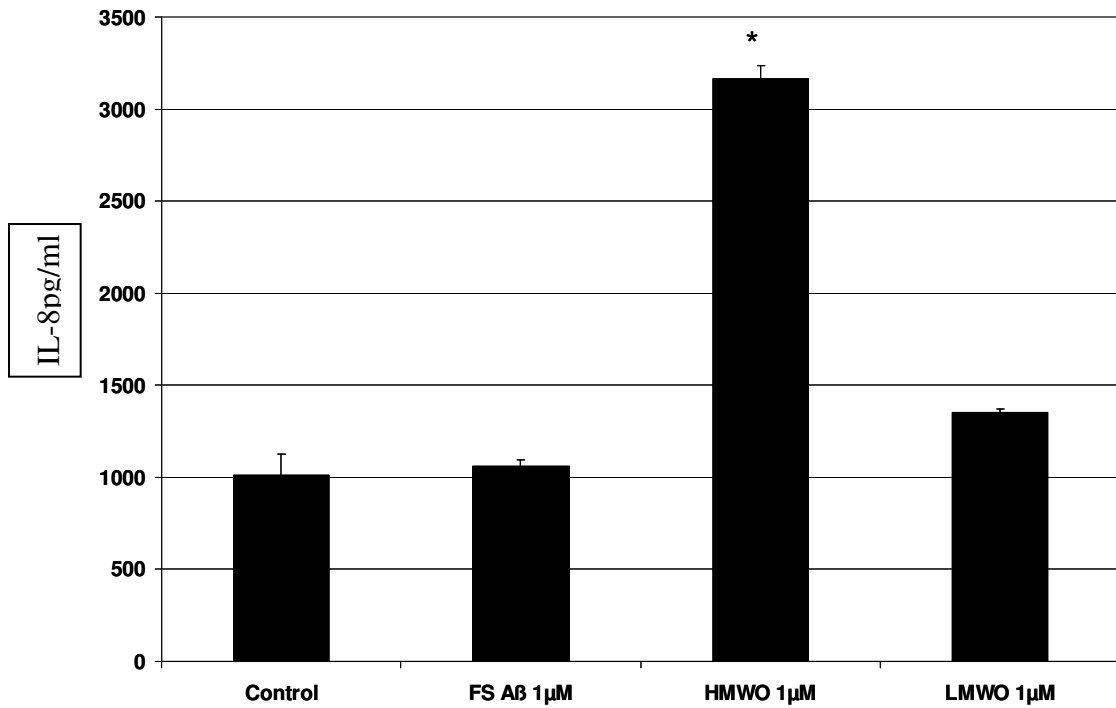


Fig. 7: a) Bar graph of IL-6 levels in media after 24h following treatment with FS, LMWO and HMWO A β 1-42 at 1 μ M dose (n=6 for each treatment group) in astrocytes. ANOVA followed by post-hoc analysis revealed significant main effects between control and HMWO (p<0.05) but no significant difference between control and LMWO (p>0.05). b) Bar graph of IL-6 levels in media after 24h following treatment with FS, LMWO and HMWO A β 1-42 at 1 μ M dose (n=6 for each treatment group) in microglia. ANOVA followed by post-hoc analysis revealed significant main effects between control and HMWO (p<0.05) but no significant difference between control and LMWO or FSA β (p>0.05). c) Bar graph of IL-8 levels in media after 24h following treatment with FS, LMWO and HMWO A β 1-42 at 1 μ M dose (n=6 for each treatment group) in astrocytes. ANOVA followed by post-hoc analysis revealed significant main effects between control and HMWO (p<0.05) but no significant difference between control and LMWO, and control or FSA β (p>0.05). d) Bar graph of IL-8 levels in media after 24h following treatment with FS, LMWO and HMWO A β 1-42 at 1 μ M dose (n=6 for each treatment group) in microglia. ANOVA followed by post-hoc analysis revealed significant main effects between control and HMWO (p<0.01) but no significant difference between control and LMWO or FSA β (p>0.01).

Figure 8a: Astrocytes

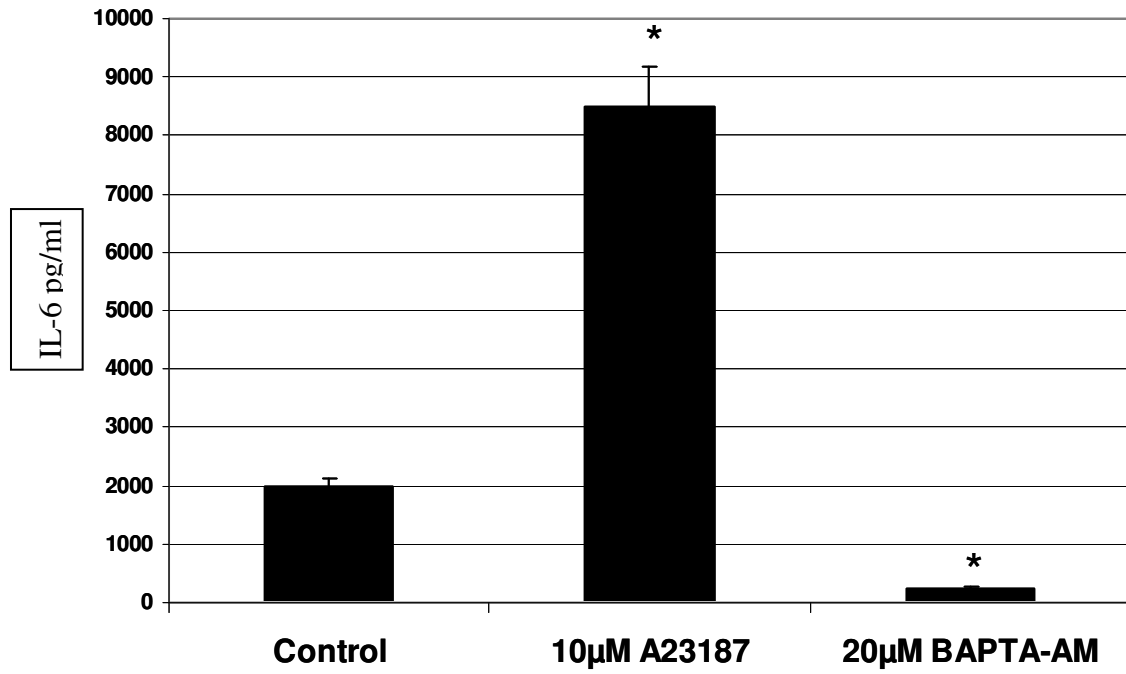


Figure 8b: Microglia

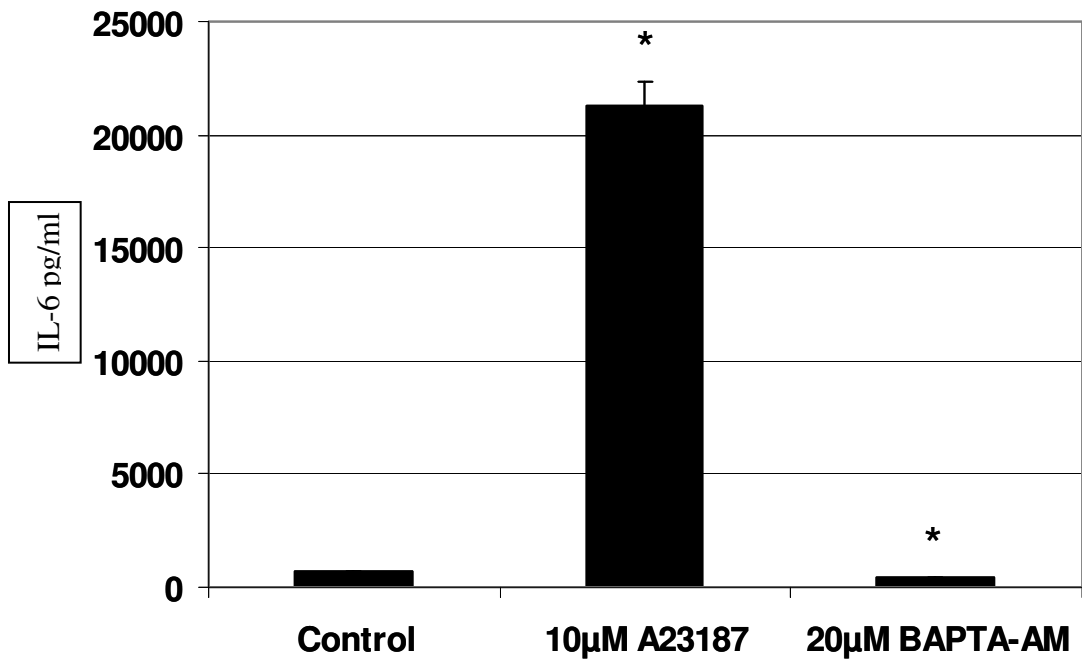


Figure 8c: Astrocytes

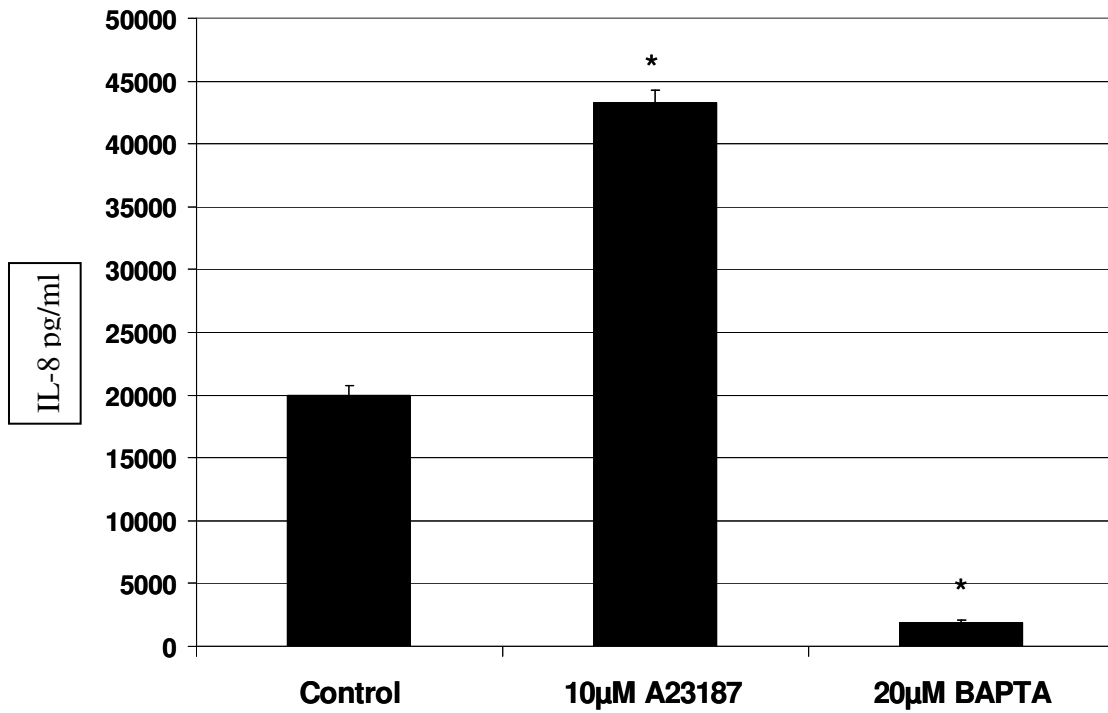


Figure 8d: Microglia

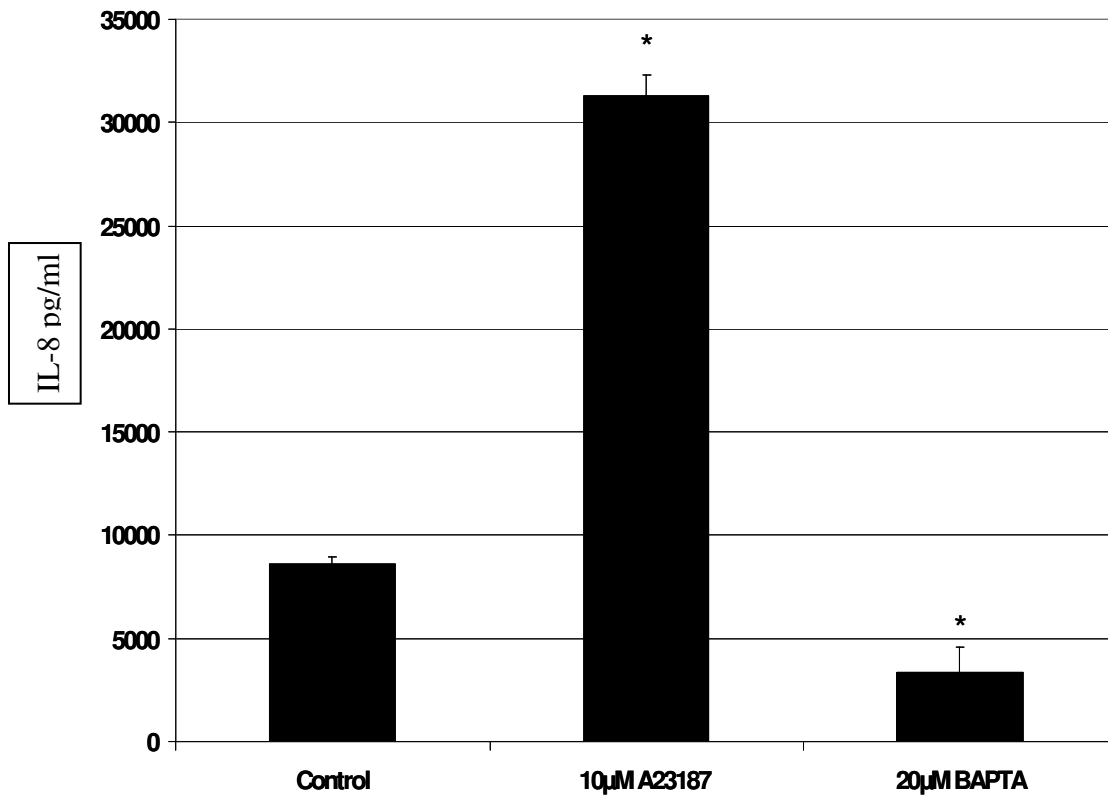


Fig. 8: a & c) Bar graph of IL-6 and IL-8 levels in media after 24h following treatment with 10 μ M A23187, 20 μ M BAPTA-AM and control (untreated cells) in astrocytes (n=6 for each treatment group). ANOVA followed by post-hoc analysis revealed significant main effects between control and A23187 (p<0.05), control and BAPTA-AM (p<0.05) and A23187 and BAPTA-AM (p<0.05) for IL-6 and IL-8. b& d) Bar graph of IL-6 and IL-8 levels in media after 24h following treatment with 10 μ M A23187, 20 μ M BAPTA-AM and control (untreated cells) in microglia (n=6 for each treatment group). ANOVA followed by post-hoc analysis revealed significant main effects between control and A23187 (p<0.05), control and BAPTA-AM (p<0.05), A23187 and BAPTA-AM (p<0.05) for both IL-6 and IL-8.

Figure 9a: Astrocytes

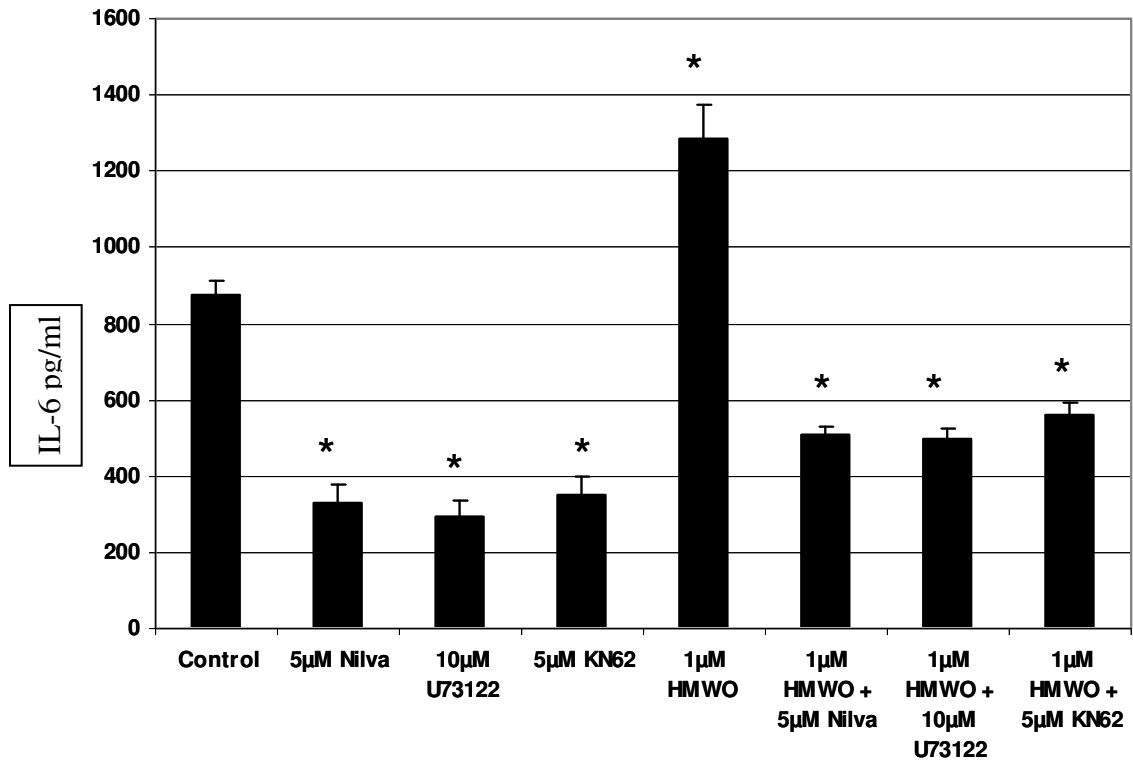


Figure 9b: Microglia

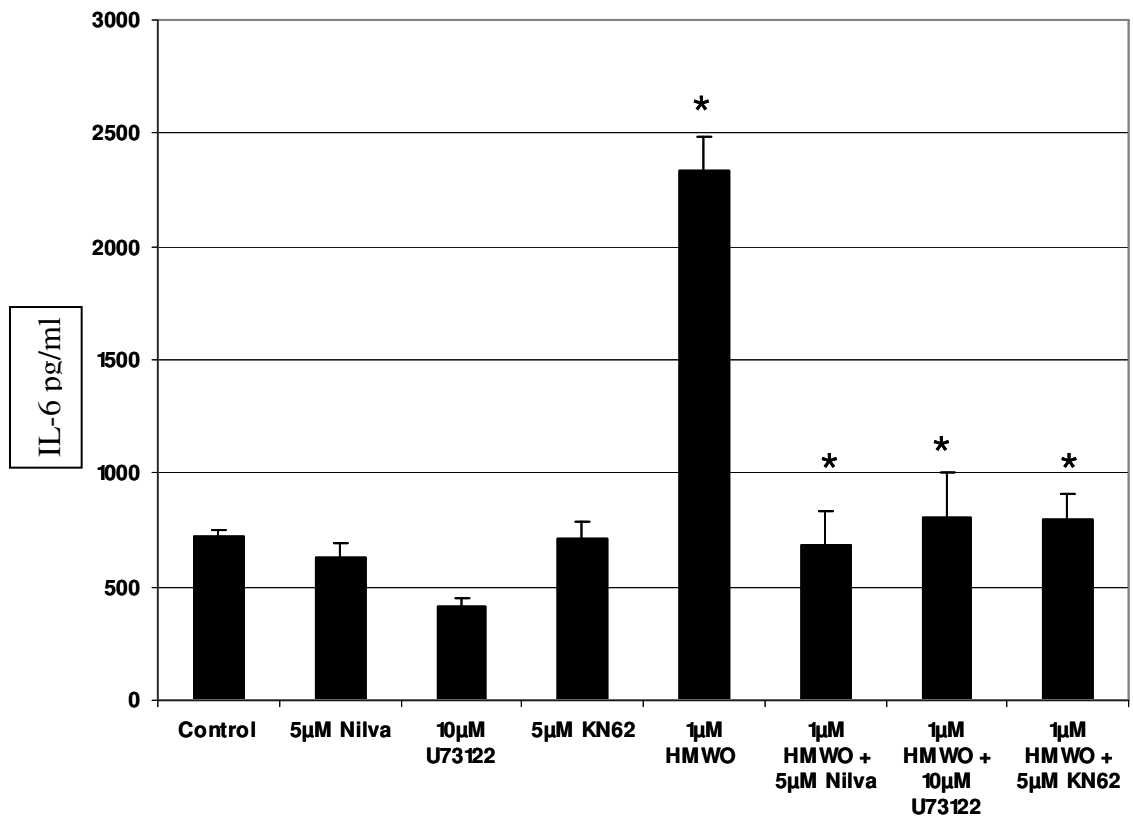


Figure 9c: Astrocytes

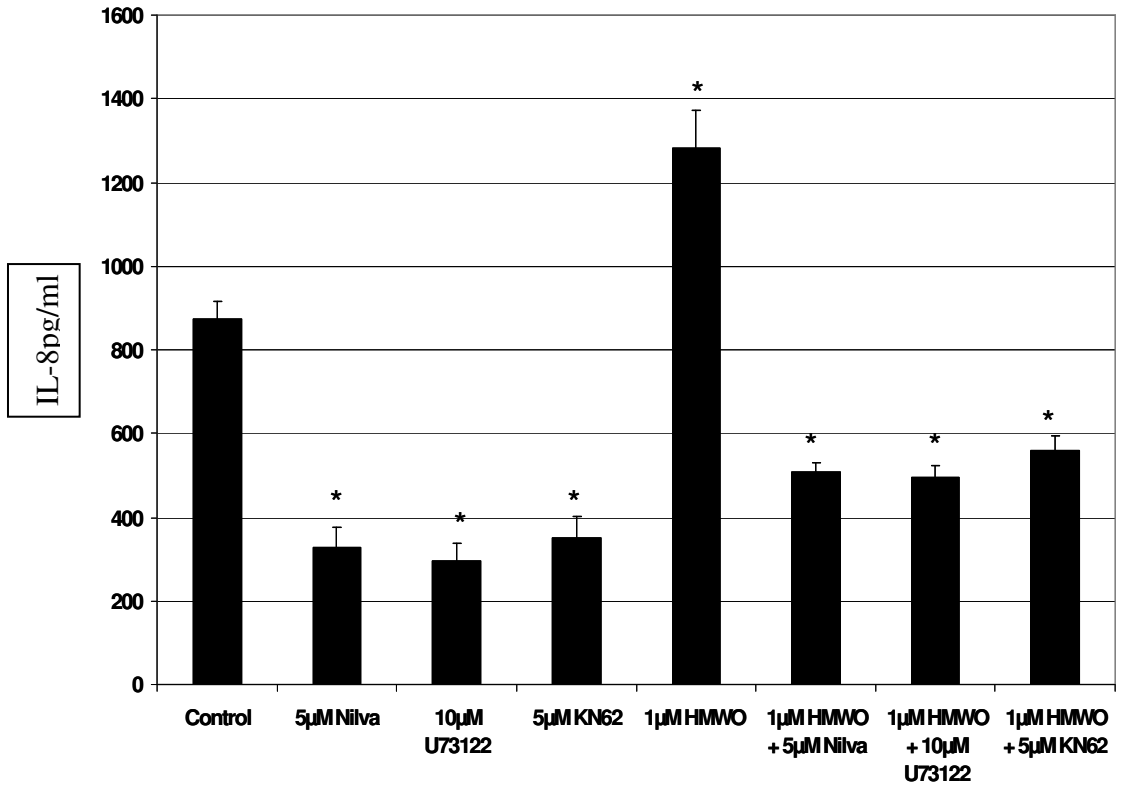


Figure 9d: Microglia

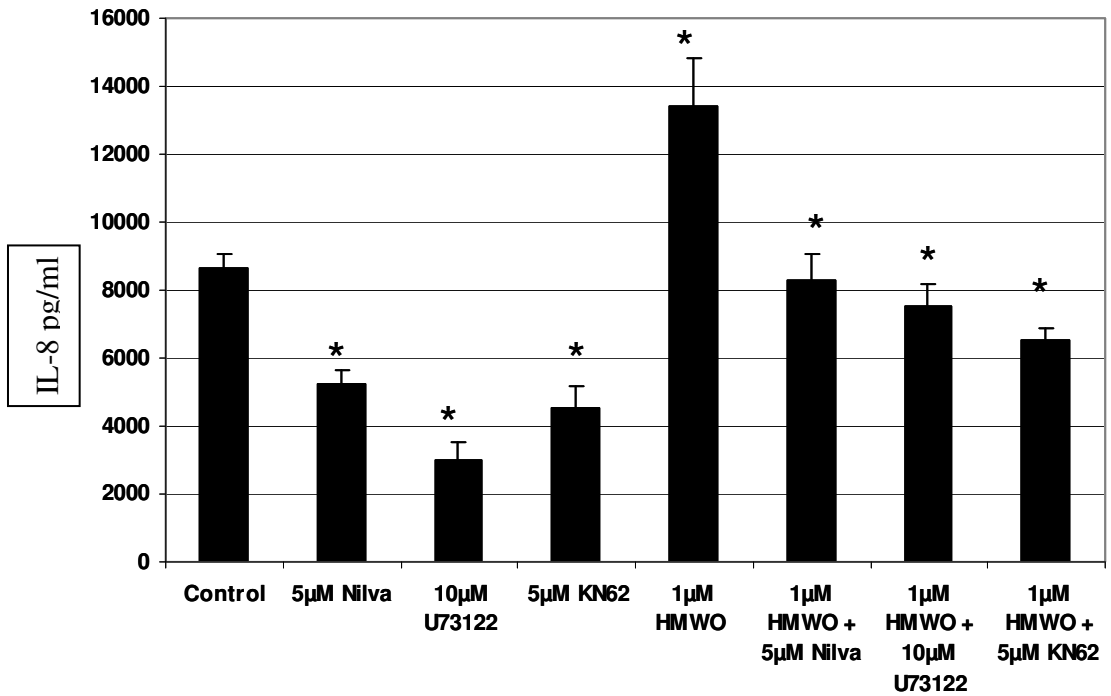


Fig. 9: a & c) Bar graph of IL-6 and IL-8 levels in media after 24h following treatment with HMWO A β 1-42 at 1 μ M, 5 μ M Nilvadipine alone, 10 μ M U73122 alone, 5 μ M KN62 alone and in combination respectively (n=6 for each treatment group) in astrocytes. ANOVA followed by post-hoc analysis revealed significant main effects between control and HMWO (p<0.05), HMWO and HMWO + Nilva (p<0.01), HMWO and HMWO + U73122 (p<0.01), HMWO and HMWO + KN62 (p<0.01) for both IL-6 and IL-8. b& d) Bar graph of IL-6 and IL-8 levels in media after 24h following treatment with HMWO A β 1-42 at 1 μ M, 5 μ M Nilvadipine alone 10 μ M U73122 alone, 5 μ M KN62 alone and in combination respectively (n=6 for each treatment group) in microglia. ANOVA followed by post-hoc analysis revealed significant main effects between control and HMWO (p<0.05), HMWO and HMWO + Nilva (p<0.05), HMWO and HMWO + U73122 (p<0.05), HMWO and HMWO + KN62 (p<0.05) for IL-6 and IL-8.

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CHAPTER FOUR

Effect of calcium channel blockers on A β induced neurodegeneration in human neurons

ABSTRACT:

Abnormalities in calcium ion homeostasis have been linked to the neurotoxic effects of A β . My previous data with human astrocytes and microglia reveal that high molecular weight oligomers (HMWO) of A β 1-42 increased the release of pro-inflammatory cytokines by stimulating calcium entry via L-type calcium channels and phospholipase C activation. My aim was to identify if HMWO A β could also increase inflammation in human neuronal precursor cells (HNPCs) via modulation of calcium influx in HNPCs. In addition, I also wanted to determine the neurotoxic effect of HMWO A β and elucidate if this neurotoxic effect could be mitigated by using L-type calcium channel blockers and other inhibitors involved in the calcium signaling pathway such as calmodulin kinase II inhibitors. My data show that HMWO A β significantly increased inflammation and neurotoxicity by stimulating calcium entry by L-type calcium channels. My results further demonstrate that the pro-inflammatory and neurotoxic effect of HMWO A β can be blocked by using calmodulin kinase II inhibitors further suggesting that HMWO A β mediated inflammation and neurotoxicity is calcium dependent. Interestingly, I also observed reduction of calcium entry in HNPCs by anti-inflammatory agents suggesting that there is a link between inflammation and altered calcium influx resulting in subsequent neurodegeneration.

Keywords: human neuronal precursor cells, calcium channel blockers, calmodulin kinase II inhibitors, and inflammation

Alzheimer's disease (AD) which is characterized by abnormal accumulation of beta-amyloid (A β) peptide in the brain is thought to be responsible for the neurodegeneration associated with AD. However, the mechanisms that contribute to neuronal degeneration in AD are not fully understood. Altered calcium homeostasis is considered to be one of the mechanisms involved in the neurodegeneration associated with normal aging, AD and related disorders (Thibault 2007). Studies with hippocampal neurons treated with aggregated A β peptide undergo neurodegeneration by a mechanism involving an increase in intracellular calcium levels (Mattson 2007). In vitro studies on cultured neurons with the calcium ionophore A23187 indicated that calcium influx resulted in microtubule disruption due to altered tau phosphorylation (Mattson 1992). In our present study we evaluated which conformation of A β was most toxic to human neuronal precursor cells (HNPC). I have previously established that HMWO A β were most potent in increasing intracellular calcium influx in glial cells and this same conformation was also able to induce the production of proinflammatory IL-6 and IL-8 (Quadros 2007). Elevated levels of IL-6 and IL-8 are found in the brain of mild cognitive impaired (MCI) patients as well as traumatic brain injury (TBI) patients as compared to non-MCI and non-TBI subjects (Magaki 2007, Kushi 2003). Elevated levels of IL-6 have also been reported in AD patients (Nelson 2001). Hence, I wanted to determine whether HMWO A β were responsible for the disruption of neurons via inflammation through a mechanism involving an increase in intracellular levels of calcium. Based on our previous results with glial cells our hypothesis for the mechanism involved in HMWO A β mediated increase in calcium entry was that A β may be affecting the activity

of L-type calcium channels or other calcium signaling pathways (Figure 1). To support my hypothesis I used Nilvadipine (a selective L-type calcium channel blocker) and KN62 (a calmodulin kinase II inhibitor). My rationale for using KN62 was because calmodulin kinase II is activated when intracellular calcium levels are elevated. I also wanted to determine if there was a link between altered levels of intracellular calcium and inflammation, so we treated HNPCs with Ibuprofen (a NSAID) and NS-398 (a selective COX-2 inhibitor).

EXPERIMENTAL PROCEDURES:

Preparation of the different forms of A β 1-42:

The different conformations of A β 1–42 peptide were prepared as per the protocol listed in the previous chapter.

Cell culture of HNPC:

Primary culture of HNPCs (Clonexpress Inc., MD) was cultured in DMEM/F12 media (Invitrogen, CA) supplemented with 5% fetal bovine serum (Invitrogen, CA), 10ng/ml basic fibroblast growth factor (Sigma-Aldrich, MO), 10ng/ml epidermal growth factor (Sigma-Aldrich, MO), 1X neuronal cell supplement (Clonexpress Inc., MD) and 1X Penicillin-Streptomycin-Fungizone mixture (Cambrex Corp., NJ). The treatment groups included 1 μ M of freshly solubilized (FS), low molecular weight oligomers (LMWO), and HMWO forms of A β 1-42. The other treatments include 5 μ M Nilvadipine (Fujisawa, Japan), 5 μ M NS-398, 25 μ M Ibuprofen, 5 μ M KN62 (Sigma-Aldrich, MO) and the various combination treatments along with control (cells treated with vehicle only). The

cell culture plates were incubated at 37°C and media from these plates was collected after 24h of treatment and tested for cytokine profiling using human cytokine 2-plex panel kits (Bio-Rad, CA) according to the recommendation of the manufacturer.

Measurement of intracellular calcium levels:

HNPC's were grown on a poly-L-lysine coated black costar 96-well plate for 24 hrs. Confluent cells were treated with 5µM of Fluo-4 (Molecular Probes) in DMEM for 30 minutes at 37°C in a cell culture incubator. Cells were subsequently washed in DMEM followed by 3 washes in calcium-free HBSS in order to remove any unincorporated Fluo-4. Intracellular calcium levels were quantified using a Bio-tek spectrofluorometer equipped with two plate microinjectors (Bio-tek Inc., VT) at specific excitation (485 nm) and emission (516 nm) wavelengths. Baseline intracellular calcium levels were continuously recorded for 1minute every 1/100 second. Immediately following treatment of the cells with 1µM FS, LMWO and HMWO Aβ1-42, intracytoplasmic calcium was continuously recorded for one minute to determine the impact of the different forms of Aβ on calcium release by intracellular calcium stores. Finally, following one minute of treatment with different forms of Aβ, 2mM of CaCl₂ was added extracellularly to the culture medium and intracellular calcium levels were continuously monitored in order to evaluate the impact of the different forms of Aβ on calcium entry.

Measurement of neurotoxicity induced by A β on HNPC:

HNPC cells were grown on a 24 well plate with the HMWO A β 1–42 alone and at a dose of 20 μ M and in combination with Nilvadipine 5 μ M, 5 μ M NS398, 25 μ M Ibuprofen, and 5 μ M KN62 over a period of 4 days. The neurotoxicity mediated by HMWO A β was monitored at different time points 24h, 48h 72h and 96h by using the Scion Image software at 10X and 20X magnification. Media was collected at the end of 96h and LDH was performed on the media and cell lysate to evaluate the level of toxicity mediated by the various conformations of A β 1-42.

Statistical analyses

For statistical analyses, ANOVA and t-tests were performed where appropriate using SPSS for Windows release 10.1. Results are represented as Mean +/- SEM. Post-hoc analysis was conducted using Bonferroni $p < 0.05$ or $p < 0.01$.

RESULTS:

Effect of FS, LMWO and HMWO A β 1–42 on the release of IL-6 and IL-8 in HNPCs:

Media from HNPC collected after 24h was used to measure the levels of IL-6 and IL-8 following treatment with 1 μ M FS, LMWO, and HMWO A β 1-42. Levels of both pro-inflammatory cytokines IL-6 and IL-8 were significantly elevated following treatment with the HMWO A β as compared to FS A β and LMWO A β (Fig 2a & 2b).

Effect of HMWO A β 1–42 on the release of IL-6 and IL-8 in HNPCs after co-treatment with calmodulin kinase II inhibitor and the L-type calcium channel blocker:

After establishing that HMWO A β 1-42 is most potent in stimulating both IL-6 and IL-8 in HNPCs, I wanted to determine if Nilvadipine (a selective L-type calcium channel blocker), and KN62 (a calmodulin kinase II inhibitor) could inhibit or reduce levels of these pro-inflammatory molecules stimulated by 1 μ M HMWO A β 1-42. My results demonstrated that 5 μ M Nilvadipine significantly reduces the production of both IL-6 and IL-8 mediated by 1 μ M HMWO A β in HNPCs (Fig.3a, 3b), suggesting that A β mediates the production of IL-6 and IL-8 in astrocytes and microglia by stimulating calcium entry via L-type calcium channels. My results also revealed a significant reduction in both IL-6 and IL-8 in astrocytes and microglia following treatment with 5 μ M KN62 (Fig. 3a, 3b), strongly suggesting that compounds affecting calcium entry and calcium signaling pathways can oppose the pro-inflammatory effect of HMWO A β on HNPCs.

Intracellular levels of calcium in HNPC following HMWO A β stimulation and in combination with Nilvadipine, Ibuprofen and NS398:

My results demonstrate that HNPCs showed an increase in intracellular calcium influx with 20 μ M HMWO A β 1-42 and this increase in calcium influx mediated by HMWO A β is blocked significantly by 5 μ M Nilvadipine (Fig. 4a & 4b), 5 μ M NS398 (Fig.5a & 5b) and 25 μ M Ibuprofen (Fig.6a & 6b) suggesting that inflammation stimulates calcium influx which can be blocked by using anti-inflammatory agents.

Effect of calcium antagonists and anti-inflammatory agents on HMWO A β 1-42 mediated neurotoxicity of HNPCs:

My results with HNPC's grown for a period of 4 days after treatment with 20 μ M HMWO A β and in conjunction with 5 μ M Nilvadipine, 5 μ M KN62, 5 μ M NS398 and 25 μ M Ibuprofen reveal that the neurodegeneration induced by HMWO A β was significantly reduced by 5 μ M Nilvadipine, 5 μ M KN62 (Fig. 7a), 5 μ M NS398, 25 μ M Ibuprofen (Fig. 7b) even after 96h. The LDH levels were measured after 96h and our results indicate that the amount of LDH released by the HMWO A β was significantly higher as compared to HNPCs co-treated with HMWO and Nilvadipine, KN62, NS-398, and Ibuprofen respectively (Fig. 8). My data suggest that these compounds may either have a direct neuroprotective effect or prevent neurodegeneration by reducing inflammation via modulation of intracellular calcium levels.

DISCUSSION:

Several studies suggest that A β may cause neurodegeneration in AD (Rasool 1986, Chiarini 2006). However, different conformations of A β have different effects on the pathophysiology of AD resulting in a cascade of events (Deshpande 2006, Standridge 2006). I used primary HNPCs to elucidate the effect of the various conformations of A β on neurotoxicity. My other aim was to determine if this neurotoxic effect of A β was mediated by an increase in intracellular calcium levels. My previous studies with human astrocytes and microglia revealed that HMWO and to some extent LMWO increased intracellular levels of calcium more than FS A β 1-42. This is supported by other studies

on hippocampal neurons revealing that A β 1-42 oligomers are more toxic to neurons than the fibrillar or monmeric/dimeric species (De Felice 2004). The oligomeric conformations significantly increased the release of pro-inflammatory interleukins IL-6 and IL-8 via modulation of calcium influx as previously seen in glial cells. Furthermore, my results with HNPC reveal that HMWO A β are toxic to neurons, and this neurotoxicity is mitigated by using 5 μ M Nilvadipine suggesting that HMWO A β increases the activity of L-type VGCC in neurons. The neurotoxic effect of HMWO A β was also blocked by using 5 μ M KN62 thereby suggesting that the neurotoxic effect is calcium dependent. It has been demonstrated that calmodulin kinase inhibitors reduce NF κ B activity in neurons (Lilienbaum 2003). My previous results in astrocytes and microglia treated with Nilvadipine and KN62, reveal that these compounds reduce the production of IL-6 and IL-8 induced by HMWO A β 1-42 (previous chapter). This suggests that the neuroprotective effect mediated by these compounds on HNPCs maybe due to their ability to decrease the pro-inflammatory effect of HMWO A β by modulating calcium entry. To further demonstrate a link between altered calcium levels and inflammation in mediating neurotoxicity of HNPCs I used two anti-inflammatory agents Ibuprofen and NS398 together with HMWO A β 1-42 and showed that even after 96h there was very little neurodegeneration (Fig. 7b). My results with these two anti-inflammatory agents on calcium influx indicated that they significantly reduced calcium entry in HNPCs following treatment with HMWO A β (Fig.5 and 6). Altogether my data suggest that elevated intracellular levels of calcium mediated by oligomeric forms of A β 1-42 promote the release of pro-inflammatory cytokines and chemokines in human neurons. This highly

elevated load of inflammatory cytokines and chemokines is responsible for the neurodegeneration associated in AD patients (Griffin 1998) and hence blocking inflammation by modulating intracellular levels of calcium could have potential therapeutic implications in various neuroinflammatory diseases including multiple sclerosis, Parkinson's disease, AIDS etc.

Figure 10: Proposed hypothesis for A β mediated neurotoxicity.

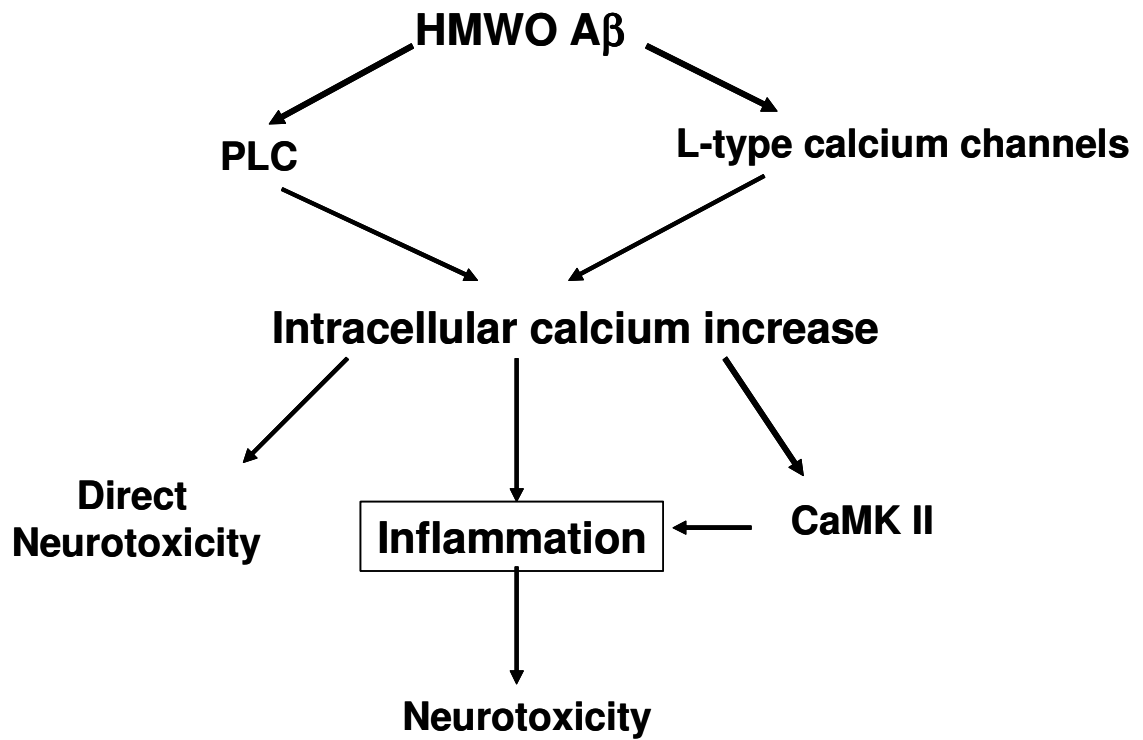


Figure 11a: IL-6 levels in HNPCs

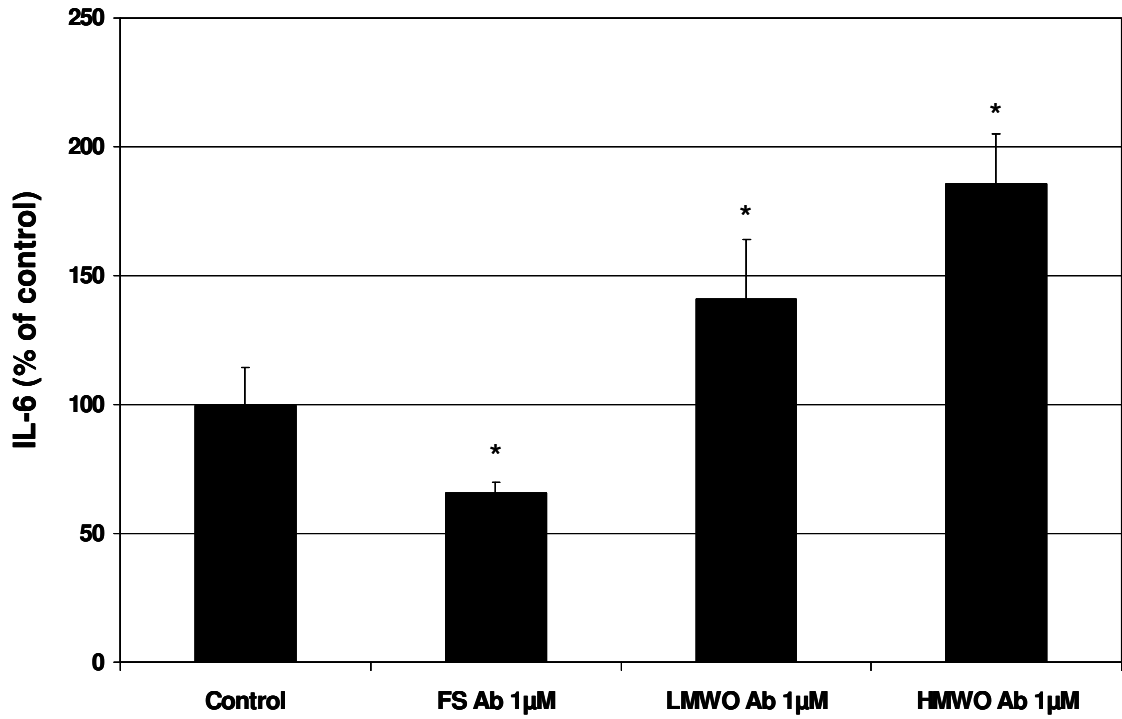


Figure 11b: IL-8 levels in HNPCs

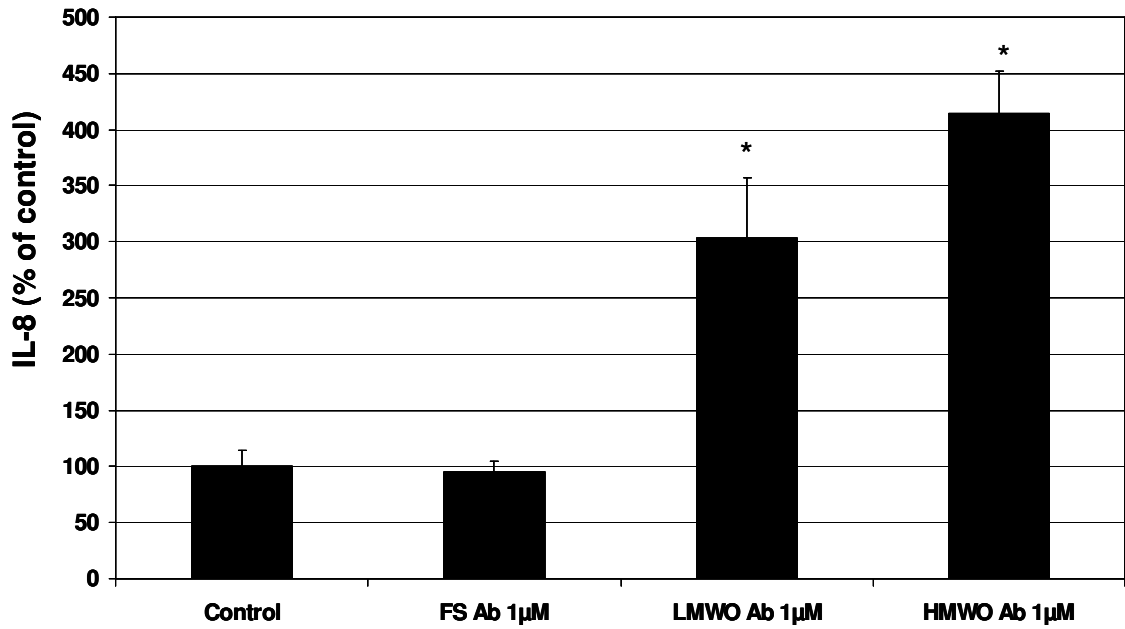


Fig. 11: a) Bar graph of IL-6 levels in media following 24h treatment with 1 μ M FS, LMWO, HMWO A β 1-42 and Control (n=6 for each treatment group) in HNPCs. ANOVA followed by post-hoc analysis revealed significant differences between control and HMWO (p<0.05), FSA β and HMWO (p<0.05), LMWO and HMWO (p<0.05).

b) Bar graph of IL-8 levels in media following 24h treatment with 1 μ M FS, LMWO, HMWO A β 1-42 and Control (n=6 for each treatment group) in HNPCs. ANOVA followed by post-hoc analysis revealed significant differences between control and HMWO (p<0.05), FSA β and HMWO (p<0.05), LMWO and HMWO (p<0.05).

Figure 12a: IL-6 levels in HNPCs

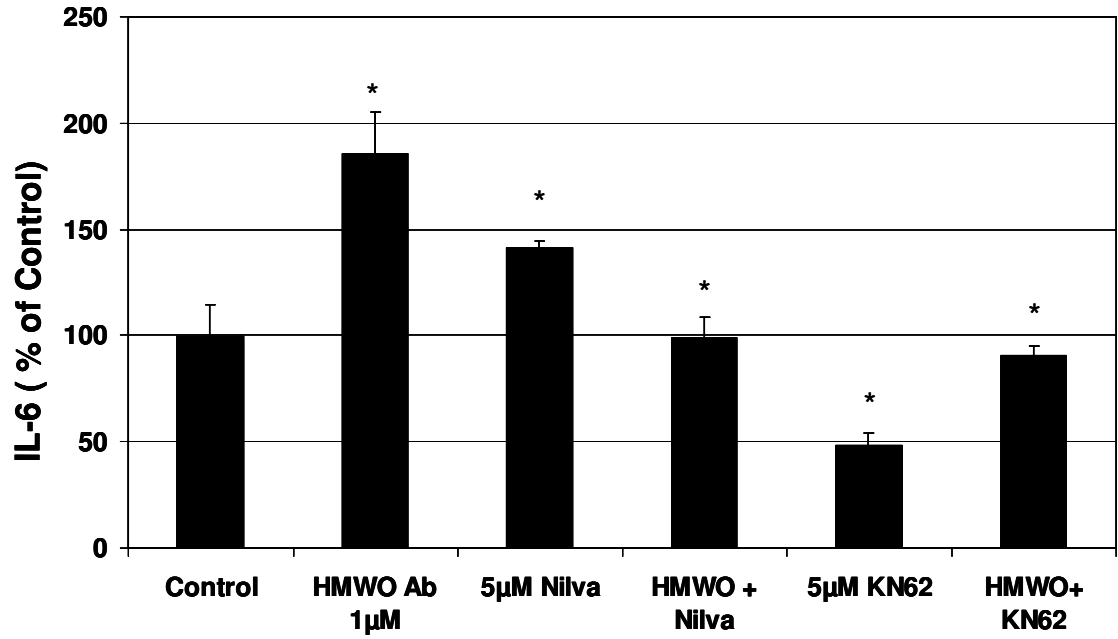


Figure 12b: IL-8 levels in HNPCs

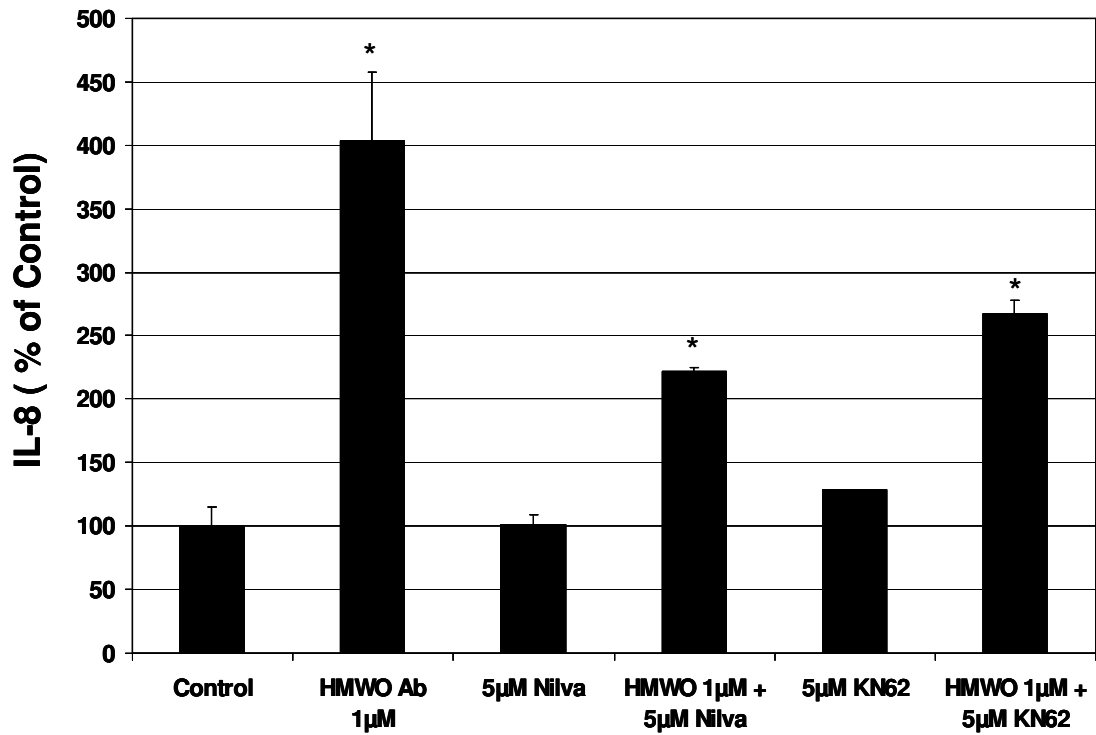


Fig. 12: a) Bar graph of IL-6 levels in media after 24h following treatment with 5 μ M Nilvadipine, 1 μ M HMWO A β 1-42, 1 μ M HMWO + 5 μ M Nilvadipine, 5 μ M KN62, and 1 μ M HMWO + 5 μ M KN62 and Control (n=6 for each treatment group) in HNPCs. ANOVA followed by post-hoc analysis revealed significant differences between control and HMWO, HMWO and HMWO + Nilvadipine ($p < 0.05$), HMWO and HMWO + KN62

b) Bar graph of IL-8 levels in media after 24h following treatment with 5 μ M Nilvadipine, 1 μ M HMWO A β 1-42, 1 μ M HMWO + 5 μ M Nilva, 5 μ M KN62, and 1 μ M HMWO + 5 μ M KN62 and Control (n=6 for each treatment group) in HNPCs. ANOVA followed by post-hoc analysis revealed significant differences between Control and HMWO, HMWO and HMWO + Nilvadipine, HMWO and HMWO + KN62 ($p < 0.05$).

Figures 13a & 13b: Calcium entry in HNPC following treatment with Nilva

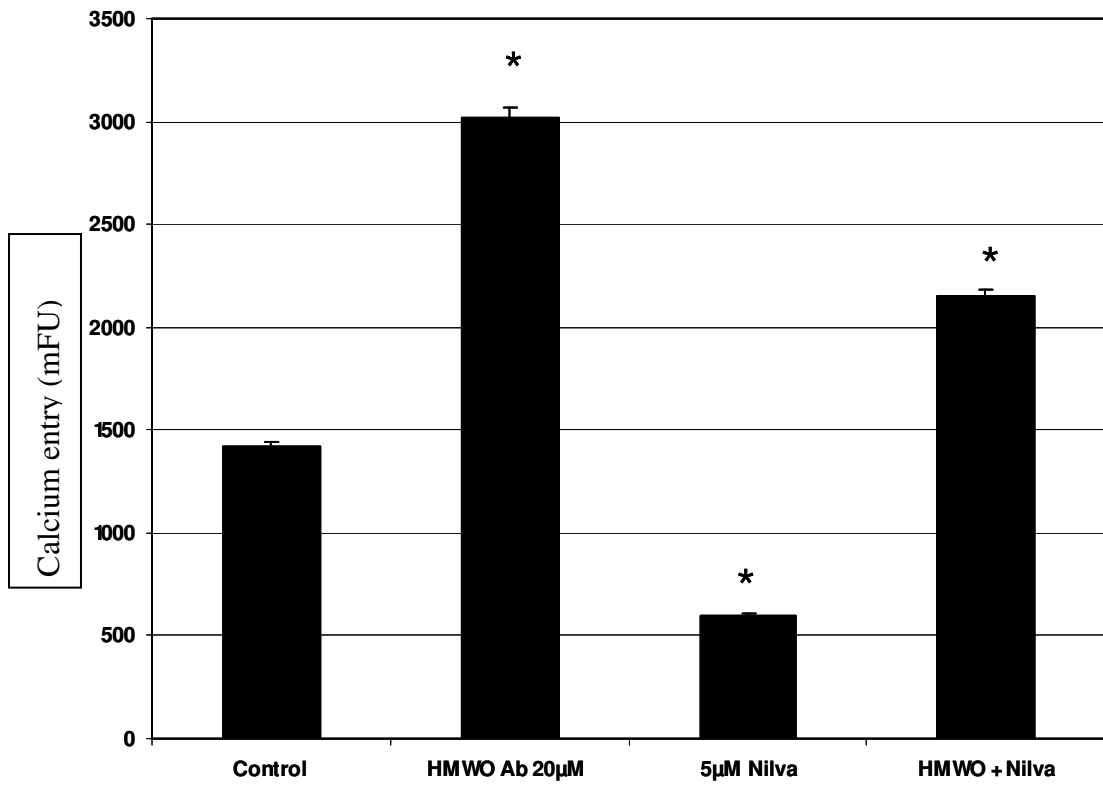
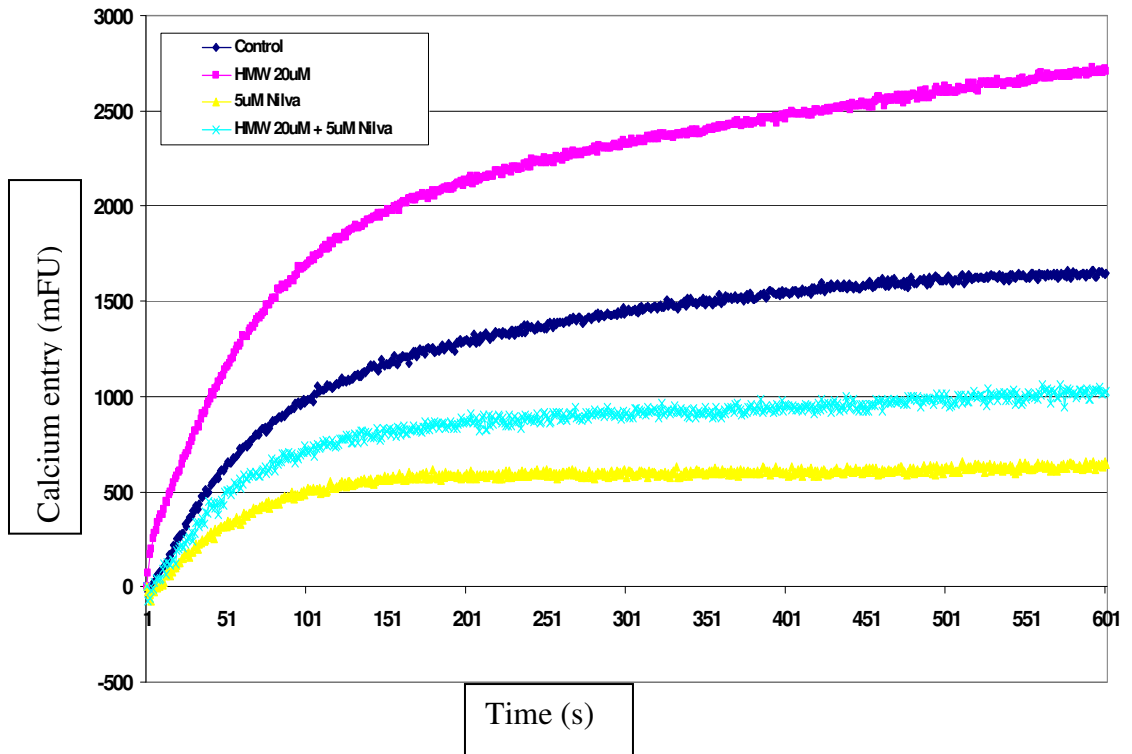


Fig. 13: a) Fluorometric measurement of calcium entry in human HNPCs following treatment with 20 μ M HMWO A β 1-42, 5 μ M Nilvadipine, HMWO + Nilvadipine (n=6 for each treatment group). b) Quantification of intracellular calcium entry in HNPCs following the above mentioned treatments. ANOVA followed by post-hoc analysis revealed significant differences between control and HMWO ($p<0.05$), HMWO and HMWO + Nilvadipine ($p<0.05$).

Figures 14a & 14b: Calcium entry in HNPC following treatment with NS-398

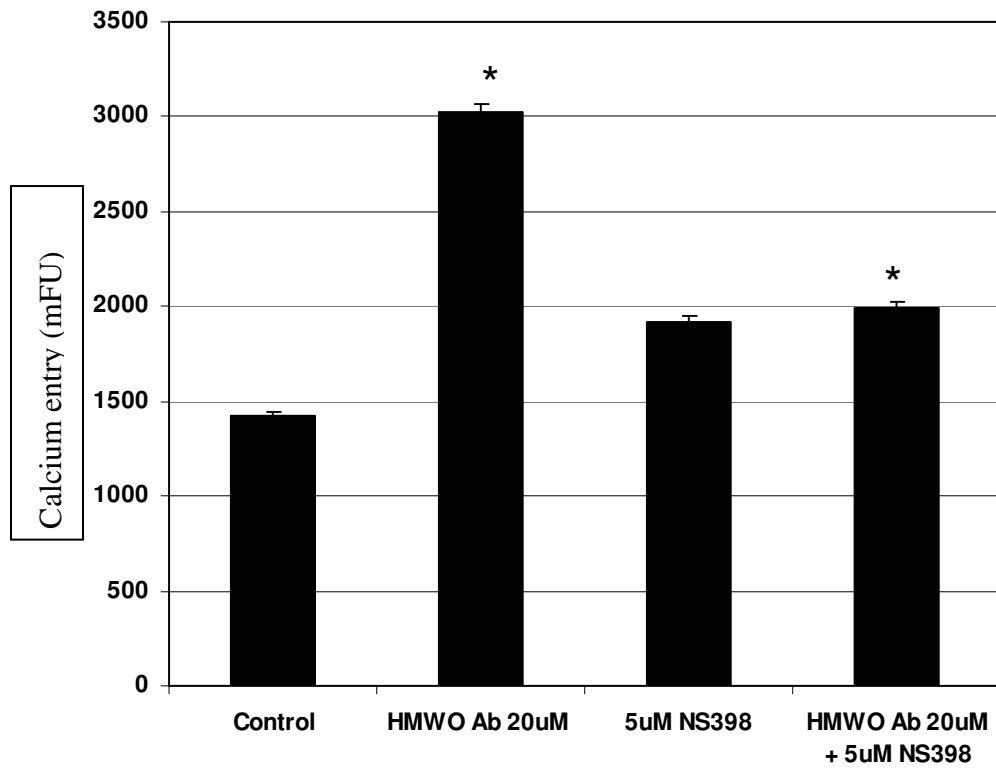
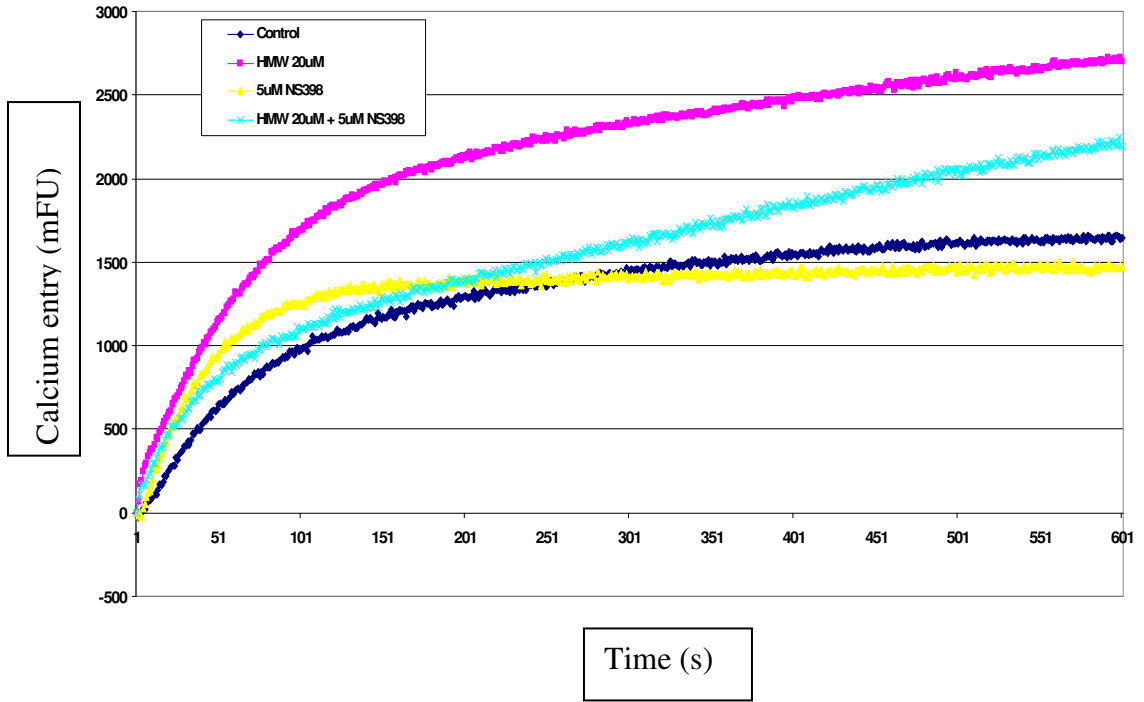


Fig. 14: a) Real time measurement of calcium entry in human HNPCs following treatment with 20 μ M HMWO A β 1-42, 5 μ M NS398, HMWO + NS398 (n=6 for each treatment group). b) Quantification of intracellular calcium entry in HNPCs following the above mentioned treatments. ANOVA followed by post-hoc analysis revealed significant main effects between control and HMWO ($p < 0.05$), HMWO and HMWO + NS398 ($p < 0.05$).

Figures 15a & 15b: Calcium entry in HNPC following treatment with Ibuprofen

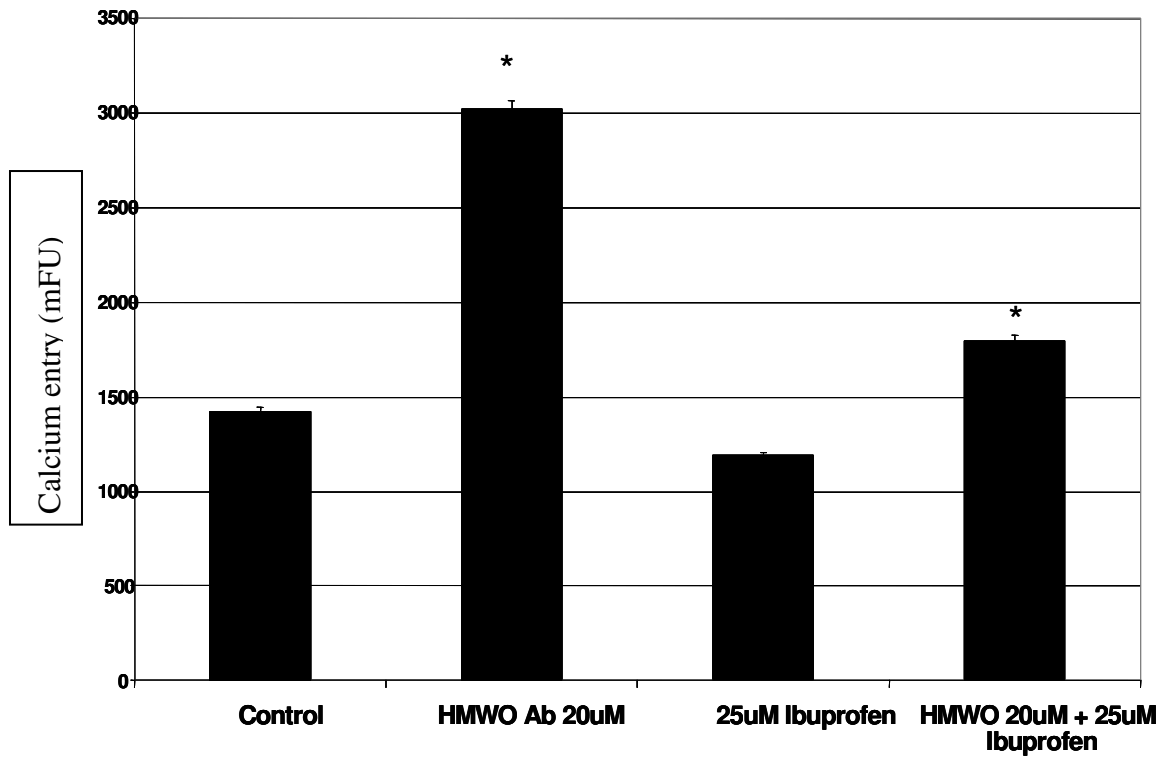
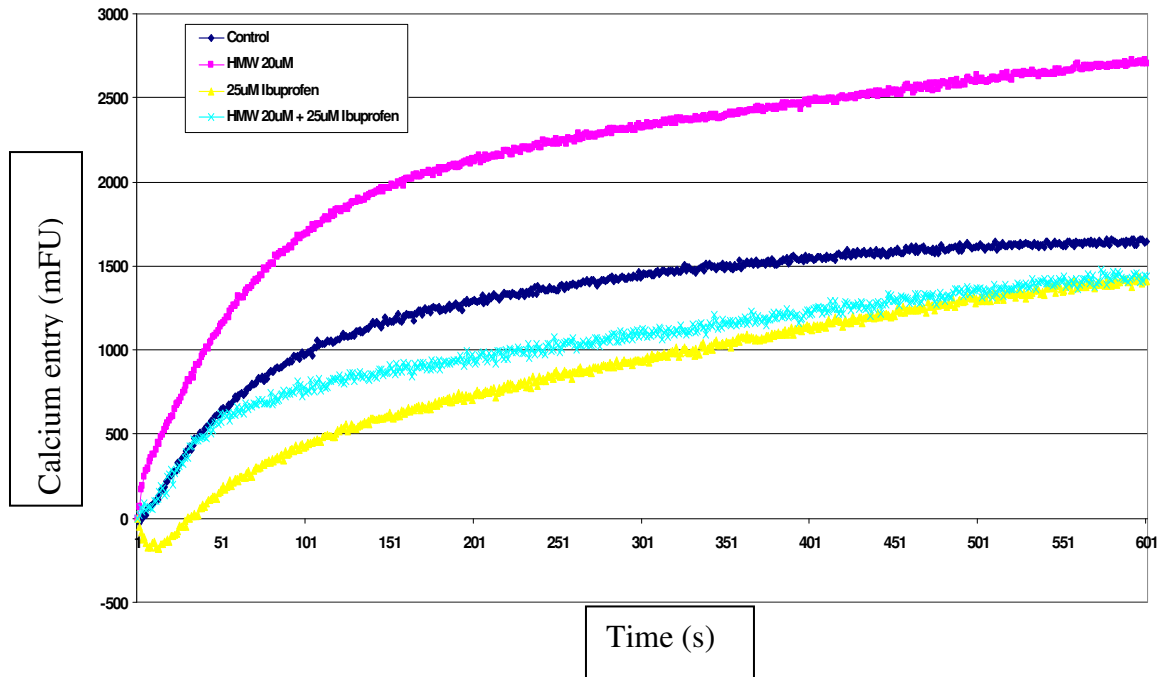


Fig. 15: a) Real time measurement of calcium entry in human HNPCs following treatment with 20 μ M HMWO A β 1-42, 25 μ M Ibuprofen, HMWO + Ibuprofen (n=6 for each treatment group). b) Quantification of intracellular calcium entry in HNPCs following the above mentioned treatments. ANOVA followed by post-hoc analysis revealed significant main effects between control and HMWO (p<0.05), HMWO and HMWO + Ibuprofen (p<0.05).

Figure 16: HNPC cells using 20X magnification – Day 4

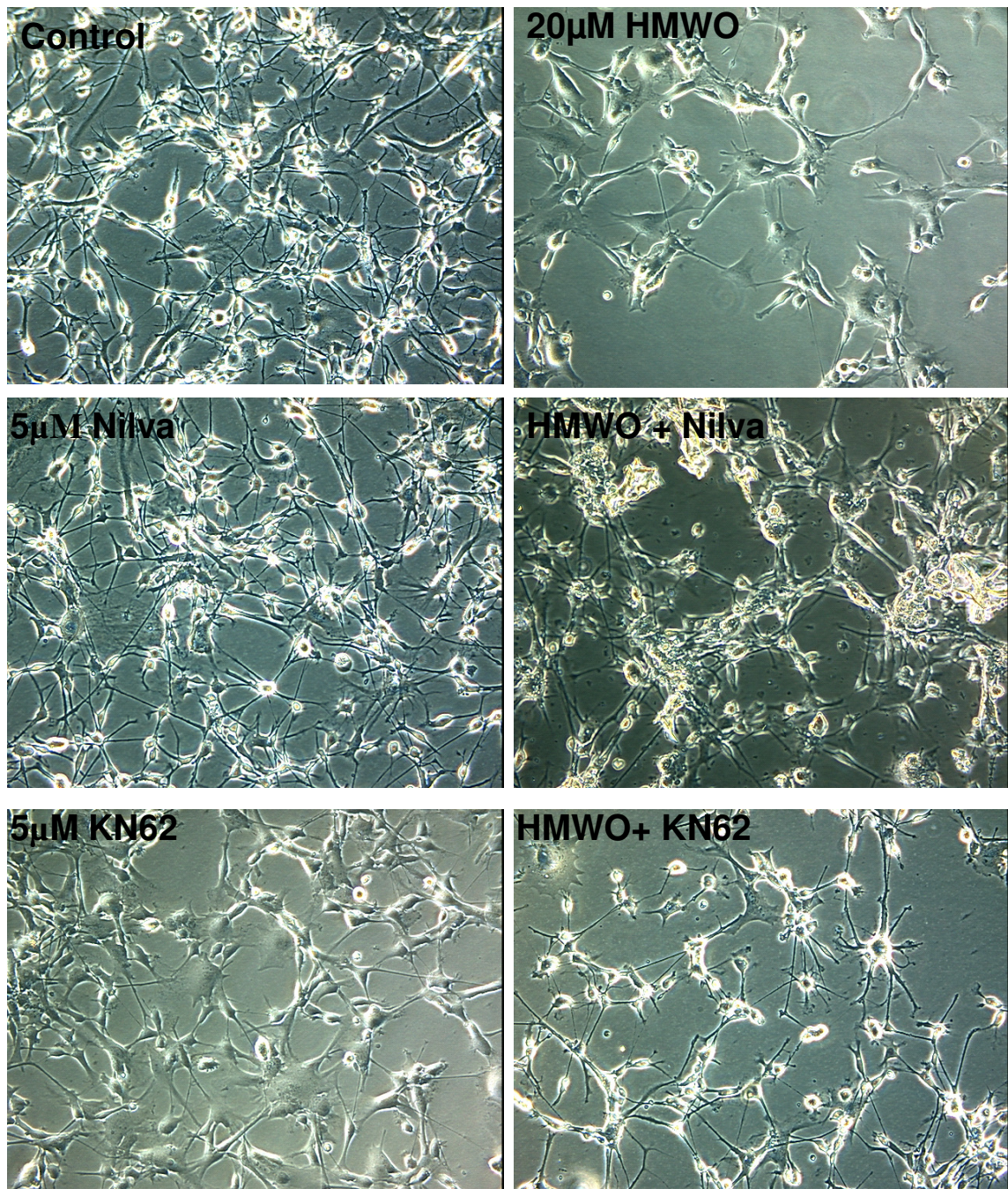


Fig. 16: Neurotoxicity observed in HNPCs following treatment with 20µM HMWO Aβ1-42 alone and in combination with 5µM Nilvadipine and 5µM KN62 after 96h as recorded using the Scion software imager at 20X magnification.

Figure 17: HNPC cells using 10X magnification – Day 4

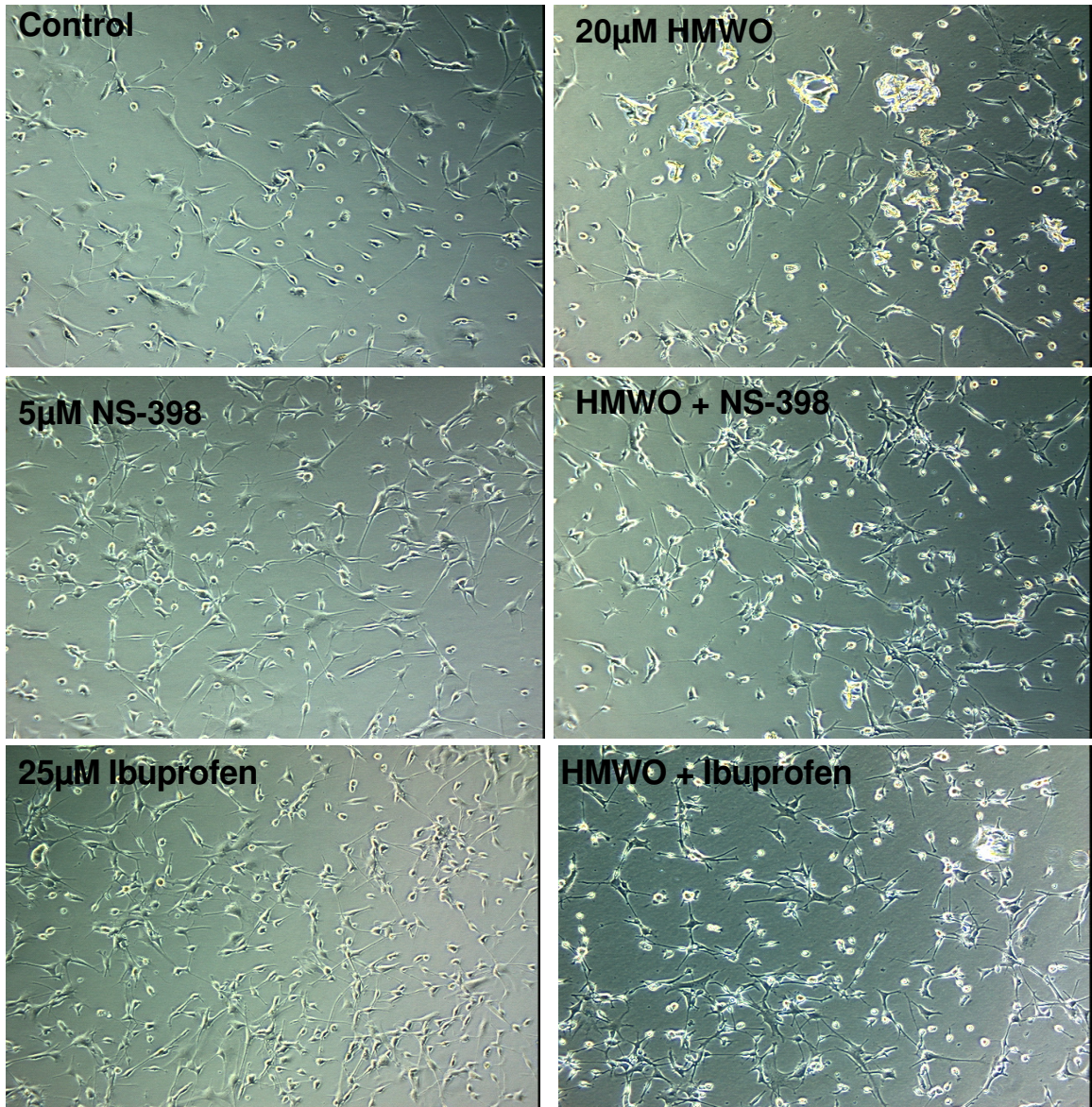


Fig. 17: Neurotoxicity observed in HNPCs following treatment with 20µM HMWO Aβ1-42 alone and in combination with 5µM NS-398 and 25µM Ibuprofen after 96h as recorded using the Scion software imager at 10X magnification.

Fig. 18: LDH in HNPCs

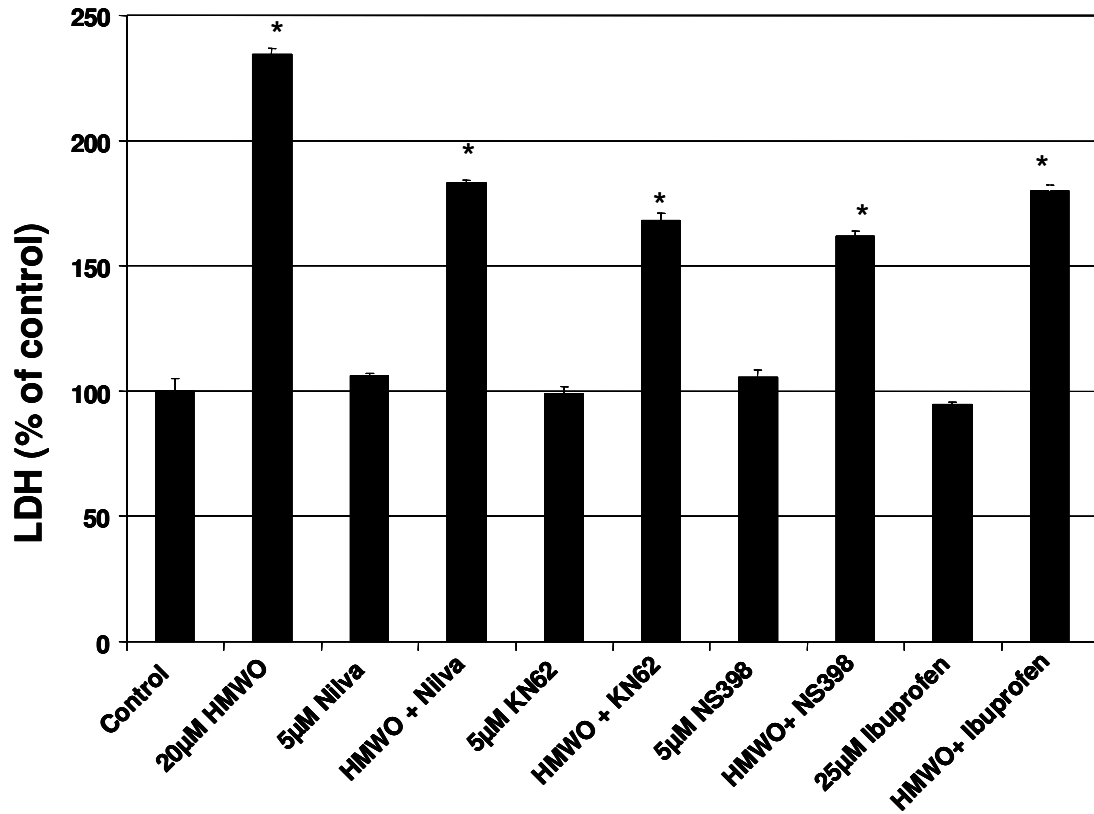


Fig. 18: LDH ratio of media versus lysate of HNPCs after treatment with 20µM HMWO Aβ1-42 alone and in combination with 5µM Nilvadipine, 5µM KN62, 5µM NS-398, 25µM Ibuprofen after 96h. ANOVA followed by post-hoc analysis revealed significant main effects between control and HMWO ($p < 0.05$), HMWO and HMWO + Nilvadipine ($p < 0.05$), HMWO and HMWO + KN62 ($p < 0.05$), HMWO and HMWO + NS-398 ($p < 0.05$), HMWO and HMWO + Ibuprofen ($p < 0.05$).

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CHAPTER FIVE

DISCUSSION and CONCLUSIONS

DISCUSSION:

My previous studies and several other reports have established that A β causes inflammation which is evident by increased activation/expression of Cox-2 enzyme (Paris et al 2002, Quadros et al 2003), activation of astrocytes and microglia in response to A β deposition (Sastre et al 2006). Other evidence supporting that inflammation is one of the pathogenic factors involved in AD is based on epidemiological studies with NSAIDs which show protective effects with long-term intake in AD patients (McGeer and McGeer 2007, Stewart et al 1997). A β which is derived from its substrate APP assumes different conformations capable of assuming different biological activities. Several earlier reports differentiated A β as soluble and aggregated forms and the soluble forms of A β were demonstrated to be more pathogenic in AD (Mclean et al 1999). Subsequent studies by atomic force microscopy revealed that the soluble pool of A β consisted of monomers/dimers and oligomers (Mastrangelo et al 2006). I have tried to determine which conformation of A β ₁₋₄₂ is most toxic to neurons by correlating its toxicity to inflammation in glial cells and its subsequent effect on neurons. My results demonstrate that A β ₁₋₄₂ in its monomer/dimer form also referred to as freshly solubilized (FS) is not toxic to neurons nor does it stimulate inflammation as observed by the release of interleukins IL-6 and IL-8 in astrocytes and microglia. However oligomeric forms of A β , more specifically the high molecular weight oligomers (HMWO) as opposed to the low molecular weight oligomers (LMWO) are more toxic to neurons and also produce

significantly higher levels of proinflammatory IL-6 and IL-8 in both astrocytes and microglia (Quadros chapter 3). IL-6 and IL-8 are both pro-inflammatory molecules found to be elevated in the brains of patients with diseases such as AD, mild cognitive impairment (MCI) and traumatic brain injury (TBI) (McGeer and McGeer 1999, Lue et al 2001, Kushi et al 2003). Studies in rat cortical neurons reveal that A β together with IL-6 activate NMDA receptors resulting in neuronal damage (Quiz and Gruol 2003, Conroy et al 2004). Similar studies in rat neurons with IL-8 treatment resulted in neurotoxicity via increase in the release of neurotoxins and pro-apoptotic proteins (Thirumangalakudi et al 2007).

The other part of my thesis was to determine if excess influx of calcium into cells is responsible for the inflammation observed in AD. It is well established that in AD there is a dysregulation of calcium within the cells (Mattson and Chan 2003). Recent reports have shown that calcium accelerates the change in conformation of A β (Isaacs et al 2006) and this change from soluble to oligomeric forms is probably responsible for the neurotoxicity observed in AD (Hartley et al 1999, Watson et al 2005). In addition, increase in cytosolic calcium also affects APP processing in rat cortical neurons resulting in an increase in intraneuronal production of A β 1-42 (Pierrot et al 2004). But I wanted to correlate this increase in intracellular calcium mediated by A β with increases in inflammation. My results with astrocytes and microglia demonstrate that oligomeric forms of A β (more specifically HMW O A β) that increased IL-6 and IL-8 were also the most potent form in increasing intracellular levels of calcium within these cells. This proinflammatory effect mediated by calcium was blocked when an intracellular calcium chelator BAPTA-AM was used and conversely was potentiated significantly with the

calcium ionophore A23187. Hence my results strongly suggest that blocking increased intracellular levels of calcium could reduce inflammation mediated by activated glia in response to A β . Acute inflammation is neuroprotective in nature, however chronic inflammation is neurotoxic. My hypothesis was that L-type calcium channel blockers and other inhibitors of the calcium signaling pathway like NF κ B inhibitors and calmodulin kinase inhibitors could block A β mediated inflammation via modulation of calcium inside the cell. The reason I chose L-type calcium channel blockers over other types of voltage gated calcium channel blockers such as N, P/Q, and R was because of previous in vitro studies on neurons indicating an increase in L-type calcium channel activity following exposure to beta amyloid (Ekinici et al 1999, Ho et al 2001). Hence blocking L-type calcium channels would reduce inflammation in glia and decrease neurodegeneration which is supported by my results shown on glia and HNPCs (Quadros et al. 2007, chapter 4). L-type calcium channel blockers and calmodulin kinase inhibitors could therefore act as dual antagonists of inflammation and calcium influx and possibly have potential therapeutic effects not only in AD but also in other neuroinflammatory diseases like multiple sclerosis, Parkinson's disease, TBI etc. Studies in rat cortical neurons revealed that L-type calcium channel blockers reduced neuronal apoptosis mediated by activation of spla2 enzyme (Yagami et al 2004). My results with known anti-inflammatory agents NS-398, a selective COX-2 inhibitor and Ibuprofen, a NSAID reveal significant reduction in neuronal loss even after day 4 compared to control and HMWO A β 1-42. Both NS-398 and Ibuprofen were capable of inhibiting the effect of HMWO A β mediated neurodegeneration. Similar effects were observed with Nilvadipine, the L-type calcium channel blocker and KN62, the calmodulin kinase

inhibitor. Interestingly, both known anti-inflammatory agents NS-398 and Ibuprofen were capable of reducing the increased calcium influx in human neuronal precursor cells (HNPCs) mediated by HMW A β 1-42 thereby suggesting that there is a correlation between calcium influx and inflammation.

CONCLUSIONS:

It is well established that several etiological factors are responsible for the pathogenesis in AD. However, my hypothesis was that there is a connection between excess calcium influx into glial cells and inflammation in AD. Regulating excess calcium influx in glial cells would decrease subsequent activation of these cells from releasing proinflammatory cytokines and chemokines which could be toxic to neurons. Several reports on astrocytes indicate that A β mediated toxicity on neurons is via changes in intracellular levels of calcium (Abramov et al 2003, Monnerie et al 2005). My results with Nilvadipine, the L-type calcium channel blocker, and KN62, a calmodulin kinase inhibitor reveal that blocking excess levels of intracellular calcium in HNPCs mediated by HMW A β and to some extent LMW A β may be responsible for the attenuation of neurotoxicity observed in these cells. Calmodulin kinase and NF κ B are actively involved in the regulation of calcium signaling across cells (Lin et al 2004, Choi et al 2006). Studies with NF κ B inhibitors demonstrate that they have anti-inflammatory effects (Lopez-Franco et al 2006, Lopez-Franco et al 2002). My data with L-type calcium channel blocker and calmodulin kinase inhibitor on glial cells reveal that they reduce the production of IL-6 and IL-8 which are both pro-inflammatory thereby suggesting that increase in intracellular calcium causes inflammation. The reason these inhibitors

mitigate neurotoxicity in HNPCs could be due to reduction in the production of these pro-inflammatory molecules. To further demonstrate that the neuroprotection offered by these calcium channel blockers or inhibitors associated with calcium binding were as a result of their anti-inflammatory effect, I used anti-inflammatory agents such as NS-398, a Cox-2 inhibitor and Ibuprofen, an NSAID on HNPCs. Previous studies with Ibuprofen on TgAPPsw mice early in the course of the disease reveal that it reduced amyloid deposition and the treatment also reduced activation of microglia around plaques resulting in a subsequent reduction in dystrophic neurites (Lim et al 2000, Heneka et al 2005). Reports indicate that increased Cox-2 activity caused neuronal damage in primary cortical neurons when induced by LPS and iron (Im et al 2006). My results with Ibuprofen and NS-398 reveal significant reduction in neurotoxicity mediated by HMW O A β . Another interesting result is that these anti-inflammatory agents (NS-398 and Ibuprofen) were capable of inhibiting the increase in calcium influx mediated by HMW O A β . This suggests that there is a correlation between inflammation and calcium influx and my results further indicate that HMW O A β 1–42 are most potent than monomers/dimers or fibrillar forms in mediating inflammation via disruption of intracellular calcium levels and causing neurotoxicity to cells. Hence, modulating intracellular calcium levels by using calcium channel blockers or calmodulin kinase inhibitors may reduce HMW O A β mediated chronic inflammation in glial cells and protect neurons in AD patients.

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BOOK CHAPTER

1. RELEVANCE OF COX-2 INHIBITORS IN ALZHEIMER'S DISEASE

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