The role of astrocytes in Alzheimer's disease

Aleksandra Parpura-Gill

Iowa State University

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The role of astrocytes in Alzheimer’s disease

by

Aleksandra Parpura-Gill

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in partial fulfillment of the requirements for the degree of
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Graduate College

Iowa State University

This is to certify that the Doctoral dissertation of

Aleksandra Parpura-Gill

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Major Professor

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For the Graduate College
DEDICATION

With love and adoration to Harry and Sebastian
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ABSTRACT

β-amyloid is the primary protein component of neuritic plaques, which are degenerative foci in brains of patients with Alzheimer's disease (AD). The effects of this naturally occurring β-amyloid on the cells of the central nervous system have not been completely understood. The effect of cytokines such as IL-1β, TNF-α, and IFN-γ on astrocytic nitric oxide is well documented; however, the effects of β-amyloid on astrocytes for their cytokine-mediated release of nitric oxide are not established. In the present study astrocytic release of nitric oxide was studied following exposure to β-amyloid peptide (B25-35) in combinations with IL-1β, TNF-α or IFN-γ. None of the cytokines induced an increase in nitrite levels (nmol/mg protein) in astrocytic cultures by themselves. However, IFN-γ combined with IL-1β or TNF-α induced a significant increase in nitrite levels. Although both IL-1β and TNF-α were effective costimulatory factors, the combination of IFN-γ and IL-1β was more effective in increasing nitrite levels in cultures than the combination of IFN-γ and TNF-α. B25-35 completely blocked the increase in nitrite levels by IFN-γ and TNF-α, whereas B25-35 partially blocked the effect of IFN-γ and IL-1β. These findings warrant a further study to determine how the modulatory action of β-amyloid on cytokine-mediated astrocytic release of nitric oxide affects neuronal functions in AD patients. β-amyloid increases the vulnerability of cultured neurons to glutamate-induced excitotoxic damage. Because astrocytes play a key role in uptake of extracellular glutamate and glutamate uptake is ATP dependent, we studied the effect of B25-35 on glutamate and glucose uptake in cultured hippocampal astrocytes following 7 days
of exposure to B25-35. Glutamate uptake by control astrocytes was time-dependent. Astrocytes exposed to B25-35, however, showed significantly lower glutamate uptake at all sampling times. Similarly, $[^{14}\text{C}]$glucose uptake by astrocytes was inhibited by B25-35. When glucose uptake was blocked by phloretin (10 mM), astrocytic $[^{3}\text{H}]$glutamate uptake was also blocked, suggesting that the inhibitory effect of β-amyloid on glutamate uptake is caused by diminished glucose uptake. Thus, our present study suggests a possible link among three proposed mechanisms of Alzheimer’s disease: astrocytic nitric oxide release, global defect in cerebral energy metabolism and glutamate neurotoxicity.
GENERAL INTRODUCTION

Alzheimer's disease (AD) is a neurodegenerative disease affecting 2% to 4% of the American population over 65 years of age (American Psychiatric Association, 1997). The prevalence of Alzheimer's disease is slightly greater in females than in males, and it is more common in individuals with Down's syndrome or a history of head trauma (American Psychiatric Association, 1997). Although the majority of Alzheimer's disease cases are sporadic, a small portion of patients shows a familial pattern of disease (Talbot et al., 1993). Linkage studies have demonstrated that the familial pattern of AD appears in early onset cases and is linked to the markers on chromosomes 21, 14 and 19. Additionally, they seem to be an autosomal dominant trait with full penetrance (Goate et al., 1990; American Psychiatric Association, 1997). The existence of a genetic component in late onset AD cases has not been confirmed, but is a possibility (Roses et al., 1990; Goate et al., 1990).

There are two subtypes of Alzheimer's disease determined by the age of onset (American Psychiatric Association, 1997). In Early Onset AD, symptoms of the disease begin before 65 years of age while in Late Onset AD they start after the age of 65. Late Onset AD is much more common than Early Onset. The course of Alzheimer's disease is characterized by an insidious beginning and progressive nature. Early deficits in memory are followed by other cognitive disturbances and personality changes, as well as loss of judgment and spatial orientation (American Psychiatric Association, 1997).
Clinical Signs of Alzheimer’s Disease

The onset of dementia is an essential clinical feature of Alzheimer’s disease and also a requirement for the diagnosis of the disease. Dementia is a syndrome characterized by progressive mental deterioration resulting from multiple cognitive deficits. The cognitive deficit must be severe enough to cause an inability of the patient to carry out occupational or social activities, and it has to represent a decline in the level of functioning from a previous existing level (American Psychiatric Association, 1997; Terry and Katzman, 1983).

Memory impairment as a component of dementia affects both short-term and long-term memory. Individuals suffering from dementia not only forget previously learned material, but also have significantly decreased ability to learn new material. Most often patients exhibit both forms of memory impairment but they can also be present independently.

Besides memory impairment, one or more other cognitive dysfunctions are present in dementia of the Alzheimer’s Type. These cognitive dysfunctions are manifested as aphasia, apraxia, agnosia or disturbance in executive functioning. Aphasia is a language dysfunction characterized by difficulty in generating the names of individuals and objects. Speech becomes vague with frequent use of words without definite reference. Apraxia is an impairment in execution of motor tasks without damage to the motor and sensory functions and comprehension of the task. Agnosia is the inability to identify or recognize objects despite intact sensory functions. Disturbances in executive functioning encompass impairment of abstract thinking and the ability to plan and execute complex behaviors. Sensory and motor functions in Alzheimer’s disease are usually conserved (Haxby, 1990).
The diagnosis of this disease depends on the symptoms described above and exclusion of other causative factors that can result in the same symptomatology. The conditions that need to be excluded encompass disorders such as other central nervous system conditions (e.g., cerebrovascular disease, brain neoplasmas, subdural hematoma), metabolic abnormalities (e.g., hypothyroidism, hypovitaminosis) and intoxications (Beal et al., 1997; American Psychiatric Association, 1997). In spite of the above diagnostic criteria, the definitive diagnosis of Alzheimer’s disease can be determined only postmortem by pathohistological examination of the brain tissue and finding the characteristic changes that are further discussed below.

**Neuropathology of Alzheimer’s Disease**

**Gross Brain Neuropathology**

Patients with Alzheimer’s disease develop more severe atrophy of frontal, temporal and parietal cortex than age-matched individuals (Jolles et al., 1990; Khachaturian and Rodebaugh, 1996). This cortical atrophy can be manifested by: 1) decrease in cortical surface area, 2) decrease in cortical thickness (‘laminar atrophy’), 3) decrease in brain weight and volume, and 4) narrowing of the gyri and enlargement of sulci and 5) enlargement of cerebral ventricles.

Cortical atrophy characterized by decreased surface area of all lobes is more pronounced in early onset than in late onset cases whereas more focal decrease in surface area was detected in late onset cases (Hubbard and Anderson, 1981). The thickness of the neocortex is significantly decreased in AD patients but it is similar to that found in normally aged brains (Kemper, 1984; Hansen et al., 1988). It has been proposed that the reported
cortical atrophy, especially in the earlier stages of AD, could be mainly due to a decrease in cortical surface area rather than decrease in cortical thickness (Hauw et al., 1990). Hauw et al. suggested that the cortical atrophy was due to the loss of neuronal columns and fibers (that are positioned perpendicular to the cortex surface) which would result in decreased cortical surface, and was not due to the loss of selective layers of the cortex (laminar loss) which would cause a decrease in cortex thickness (Hauw et al., 1990). In fact, it was demonstrated that mental decline correlated with the decrease of cortical surface, not the thickness of the cortex (Duyckaerts et al., 1985; Hauw et al., 1987). However, in most severe cases of AD, when compared to age-matched controls, cortical thickness is significantly decreased; furthermore, it is the most prominent in the temporal cortex which appears to be significant because AD pathologic changes in the temporal region are well documented (Hauw et al., 1990).

It has been suggested that ventricular and sulcal enlargement in AD patients is somewhat greater compared with elderly controls (Arai et al., 1983). Regional cortical atrophy manifested by enlargement of sulci and ventricles has been proposed to be more specific and more important in AD (Jolles et al., 1990) than diffuse, more generalised enlargement of sulci and ventricles which can also be present in a variety of neurological and systemic conditions (TerBrugge et al., 1987; Jolles et al., 1990). The importance of these regional changes is supported by studies that reported greater temporal lobe atrophy as revealed by dilation of the hippocampal fissure and ventricular enlargement in AD brains than in control subjects (George et al., 1987; Kido et al., 1987). Additionally, it was proposed that this focal change is the earliest sign of AD (George et al., 1987; Kido et al.,
Indeed, the severity of dementia have been shown to be highly correlated with the degree of this focal ventricular and sulcal enlargement (de Leon et al., 1980; Earnest et al., 1979; George et al., 1983; Jacoby et al., 1980).

Regional specificity of the cortical atrophy was further supported by findings of the changes in volume of the cerebral cortex. In AD patients cortical volume was significantly reduced when compared to age-matched individuals, with the most prominent reduction in volume noted in medial temporal structures such as the hippocampus (Double et al., 1996). This particular volume reduction was related to severity and progression of the disease.

Several types of changes in white matter demonstrated by computerized tomography (CT) and magnetic resonance imaging (MRI) are collectively referred to as leukoaraiosis. Leukoaraiosis encompasses: 1) periventricular low-density lesions on CT, 2) periventricular hyper-intensity lesions on CT, and 3) periventricular hyper-intensity changes on MRI (Ishijima et al., 1996). CT studies have shown that white matter changes in the form of lucencies (low density lesion) were more common in AD patients than in control subjects (George et al., 1986; Leys et al., 1990). Furthermore, MRI investigations of AD brains found halo-shaped hyper-intensity foci of white matter (suggestive of water accumulation and/or demyelination) surrounding the lateral ventricle (Fazekas et al., 1987; Petrovic et al., 1990). Subsequent examinations of periventricular white matter have shown diffuse loss of myelin sheets, axons, oligodendroglia and astrocytic gliosis (Brun et al., 1990; Leys et al., 1990). These changes of white matter were also shown to be associated with the degree of cognitive impairment in AD patients (Brun et al., 1990; Ishijima et al., 1996).
Pathohistology

Although assessment of the gross cerebral features may suggest AD, the final neuropathological diagnosis depends on microscopic examination of the brain tissue. The pathological hallmarks of Alzheimer’s disease are neuronal loss, extracellular neuritic plaques, intracellular neurofibrillary tangles, and amyloid angiopathy.

Two major subtypes of neuritic plaque are recognized: classic neuritic plaque and diffuse plaque. Classic neuritic plaque consists of a dense central core of radiating amyloid fibrils surrounded by a rim of abnormal axonal and dendritic processes, reactive microglia and astrocytes. The diffuse plaques that can not be detected using routine stains (Congo red, silver) have been observed after an immunohistochemical reaction with antibodies to β-amyloid. Most diffuse plaques are not associated with abnormal neurites or reactive glia. Ultrastructural observations revealed little or no fibrillary amyloid in these lesions suggesting that diffuse plaques are an early stage in classic neuritic plaque formation (Yamaguchi et al., 1990).

The second pathohistological hallmark of AD is the presence of neurofibrillary tangles that appear in neuronal cell bodies and in altered neurites as dense bundles of fibers. Tangles consist of paired helical filaments (PHFs) which are composed of tau protein (Kosik et al., 1986; Nukina and Ihara, 1986; Kosik et al., 1988). Immunocytochemical studies have shown that antibodies raised against tau protein have recognized PHFs in tissue sections and, conversely, that antibodies raised against isolated preparations of PHFs recognized the tau protein. The observation that some antibodies to tau protein react with PHFs much more
strongly after dephosphorylation of tissue sections suggests that certain epitopes within PHFs may be abnormally phosphorylated (Grundke-Iqbal et al., 1986).

The third neuropathological feature of AD is amyloid angiopathy or deposition of β-amyloid protein in walls of meningeal and cortical blood vessels and around cerebral vasculature (Wisniewski et al., 1992b). These vascular β-amyloid deposits assume three distinct morphological types: 1) a semicircular thickening of the vascular wall caused by a large amount of fibrilar β-amyloid deposits, 2) tuberlike amyloid deposits, and 3) amyloid stars with a radial arrangement of β-amyloid fibrils (Wisniewski and Wegiel, 1992). Such perivascular depositions of β-amyloid cause amyloid angiopathy characterized by endothelial degeneration and obliteration of the vessel lumen (Wisniewski and Wegiel, 1992). β-amyloid angiopathy is also accompanied by neuronal degeneration and appearance of reactive astrocytes (Wisniewski et al., 1992).

**β-Amyloid Protein**

Amyloid is a general term in pathology that designates deposits of 5-10 nm proteinaceous fibrils which accumulate progressively in the extracellular spaces of tissue and vasculature. In the case of AD a specific protein that is deposited in the core of neuritic plaques and the walls of cerebral vasculature is designated as β amyloid protein (βA4).

Amino acid sequencing has shown that βA4, originally isolated from meningeal blood vessels (Glenner and Wong, 1984), is essentially the same as the senile plaque βA4 (Masters et al., 1985). Molecular cloning has revealed that βA4 is a small fragment of a much larger amyloid precursor protein (APP). The βA4 molecule is a 32- to 42-amino acid
peptide, part of which forms the hydrophobic transmembrane domain in the COOH- terminal portion of APP (Kang et al., 1987).

APP is normally cleaved by protease-APP secretase within 43-amino acid of βA4 to produce a large secreted derivative ending at amino acid 16 of βA4 and a small, membrane associated, COOH- terminal derivative beginning at amino acid 17 of βA4 (Esch et al., 1990). A complex set of 8- to 12-kD COOH- terminal derivatives of APP have been shown to exist in human brain and peripheral tissues. The two largest COOH-derivatives have the entire βA4 insert at, or near, their NH2- terminus and are likely the intermediates in the pathway leading to amyloid deposition. Different brain regions have roughly equivalent levels of the various 8- to 12-kD derivatives, whereas each peripheral tissue has relatively low levels of the larger, potentially amyloidogenic forms (Estus et al., 1992). The discovery of the production of these complex 8- to 12-kD COOH- terminal derivative sets as a result of APP degradation suggested that APP can be cleaved in some alternative proteolytic pathways.

Three distinct pathways of APP proteolysis have been demonstrated using APP 695 deletion mutant that produces the normal set of COOH- terminal derivatives and shortened secreted derivatives (Golde et al., 1992; Haass et al., 1993a; Haass et al., 1993b). These researchers found that, in the secretory pathway, mediated by APP secretase, cleavage of the APP occurred at a single site within the βA4 to generate one secreted derivative and one nonamyloidogenic COOH- terminal fragment. On the other hand, in the endosomal-lysosomal pathway, a complex set of COOH- terminal derivatives that includes the potentially amyloidogenic forms was produced (Todd et al., 1992). The previous study
which demonstrated elevated levels of lysosomal proteases in neuritic plaques (Cataldo and Nixon, 1990) supported the feasibility of this finding and the importance of the lysosomal pathway in amyloidogenesis and pathogenesis of AD.

Subsequent work has shown that cultured cells produced βA4 during normal metabolism (Haass et al., 1992), demonstrating that an aberrant pathway may not be necessary for amyloidogenesis. Thus, it has been suggested that lysosomes may not be directly required and may not play a predominant role in formation of βA4 (Haass et al., 1993a, 1993b). Such findings favor the current hypothesis that βA4 may be generated in acidic intracellular compartments (Haass et al., 1993a, 1993b).

Different forms of APP that contain βA4 are derived from a single gene by alternative mRNA splicing. These βA4 precursors contain 695, 751 and 770 amino acids and differ from one another primarily by the presence (in case of APP 770 and APP 751) or absence (in case of APP 695) of a Kunitz-type protease inhibitor insert (Kithaguchi et al., 1988; de Sauvage and Octave, 1989; Ponte et al., 1988; Tanzi et al., 1988). APP is expressed at high levels in many different tissues throughout the body. In most tissues, APP 770 is the most abundant, whereas in the CNS, the transcript lacking the protease inhibitor insert (APP 695) is predominant. The ratio of amyloidogenic and nonamyloidogenic fragments released by APP degradation may depend on the balance of APP's intrinsic protease inhibitory property, exposure to the proteases in the microenvironment (Aisen and Davis, 1994), and overexpression of APP. The significance of overexpression of the APP gene was first elucidated in patients with Down's syndrome (trisomy 21) when the gene encoding APP was mapped to chromosome 21 (Davies and Tilghman, 1993). Early onset of dementia in
patients with Down’s syndrome is characterized by β-amyloid deposition and development of diffuse and neuritic plaques. Such observations led to the conclusion that the process of β-amyloid deposition is a central event in AD pathogenesis (Davies and Tilghman, 1993).

The evidence further supporting the theory of β-amyloid deposition as the primary pathological process was the identification of pathogenic mutations in the β-amyloid precursor protein gene that were linked to autosomal dominant forms of familial AD (Hardy, 1994; Hanger et al., 1992). Transgenic mice overexpressing human mutant β-amyloid precursor protein progressively developed pathological hallmarks similar to AD including β-amyloid deposits, formation of neuritic plaques and association of reactive microglia and astrocytes with amyloid deposits (Games et al., 1995). Although synaptic loss and degenerating neurons in transgenic mice accompany the β-amyloid deposition, the mechanisms involved in β-amyloid mediated neuronal degeneration are not well understood. However, it is known that the neurotoxicity of β-amyloid is related to amino acids 25-35 (β25-35) of the full-length β-amyloid peptide (β1-43) (Mattson et al., 1992; Yanker et al., 1989). Additionally, toxicity of β-amyloid depends, at least in part, on its aggregation state (Pike et al., 1991; Pike et al., 1993).

Astrocytes and Alzheimer’s Disease

The morphological observations of a close association between reactive glia cells and neuritic plaques suggest reactive glial cells as a prominent component of Alzheimer’s disease pathology. Astrogliosis manifested by activation of astrocytes is a common form of astrocytic response to injury and cellular necrosis in the CNS (Montgomery, 1994). The most distinctive characteristic of activated astrocytes, also referred to as reactive astrocytes,
is an increase in glial fibrils composed of glial acidic fibrillary protein (GFAP), demonstrated by an increased immunocytochemical staining for GFAP (Montgomery, 1994). Interestingly, a marked increase in astrocytic GFAP levels has also been detected in the brain of AD patients (Haga et al., 1989; Ogata et al., 1992). One of the sites found to have a significantly high GFAP immunoreactivity is neuritic plaque (Farinelli and Nicklas, 1992). It has been suggested that glial cells surrounding neuritic plaques are also involved in the removal of debris from degenerated neurites (Rozemuller et al., 1989; Wisniewski and Wegiel, 1992), the synthesis and deposition of β-amyloid (Wisniewski and Wegiel, 1992), or the isolation of β-amyloid from neuropil by astrocytic processes (Wisniewski and Wegiel, 1992).

A variety of cellular metabolic processes in reactive astrocytes are known to be upregulated. An example is the synthesis of proteins (e.g., cytokine synthesis) with potent biological effects (Montgomery, 1994; Norenberg et al., 1979). Various factors have been identified to have a regulatory effect on activation of astrocytes (Montgomery, 1994). For example, an in vitro study of astrocytic cultures has shown that β-amyloid stimulates astrocytic release of interleukin-1 (IL-1) and basic fibroblast growth factor (bFGF) (Araujo and Cotman, 1992). This study suggests the possibility that reactive astrocytes may be responsible for high levels of cytokine immunoreactivity observed in AD (Gomez-Pinilla et al., 1990; Griffin et al., 1989; Hara et al., 1989). Therefore, it is possible that β-amyloid may also affect some other astrocytic functions.
Astrocytic Role in Nitric Oxide (NO) Production

Astrocytes isolated from animals are uniquely equipped with both constitutive Nitric Oxide Synthase (cNOS) and inducible NOS (iNOS). Astroglial cNOS can be activated by glutamate, noradrenalin and calcium ionophores, followed by a significant increase in cGMP. In contrast, astroglial iNOS is stimulated by cytokines such as IL-1β, IFN-γ and TNF-α. Human astrocytes have also been reported to release nitric oxide (NO) upon stimulation with a combination of IFN-γ and IL-1β (Lee et al., 1993).

IL-1 immunoreactivity levels in temporal lobe homogenates of AD patients have been shown to be elevated in comparison to age-matched controls. An immuno-histological study also showed a 30 fold increase in the number of IL-1 immunoreactive glial cells in AD brains. Most of the immunoreactive glia were microglia, but numerous astrocytes associated with neuritic plaques were also intensely IL-1 immunoreactive (Griffin et al., 1989).

Association of IL-1, bFGF and T-cells with neuritic plaques suggests some specific roles played by glial cells in the pathogenesis of Alzheimer's disease (AD) (Gomez-Pinilla et al., 1990; Griffin et al., 1989; Haga et al., 1989). One of the suggested specific astrocytic roles in AD pathogenesis is their capacity to release nitric oxide as a response to cytokine (IL-1β, IFN-γ and TNF-α) stimulation (Murphy et al., 1993). At high concentrations, NO has been shown to exhibit cytotoxic effects on a variety of cell types including neurons (Bruhwiler et al., 1993). However, NO also plays an important role in the regulation of cerebrovascular blood flow. The coupling between cerebral metabolic function and cerebral blood flow is one important regulatory feature of the cerebral blood flow. NO released by neurons, vascular endothelium and astrocytes appears to be the key factor in this coupling.
process (Bruhwiler, 1993; Whal et al., 1993). Therefore, interactions of β-amyloid and various cytokines implicated in AD pathogenesis and their effect on astrocytes could be very important for understanding of β-amyloid neurotoxicity in AD. Furthermore, possible β-amyloid modulation of astroglial release of NO is also important because nitric oxide plays a role in the regulation of cerebrovascular function.

Direct neuronal effects of β-amyloid have been extensively studied; however, less work has been done describing β-amyloid effect on astrocytes and microglial cells which are observed as a prominent component of the neuritic plaques. In vitro studies have shown that astrocytes and microglial cells react to β-amyloid by changing morphology and releasing IL-1 and bFGF (Araujo and Cotman, 1992). In addition, it has been shown that β-amyloid significantly enhances microglial release of nitric oxide (NO) (Goodwin et al., 1995). Astrocytes are also capable of releasing NO when stimulated by various cytokines (IL-1β, IFN-γ and TNF-α) and glutamate. However, it is not well known whether β-amyloid modulates cytokine-induced astroglial release of NO.

Considering the anatomical location and abundance of astrocytes in the CNS, their change of cellular metabolism in response to a wide variety of environmental changes in the CNS, and their capability for release of nitric oxide, the effects of interaction among astrocytes, cytokines, and β-amyloid would have significant implications for CNS pathophysiology.

**Astrocytic Role in Glutamate and Glucose Metabolism**

Glutamate is the most abundant excitatory neurotransmitter in the mammalian CNS. It has been implicated in the processes of learning and memory (Maragos et al., 1987) as
well as the pathogenesis of AD (Palmer et al., 1990). Neurochemical studies of glutamatergic neurons in Alzheimer’s disease revealed alterations in the glutamatergic systems (Maragos et al., 1987). More specifically, a decreased number of glutamate receptors in AD patients was detected when compared to age-matched controls (Greenmayre et al., 1985). The largest decrease of glutamate receptors was seen in the outer cortical layers and hippocampus (Greenmayre et al., 1985) where the greatest concentration of neuritic plaques was noted. Additional evidence supporting the loss of the glutamatergic nerve terminals came from post-mortem studies of the neocortex. In advanced AD patients, both cerebral glutamate concentrations and sodium-dependent aspartate binding were decreased compared to age-matched controls (Maragos et al., 1987). The connection between the temporal nature of neuronal death and changes in the glutamatergic system of AD patients was established with the discovery of a correlation between the decline in cognitive test scores of AD patients and the concentration of glutamate in their cerebrospinal fluid (Maragos et al., 1987).

The exact cause of neuronal degeneration in AD is unknown, but the dysfunction of the glutamatergic system is a strong candidate (Maragos et al., 1987). When the glutamate receptor agonist (NMDA) was applied to the cerebral cortex of rats, a retrograde neuronal degeneration was found in areas that are known for the presence of histopathological features in AD patients (Sofroniev and Pearson, 1985). Furthermore, in human fetal spinal cord cultures, glutamate induced the formation of various paired helical filaments with some of them nearly identical to paired helical filaments found in AD (DeBonni and McLachlan,
1985). These studies support the neurotoxic hypothesis of AD pathogenesis that is based on glutamate action as endogenous excitatory amino acid toxin (Maragos et al., 1987).

Two types of toxic changes induced by glutamate in vivo are acute swelling of neuronal bodies and dendrites and delayed neuronal degeneration (Choi, 1988). A NMDA receptor, one of the three types of glutamate membrane receptors, plays the key role in mediating glutamate toxicity (Choi, 1988). Acute neuronal swelling occurs early in the glutamate exposure and it is dependent on extracellular Na\(^+\) and Cl\(^-\) because Na\(^+\) influx leads to membrane depolarization and secondary influx of Cl\(^-\) and water, resulting in neuronal swelling (Choi, 1987). Delayed neuronal degeneration occurs later, and it is mediated by excessive Ca\(^{2+}\) influx and Ca\(^{2+}\) release from intracellular stores which elevate cytosolic Ca\(^{2+}\) that, in turn, can activate numerous intracellular processes (e.g. protein kinases, endonucleases) (Choi, 1987; Meldrun and Garthwaite, 1990). Both types of glutamate induced toxic changes can lead to irreversible neuronal injury, but the Ca\(^{2+}\)-dependent delayed neuronal degeneration is more important at lower exposures to glutamate and hence more likely to predominate in pathological conditions (Choi, 1987, 1988).

Normally, extracellular levels of glutamate rise to high levels only briefly during synaptic transmission, with this rise being localized within the synaptic cleft (Choi, 1988; Nichols and Attwell, 1990). After its release from the nerve terminal, glutamate is removed from extracellular space by 1) transport back to the nerve terminal, and 2) uptake into astrocytes (Nichols and Attwell, 1990; Palmer et al., 1990). Plasma membrane glutamate carriers that are present in neuronal presynaptic membrane, and astrocytic plasma membrane are very similar (Kanner, 1988). Plasma membrane glutamate transporters have low
specificity for glutamate as they can also transport L- and D-aspartate. Nevertheless, they possess a high affinity for glutamate which makes them ideal carriers at the physiological levels of glutamate (Nichols and Attwell, 1990).

It seems that astrocytes play a more prominent role in the removal of extracellular glutamate. A study with radioactive markers showed that most of the extracellular glutamate is taken up by astrocytes rather than by neurons (McLennan, 1976). The glutamate taken up by astrocytes is then converted by a glia cell-specific enzyme, glutamine synthetase, into glutamine which can, in turn, be used by neurons for glutamate synthesis (Farinelli and Nicklas, 1992). The essential role of astrocytes in protection against glutamate neurotoxicity and maintenance of low extracellular levels of glutamate was further supported by in vitro experiments (Rosenberg et al., 1992). This study clearly demonstrated a significant increase in neuronal susceptibility to glutamate toxicity when neurons were co-cultured with relatively few astrocytes compared to astrocyte-rich neuronal co-cultures (Rosenberg et al., 1992). Neuronal death, however, occurred even in astrocyte-rich co-culture when glutamate uptake into astrocytes was blocked, suggesting the importance of astrocytic glutamate uptake in preventing the glutamate neurotoxicity (Rosenberg et al., 1992). These findings are important in view of the evidence that glutamate uptake decreases in the brains of AD patients (Kanai et al., 1993).

The accumulation of glutamate in astrocytes, against its concentration gradient due to glutamate uptake transport, is an energy dependent process (Swanson, 1992). Consequently, astrocytes require ATP energy to maintain the glutamate uptake process (Swanson, 1992; Nichols and Attwell, 1990; Ogata et al., 1992). A close relationship between glutamate
uptake and glucose availability has been shown in both conditions, in vitro and in situ (Swanson, 1992; Swanson et al., 1994). As long as glucose is available, astrocytes can maintain the production of sufficient ATP levels and continue glutamate uptake (Swanson, 1992; Swanson et al., 1994). The presence of glutamate-coupled glucose utilization in astrocytes (Swanson et al., 1990; Pellerin and Magistretti, 1994) further suggests the interdependency of glucose and glutamate metabolisms in astrocytes.

In summary, reactive astrocytes are a prominent component of the neuritic plaque, a pathohistological hallmark of AD. Reactive astrocytes respond to extracellular environment changes and insults by changing their cellular metabolism. In response to β-amyloid, astrocytes are known to release IL-1, and IL-1 immunoreactivity was detected around neuritic plaques. Astrocytes are also capable of NO production when stimulated with cytokines. This is important because NO is a key factor in regulation of cerebral blood flow and, in addition, it can be neurotoxic when present in high concentrations. Given the importance of NO in regulation of blood flow and astrocytic capability to release NO as well as their responsiveness to various stimuli (e.g. β-amyloid), the first study of this thesis examined the β-amyloid modulation of cytokine induced NO release from astrocytes by exposing cultured astrocytes to β-amyloid peptide (β25-35) alone or in combinations with IL-1β, TNF-α and IFN-γ.

Another astrocytic function that can claim a pivotal role in development of AD pathology is maintenance of extracellular glutamate levels. Glutamatergic system dysfunction and glutamate neurotoxicity have been implicated in AD pathology. β-amyloid has been shown to increase the vulnerability of cultured neurons to glutamate-induced
excitotoxic damage, and it is likely that the interaction between \( \beta \)-amyloid and astrocytes would lead to dysfunction of the astrocytic glutamate uptake system, hence increased glutamate toxicity. In light of the importance of astrocytes described above, in the glutamate uptake process and the evidence supporting the accumulation of \( \beta \)-amyloid as a primary pathological process in AD, the second study of this thesis explored the effect of \( \beta \)-amyloid on astrocytic glutamate and glucose uptakes in vitro.

**Explanation of dissertation format**

This dissertation is composed of two papers. The papers are preceded by a general introduction and followed by a general discussion which contains a summary and interpretation of the entire body of work. References from the general introduction, discussion as well as both papers are located at the end of the general discussion chapter.

The first paper has been submitted to Glia by Parpura-Gill, A. and Uemura, E. The second paper has been published in Brain Research where it was submitted by Parpura-Gill, A., Beitz, D. and Uemura, E.
THE EFFECTS OF β-AMYLOID ON ASTROGLIAL RELEASE OF NITRIC OXIDE MEDIATED BY IL-1β, TNF-α, AND IFN-γ

A paper submitted to Glia

Aleksandra Parpura-Gill and Etsuro Uemura

Abstract

The effect of cytokines such as IL-1β, TNF-α, and IFN-γ on astrocytic nitric oxide is well documented; however, the effects of β-amyloid on astrocytes for their cytokine-mediated release of nitric oxide is not established. In the present study, astrocytic release of nitric oxide was studied following exposure to β-amyloid peptide (β25-35) in combinations with IL-1β, TNF-α or IFN-γ. None of the cytokines induced an increase in nitrite levels (nmol/mg protein) in astrocytic cultures by themselves. However, IFN-γ combined with IL-1β or TNF-α induced a significant increase in nitrite levels. Although both IL-1β and TNF-α were effective costimulatory factors, the combination of IFN-γ and IL-1β was more effective in increasing nitrite levels in cultures than the combination of IFN-γ and TNF-α. β25-35 completely blocked the increase in nitrite levels by IFN-γ and TNF-α, whereas β25-35 partially blocked the effect of IFN-γ and IL-1β. The effect of cytokines (IFN-γ + IL-1β, IFN-γ + TNF-α) on nitrite levels in the astrocytic supernatant was substantially reduced by NOS-monomethyl-L-arginine (NMMA), suggesting that the effect of β25-35 and cytokines was due to a change in nitric oxide synthase activity and the release of nitric oxide. These findings warrant a further study to determine how the modulatory action
of β-amyloid on cytokine-mediated astrocytic release of nitric oxide affects neuronal functions in AD patients.

1. Introduction

In Alzheimer’s disease (AD), the accumulation of β-amyloid is a characteristic feature seen in certain areas of the brain (Beyreuther et al., 1991; Marotta et al., 1992). β-amyloid deposition can occur in the absence of neuronal degeneration in patients with AD. Non-compacted, diffuse deposits of β-amyloid are not always associated with dystrophic neurites and reactive astrocytes. In contrast, the dense-core type (or matured) neuritic plaques are almost always associated with dystrophic neurites (Hardy and Allsop, 1991; Ikeda et al., 1990). These observations suggest that neuritic plaques progress from the diffuse inert form to the β-pleated amyloid plaque. β-amyloid deposits also occur around cerebral vasculature (Wisniewski et al., 1992). Such perivascular depositions of β-amyloid cause amyloid angiopathy characterized by endothelial degeneration and obliteration of the vessel lumen (Wisniewski and Wegiel, 1992). β-amyloid angiopathy is also accompanied by neuronal degeneration and reactive astrocytes (Itagaki et al., 1989; Wisniewski et al., 1992).

At the site of β-amyloid deposits in the AD brain, reactive astrocytes and their processes are at the margins of the deposits (Wisniewski and Wegiel, 1992). Such morphological observations led to the suggestion that astrocytes surrounding β-amyloid are involved in the dispersal, removal or isolation of β-amyloid from the neuropil. However, reactive astrocytes are known to upregulate many proteins with potent biological effects (Norenbert, 1994), and astrocytic neurotrophic factors appear to support regeneration and recovery of damaged neurons (Murphy and Grzybicki, 1996). Therefore, it is possible that
β-amyloid changes the intercellular environment by altering astrocytic functions. For example, it was shown that β-amyloid stimulates astrocytic release of interleukin-1 (IL-1) and basic fibroblast growth factor (bFGF) (Araujo and Cotman, 1992). This *in vitro* study suggests the possibility that reactive astrocytes are responsible for the high IL-1 and bFGF immunoreactivity observed in AD, particularly around neuritic plaques (Gomez-Pinilla et al., 1990; Griffin et al., 1989). Expression of potential mediators of reactive gliosis, such as bFGF, which astrocytes synthesize and respond to by increased GFAP levels, has also been shown in cultured astrocytes (McMillian et al., 1994). Interestingly, a marked increase in GFAP levels has also been detected in the brain of AD (Harpin et al., 1990; Panter et al., 1985). One of the sites found to have high GFAP immunoreactivity was neuritic plaques (Duffy et al., 1980).

Astrocytes proliferate in response to IL-1β and release TNF-α (Lee et al., 1993). Cytokines, such as IL-1β, TNF-α, and IFN-γ, have been shown to induce release of nitric oxide from a variety of glial cells including astrocytes (Lee et al., 1993; Murphy et al., 1993). This cytokine-mediated release of nitric oxide by astrocytes is important because nitric oxide is a potent vasodilator of cerebral blood vessels (Moncada et al., 1991) and levels of nitric oxide released by activated astrocytes are sufficient for induction of smooth muscle relaxation and subsequent dilation of cerebral vasculature (Faraci and Brian, 1994).

Astrocytes have their endfeet processes in close proximity to the endothelial cells of cerebral capillaries and are believed to play a role in regulation of endothelial cells (Montgomery 1994; Risau and Wolburg, 1990). Thus, astrocytic endfeet processes are an important element that regulates nitric oxide-mediated relaxation of cerebral vasculature.
Although the effect of cytokines such as IL-1β, IFN-γ and TNF-α on astrocytic release of nitric oxide is well documented (Murphy et al., 1993), the effect of the particular cytokine combination (IFN-γ + TNF-α) on induction of astrocytic nitric oxide is not known. Also, the effects of β-amyloid on astrocytes for their cytokine-mediated release of nitric oxide is not established. In the present study astrocytic release of nitric oxide was studied following exposure to β-amyloid peptide (β25-35) in combinations with IL-1β, TNF-α or IFN-γ.

2. Materials and Methods

2.1 Synthesis of β-amyloid (β25-35) and Scrambled Peptide

Scrambled peptide and β-amyloid (β25-35) peptides were synthesized and purified by the University of Iowa Protein Structure Facility. The scrambled peptide has the following sequence: H-IIe-Met-Leu-Gly-Asn-Gly-Asn-Gly-Ala-Ser-IIc-Gly-OH (Mattson et al., 1992). Cultured neurons were exposed to β-amyloid by the method described previously (Goodwin et al., 1997; Parpura-Gill et al., 1997). Briefly, scrambled peptide and β25-35 peptides were dissolved in sterile tissue culture water (Sigma, pH 7.2) at a concentration of 1mg/ml, and aliquots were stored at -20°C. Culture wells were coated with β25-35 or scrambled peptide by placing the peptide solutions (20 µl of a stock solution) onto the culture wells. Tissue culture wells were left to dry overnight at room temperature after which they were used for astrocyte cultures.

2.2 Astrocyte Culture

Primary glial cultures were prepared from the hippocampus of P2-5 Sprague-Dawley rats. The hippocampus was dissected and mixed glial cultures were grown in flasks containing: Eagle's minimum essential media supplemented with 10% heat inactivated fetal
bovine serum, 40 mM glucose, 2 mM L-glutamine, 1 mM pyruvate, 14 mM sodium bicarbonate, penicillin 100 i.u./ml and streptomycin 100 μg/ml (pH 7.35) (Parpura-Gill et al., 1997). At confluency, flasks were shaken manually and the media containing mainly non-adherent microglial cells were removed. Fresh media were added to the adherent cells and flasks were shaken overnight (200 rpm @ 37°C). The media were removed once again and adherent cells were scraped off the bottom of the flasks and mechanically dissociated. This cell suspension was plated into new flasks for 30 min. to allow the remaining microglia to attach. Non-adherent cells were collected and plated onto 96-well tissue culture plates. Purity of the astrocyte cultures, as determined by GFAP immunocytochemistry staining, was greater than 95%.

Astroglial release of nitric oxide was examined by exposing astrocytes to β25-35 coated on to the surface of culture wells and cytokines (IL-1β, TNF-α and IFN-γ) in the media. Although β-amyloid found in the AD neuritic plaques is immobile and insoluble, most previous tissue culture studies have sought to determine β-amyloid’s effects by solubilizing it or suspending it in culture medium. The alternative method used in our study (Goodwin et al., 1995; Parpura-Gill et al., 1997) not only ensures that most cells are exposed to β-amyloid, but it also prevents any possible physical cellular damage by aggregated and mobile β-amyloid. At postplating day 6, IL-1β (100 U/ml, Boehringer-Mannheim), IFN-γ (100 U/ml, Gibco BRL), TNF-α (100 U/ml, Genzyme) and their combinations (IFN-γ + IL-1β or IFN-γ + TNF-α) were added to the astrocyte cultures. At postplating day 8, the culture media were collected for nitrite analysis. Concentrations of nitrite, a nitric oxide metabolite,
in the media were used as a measure of nitric oxide generation. Each experiment was performed in a minimum of two wells per condition and was replicated at least four times.

To investigate whether nitrite measured in the media was of nitric oxide origin, astrocytes were exposed to N\textsuperscript{G}-Monomethyl-L-arginine (NMMA) (Calbiochem), a competitive inhibitor of nitric oxide synthetase (Hibbs et al., 1988). At postplating day 6, cultured astrocytes were exposed to cytokines [IL-1\(\beta\) (100 U/ml), IFN-\(\gamma\) (100 U/ml), TNF-\(\alpha\) (100 U/ml) and their combinations (IFN-\(\gamma\) + IL-1\(\beta\) or IFN-\(\gamma\) + TNF-\(\alpha\))] with addition of 500 nM NMMA. At postplating day 8, tissue culture media were tested for nitrite levels. Each experiment was performed in a minimum of two wells per condition and was replicated at least three times.

Nitric oxide release from astrocytes was initially studied by plating cells onto culture wells coated with or without 825-35. In the subsequent experiment, the effects of scrambled control peptide on astrocytic release of nitric oxide was assessed. Astrocytes were cultured in culture wells either coated with scrambled peptide or uncoated. At postplating day 6, IL-1\(\beta\) (100 U/ml), IFN-\(\gamma\) (100 U/ml), TNF-\(\alpha\) (100 U/ml) and their combinations (IFN-\(\gamma\) + IL-1\(\beta\) or IFN-\(\gamma\) + TNF-\(\alpha\)) were added into the culture media for 2 days after which, media were collected and assayed for nitrite content. Each experiment was performed in a minimum of two wells per condition and was replicated at least four times.

2.3. Nitric Oxide Assay

Nitrite levels were determined by a colorimetric assay based on the Griess reaction. Equal volumes of Griess reagent (0.05% N-(1-naphthyl)ethylenediamine dihydrochloride and 0.5% sulfanilamide in 2.5% phosphoric acid) and either samples or standards were mixed
and their absorption read on an Elisa reader at 550 nm (Molecular Devices, Vmax). Concentrations were expressed as a ratio of nitrite level per milligram of total cell protein in a culture. As an additional precaution, percent change of each nitrate/protein ratio of experimental groups from the nitrite/protein ratio of the control cultures was calculated and used in statistical analyses. The percent change was calculated to remove the potential artifactual effects due to differences in cell proliferation among cultures.

2.4. Cellular Protein Assay

Cellular protein assay was used as an index of cell proliferation. In the first experiment, after media collection, astrocyte cultures were washed three times with 0.1 M PBS and subsequently air dried. Total cell protein content was determined using the Bio-Rad protein assay kit. Absorption was read on an Elisa reader at 630 nm with reference to 490 nm. In the subsequent experiments astrocyte cultures were washed three times with 0.1 M PBS and were subsequently lysed with 0.1N NaOH overnight. Total cell proteins were determined in cell lysates using the standard protocol for microtiter plates of the Bio-Rad protein assay kit. Absorption was read on an Elisa reader at 595 nm. Total cell proteins were expressed in milligrams and treatment related changes were statistically evaluated.

2.5. Statistical Analysis

Two way analysis of variance (ANOVA) was performed. Levels of nitrate production were expressed as percentage of reduction or increase from the control. Tukey's B posthoc procedure was used to determine any intertreatment differences.
3. Results

3.1. Effect Of β25-35 On Astrocytic Release Of Nitric Oxide Mediated By IL-1β, TNF-α And IFN-γ

To examine whether β25-35 affects cytokine-induced astroglial release of nitric oxide, astrocytes cultured on β25-35 peptide for 6 days were subsequently exposed to cytokines (IL-1β, TNF-α, and IFN-γ). Astrocytes not exposed to either β25-35 peptide or cytokines served as controls. Astrocytes exposed to β25-35 had significantly higher content of cellular protein than control cultures [F(1, 248) = 137.22, p < 0.0001]. However, none of the cytokines tested affected the cellular protein content (Fig. 1).

![Graph showing the effect of β25-35, IL-1β, TNF-α, and IFN-γ on astrocyte protein.](image-url)

**Fig 1.** Effect of β25-35, IL-1β, TNF-α, and IFN-γ on astrocyte protein. Astrocytes exposed to β25-35 for 8 days had significantly higher content of cellular protein than control cultures. However, none of the cytokines tested affected the cellular protein content. Values represent mean ± S.E.M.
None of the cytokines induced an increase in nitrite levels (nmol/mg protein) in astrocytic cultures by themselves. However, IFN-γ combined with IL-1β or TNF-α induced a significant increase in nitrite levels \(F(4,203)=75.26, p<0.0001\) (Fig. 2). Although both IL-1β and TNF-α were effective costimulatory factors, the combination of IFN-γ and IL-1β was more effective in increasing nitrite levels in cultures than the combination of IFN-γ and TNF-α \(p<0.01\). B25-35 significantly affected cytokine-mediated increase in nitrite levels \(F(1,203)=13.64, p<0.0003\), i.e., B25-35 blocked the increase in nitrite levels by IFN-γ and TNF-α \(p<0.01\),

![Fig 2. Effect of B25-35 on cytokine-mediated astrocytic release of nitric oxide. Astrocytes were cultured on B25-35 peptide for 6 days, then exposed to cytokines for 2 days before measuring nitrite. None of the cytokines induced an increase in nitrite levels (nmol/mg protein) in astrocytic cultures by themselves. However, IFN-γ combined with IL-1β or TNF-α induced a significant increase in nitrite levels. B25-35 completely blocked the increase in nitrite levels by IFN-γ and TNF-α, whereas B25-35 only partially blocked the effect of IFN-γ and IL-1β. Values represent mean ± S.E.M.](image-url)
whereas β25-35 only partially decreased the effect of IFN-γ and IL-1β (p<0.01). Thus, nitrite levels detected in these cultures were still greater than the levels found in the control (p<0.01). This differential effect of β-amyloid on cytokine-mediated release of nitric oxide was evidenced by the presence of a significant interaction between β-amyloid and cytokine related effects [F(4,203)=3.35, p<0.01].

To examine whether nitrite accumulation in the media was nitric oxide synthase-specific, astrocytes were exposed to N⁵-monomethyl-L-arginine (NMMA: 500 nM). The effect of cytokines (IFN-γ + IL-1β, IFN-γ + TNF-α) on nitrite levels in the astrocytic supernatant was substantially reduced by the inclusion of NMMA in the culture, and no significant effects of β25-35 or cytokines were detected (Fig. 3). The result indicates that the previously described β25-35 and cytokine effects were indeed due to a change in nitric oxide synthase activity and the release of nitric oxide, not some other source of nitrite.

![Fig 3. Effect of N⁵-Monomethyl-L-Arginine (NMMA) on astrocytic release of nitric oxide.](image)

The effect of cytokines (IFN-γ+IL-1β, IFN-γ+TNF-α) on nitrite levels in the astrocytic supernatant was substantially reduced by the inclusion of NMMA, suggesting that the effect of β25-35 and cytokine shown in Fig. 2 was due to a change in nitric oxide synthase activity. Values represent mean ± S.E.M.
3.2. Effect of Control Peptide On Astrocytic Release Of Nitric Oxide Mediated By IL-1β, TNF-α And IFN-γ

Since our initial experiment did not include scrambled peptide as the control, a separate experiment was performed to assess the effect of scrambled peptide on astrocytic release of nitric oxide mediated by IL-1β, TNF-α and IFN-γ. Astrocytes cultured for 6 days on scrambled peptide were exposed to each cytokine and their combinations for 2 days after which, media were collected and assayed for nitrite content. No significant effects of scrambled control peptide or cytokines on cellular protein content were detected (data not shown). As in the preceding experiment, IFN-γ in combination with IL-1β or in combination with TNF-α were effective in increasing nitrite levels \(F(4,140)=23.14, p<0.0001\) (Fig. 4).

![Figure 4](image_url)

**Fig 4.** Effect of control peptide on astrocytic release of nitric oxide. Since our initial experiment (Figs. 1, 2) did not include scrambled peptide as the control, a separate experiment was performed to assess the effect of control peptide on astrocytic release of nitric oxide. IFN-γ in combination with IL-1β or in combination with TNF-α were effective in increasing nitrite levels. However, scrambled B25-35 peptide did not affect nitrite levels neither alone nor in combination with cytokines. Values represent mean ± S.E.M.
However, scrambled control peptide did not affect nitrite levels either alone or in combination with cytokines. Furthermore, no significant interaction was detected, demonstrating specificity of B25-35 peptide effect in the modulation of the nitric oxide release from astrocytes.

4. Discussion

Previous studies have shown that astrocytes are capable of releasing nitric oxide and that certain cytokines including IFN-γ, IL-1β, and TNF-α are inducers of inducible nitric oxide synthase (iNOS) in astrocytes (Murphy et al., 1993). Our present study demonstrated that IFN-γ (100U/ml) in combination with IL-1β (100U/ml) or TNF-α (100U/ml) is a potent inducer of astroglial release of nitric oxide. Furthermore, we found that IL-1β is a more potent cofactor in inducing nitric oxide release from hippocampal astrocytes than TNF-α. Since IFN-γ, IL-1β, TNF-α and the combination of IL-1β and TNF-α (results not shown) failed to induce nitric oxide release, the synergistic action of IFN-γ appears to be necessary for astroglial release of nitric oxide. Unlike other studies that showed TNF-α alone as an iNOS inducer on C6 glioma cells (Murphy et al., 1993; Rossi and Bianchini, 1996), TNF-α alone in our study did not enhance the release of nitric oxide from hippocampal astrocytes. Similarly, hippocampal microglial cells were also reported not to respond to TNF-α alone (Goodwin et al., 1995).

B25-35 did not alter the astrocytic release of nitric oxide in the presence of cytokines IFN-γ, TNF-α, or IL-1β. However, it significantly reduced the release of nitric oxide mediated by IFN-γ in combination with IL-1β or TNF-α. In contrast, a recent report has shown that β-amyloid stimulates the production of nitric oxide from C6 glioma cells in the...
presence of cytokines IFN-γ, TNF-α, or IL-1β (Rossi and Bianchini, 1996). It is possible that
this difference in the effect of B25-35 reflects the method used in each study. Our study was
based on astrocytes from postnatal (P2-5) rat hippocampus and exposing the cultured
astrocytes to B25-35 for 8 days. This method differs from the previous study (Rossi and
Bianchini, 1996) in which C6 glioma cells were used in a shorter exposure time (24 hrs) to
an aqueous solution of B25-35. Since the importance of chronic exposure has been shown in
inducing significant neuronal responses, e.g., apoptosis (Forloni et al., 1993) and oxidative
stress (Cafè et al., 1996), our present study was designed to examine a longer-term (8 days)
effect of β-amyloid on astrocytic release of nitric oxide.

The other factor considered in the present study was the effect of β-amyloid on cell
population. In the previous study by Rossi and Bianchini (1996), the nitrite level in C6 cell
culture was expressed as nitrite per cell population at the time of cell plating in cultures
exposed to β-amyloid for 24 hrs. β-amyloid is known to induce significant astrocytic
proliferation within 24 hours (Araujo and Cotman, 1992). Our study also showed a
significant increase in the total cellular protein by B25-35, suggesting proliferation of
astrocytes. This effect was specific for β-amyloid as scrambled peptide did not induce a
similar effect. To account for changes in plated cell population, nitrite levels in the present
study were expressed as nitrite (nmol) per milligram of cellular protein, and the percent
change of nitrite levels from the control cultures was analyzed statistically. Thus, the effect
of β-amyloid on cytokine-mediated astrocytic release of nitric oxide was not due to astrocytic
proliferation, but rather to a specific response of each astrocyte.
The inhibitory effect of B25-35 on cytokine-mediated release of astrocytic nitric oxide was significant, but not equally effective for all cytokines: the inhibitory effect of B25-35 on the release induced by IFN-γ in combination with TNF-α was complete, while that on IFN-γ in combination with IL-1β was not. The reason for this difference is not known.

As shown in the rat astrocyte cell line C6 (Rossi and Bianchini, 1996), IL-1β could be a more potent inducer of astrocytic nitric oxide than TNF-α. However, IL-1β alone is known to induce nitric oxide release from C6 astrocytes, but not from hippocampal astrocytes (Murphy et al., 1993; Simmons and Murphy, 1993). Similarly, hippocampal astrocytes in our present study did not respond to IL-1β alone. Since, cultured astrocytes respond to β-amyloid by releasing IL-1β (Araujo et al., 1992), it is likely that this additional IL-1 may account for the incomplete suppressive action of β-amyloid on astrocytic release nitric oxide.

At present, the importance of astrocyte-derived nitric oxide in the CNS pathophysiology is not well understood. Neuronal nitric oxide has been shown to mediate glutamate-induced dilation of cerebral arterioles (Faraci and Breese, 1993; Faraci and Brian, 1994), and inhibitors of nitric oxide synthase block increases in blood flow in neuronally active regions of the brain (Arisztid et al., 1992). These observations support the notion that nitric oxide activation of soluble guanylyl cyclase in vascular smooth muscle or pericytes may modulate cerebral blood flow (Murphy et al., 1993). Similarly, increased astroglial release of nitric oxide by cytokines has been proposed to transduce metabolic or intrinsic neuronal signaling, and to dilate cerebral vessels and alter cerebral blood flow (Kovach et al., 1992; Ladecola, 1992; Murphy et al., 1993; Wang et al., 1992). If both neuronal and astrocytic nitric oxide play a role in regulating local blood flow, neuronal losses reported in certain
areas of the AD brain (Coleman and Flood, 1987) imply that cerebral vasculature may have to rely on astrocytes for regulation of the local cerebral blood flow.

It is conceivable that suppressive action of β-amyloid on astrocytic release of nitric oxide may interfere with regulation of regional cerebral blood flow and could be responsible for the reduction in regional cerebral blood flow known to occur in AD patients (Bonte et al., 1990; Bonte et al., 1993; Holman et al., 1992). Human astrocytes release nitric oxide upon stimulation with a combination of IL-1β and IFN-γ (Lee et al., 1993), and increased levels of cytokines (IL-1, TNF-α, IFN-γ) have been detected in the brain of AD patients (Dickson et al., 1993; Griffin et al., 1989; Haga et al., 1989; Huberman et al., 1994). Therefore, it is possible that β-amyloid also exerts a similar suppressive effect on human astrocytes. However, it remains to be determined how the modulatory action of β-amyloid on astrocyte-generated nitric oxide induced by cytokines may affect vascular functions and local cerebral blood flow in situ.

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THE INHIBITORY EFFECTS OF β-AMYLOID ON GLUTAMATE AND GLUCOSE UPTAKES BY CULTURED ASTROCYTES

A paper published in Brain Research

Aleksandra Parpura-Gill\(^2\), Donald Beitz\(^3\), and Etsuro Uemura\(^2\)^5

\(^1\)Department of Anatomy and Neuroscience Program, \(^2\)Department of Animal Science, Iowa State University, Ames IA 50011

Abstract

β-amyloid is the primary protein component of neuritic plaques, which are degenerative foci in brains of patients with Alzheimer’s disease (AD). The effects of this naturally occurring β-amyloid on the cells of the central nervous system have not been completely understood. β-amyloid increases the vulnerability of cultured neurons to glutamate-induced excitotoxic damage. Because astrocytes play a key role in uptake of extracellular glutamate and glutamate uptake is ATP dependent, we studied the effect of β25-35 on glutamate and glucose uptake in cultured hippocampal astrocytes following 7 days of exposure to β25-35. Astrocytic glutamate uptake was studied at 1, 5, 10, 15, 20, and 60 min following the addition of \(^{1}\)H]glutamate (5 nM) to the culture media.

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\(^2\) Graduate student and Associate Professor, respectively, Department of Anatomy and Neuroscience Program, Iowa State University.

\(^3\) Associate Professor, Department of Animal Science, Iowa State University

\(^4\) Primary researcher and author

\(^5\) Author for Correspondence
and astrocytic glucose uptake was assessed at 60 min after the addition of [\(^{14}\text{C}\)]glucose (600 nM and 640 nM) to the media. Glutamate uptake by control astrocytes was time-dependent. Astrocytes exposed to β25-35, however, showed significantly lower glutamate uptake at all sampling times. Similarly, [\(^{14}\text{C}\)]glucose uptake by astrocytes was inhibited by β25-35. When glucose uptake was blocked by phloretin (10 mM), astrocytic [\(^{3}\text{H}\)]glutamate uptake was also blocked, suggesting that the inhibitory effect of β-amyloid on glutamate uptake is caused by diminished glucose uptake. Thus, our present study suggests a possible link between two proposed mechanisms of pathogenesis of the Alzheimer’s disease: glutamate neurotoxicity and global defect in cerebral energy metabolism.

1. Introduction

β-amyloid is the primary protein component of neuritic plaques, which are degenerative foci in brains of patients with Alzheimer’s disease (AD) (Marotta et al., 1992). The morphology of neuritic plaques varies from diffuse deposits of β-amyloid to dense core β-amyloid plaques that usually are associated with dystrophic neurites, reactive astrocytes, and microglial cells (Hardy and Allsop, 1991, Ikeda et al., 1990). These observations suggest that neuritic plaques progress from a diffuse inert form to β-pleated amyloid plaques accompanied by dystrophic neurites. To date, the effects of this naturally occurring β-amyloid on cells of the central nervous system have not been determined; however, when β-amyloid plaque cores, isolated from brain tissue of AD patients, were injected into the hippocampus of rats, neuronal loss was observed by one month post-injection (Frautschy et al., 1991). Although the injected material contained all of the constituents of plaques, not just
β-amyloid, it is suggestive that β-amyloid from brains of AD patients has neurotoxic properties.

The evidence supporting β-amyloid deposition as the primary pathological process is the identification of pathogenic mutations in the β-amyloid precursor protein gene and neuropathology similar to AD in transgenic mice overexpressing β-amyloid precursor proteins (Games et al., 1995). Although degenerating neurons in transgenic mice accompany the β-amyloid deposition, the mechanisms involved in β-amyloid-mediated neuronal degeneration are not well understood. However, it is known that the neurotoxic portion of β-amyloid is amino acids 25-35 (β25-35) of the full-length β-amyloid peptide (β1-43) (Mattson et al., 1992, Yanker et al., 1989), and toxicity of β-amyloid depends at least in part, on its aggregation states (Pike et al., 1991, Pike et al., 1993). In vitro studies have shown that β-amyloid increases the vulnerability of cultured neurons to glutamate-induced excitotoxic damage (Kanai et al., 1993) by increasing the influx of extracellular Ca^{2+} and subsequently elevating the intraneuronal free Ca^{2+} concentration (Joseph and Han, 1992, Mattson et al., 1992). These in vitro studies suggest that any condition that elevates focal glutamate levels could enhance neuronal susceptibility to excitotoxic cell injury.

Normally, the extracellular levels of glutamate rise to high levels only briefly and in a spatially localized manner during synaptic transmission (Choi, 1988, Nichols and Attwell, 1990). Extracellular glutamate is taken up rapidly by astrocytes, where it is converted chiefly to glutamine by glutamine synthetase (Farinelli and Nicklas, 1992). The essential role of astrocytes in maintaining low extracellular levels of glutamate was demonstrated clearly in studies showing a significant increase in neuronal susceptibility to glutamate toxicity under
neuron-astrocyte co-culture with relatively few astrocytes (Rosenberg and Aizenman, 1989). Neuronal death, however, occurs even in astrocyte-rich co-culture when glutamate uptake into astrocytes is blocked, suggesting the importance of astrocytic glutamate uptake in glutamate neurotoxicity (Rosenberg et al., 1992). These findings are important in view of the evidence that showed an acute inhibitory effect of β-amyloid on glutamate uptake into cultured astrocytes (Harris et al., 1995; Harris et al., 1996; Parpura-Gill et al., 1995) and that glutamate uptake decreases in the brains of AD patients (Kanai et al., 1993).

Astrocytes require ATP to maintain the glutamate uptake process (Nichols and Attwell, 1990; Ogata et al., 1992; Swanson, 1992). As long as glucose is available, astrocytes can maintain ATP levels and continue glutamate uptake in both in vitro and in situ conditions (Swanson, 1992; Swanson et al., 1994). The presence of glutamate-coupled glucose utilization in astrocytes (Pellerin and Magistretti, 1994; Swanson et al., 1990) further suggests the interdependency of glucose and glutamate metabolisms in astrocytes. Therefore, the purpose of the present study was to explore the effect of β-amyloid on astrocytic glutamate and glucose uptakes in vitro.

2. Materials and methods

2.1. Synthesis of β-amyloid and scrambled peptide

β-amyloid (B25-35) and scrambled peptide (Mattson et al., 1992) were synthesized and purified by the University of Iowa Protein Facilities (Iowa City, IA). The scrambled peptide has the following sequence: H-Ile-Met-Leu-Gly-Asn-Gly-Ala-Ser-Ile-Gly-OH. B25-35 and the scrambled peptide were dissolved in sterile tissue culture water (Sigma) in a stock concentration of 1 mg/ml (pH 7.2). Aliquots were stored at -20°C. Culture wells were coated
with β25-35 or scrambled peptide by placing a drop of peptide solution (50 μl of stock solution) on to the culture wells. Culture wells were left to dry over night at room temperature.

2.2. Astrocyte cultures

Astrocytes were obtained from the primary glial cultures prepared from the hippocampi of P2-5 Sprague-Dawley rats. The hippocampi were dissected and mixed glial cultures were grown in flasks containing Eagle's minimum essential medium (MEM) supplemented with 10% heat-inactivated fetal bovine serum, 40 mM glucose, 2 mM L-glutamine, 1 mM pyruvate, 14 mM sodium bicarbonate, penicillin (100 i.u./ml), and streptomycin 100 μg/ml (pH 7.35). At confluency, flasks were shaken manually and the medium containing non-adherent microglial cells was removed. Fresh medium containing additional 5 mM L-leucine-methylester was added to flasks with adherent cells, and the flasks were shaken overnight (200 rpm @ 37°C). Then, the medium was removed, and adherent cells were scraped from flasks and then mechanically dissociated from each other. Cell suspensions were plated into new flasks for 30 min to allow any remaining microglia to attach. Non-adherent cells were collected and plated onto 24-well tissue culture plates coated with either β25-35 or scrambled control peptide, or directly onto culture wells. More than 98% of the cells stained positive for GFAP.

2.3. Glutamate uptake

The effect of β-amyloid on glutamate uptake into cultured astrocytes was measured by use of L-[G-³H]glutamate (46 Ci/mmol, Amersham). Astrocytes plated directly onto culture wells served as the control. At 7 days postplating, the complete culture media were
replaced with MEM supplemented with 10 mM HEPES buffer, and [\textsuperscript{3}H]glutamate was added to the cultures at 5nM final concentration. To determine background glutamate uptake for each experimental group, some cultures were incubated with the glutamate uptake blocker, D,L-threo-ß-hydroxyaspartate (TBA; 1 mM), starting 15 min before the addition of [\textsuperscript{3}H]glutamate to the culture media. Glutamate uptake was stopped at 1, 5, 10, 15, 20, and 60 min by three washes with ice-cold PBS (0.1M Na2HPO4, 0.1M NaH2PO4), and cells were lysed in 400 µl of 0.1N NaOH overnight. Cell lysates were used for determination of radioactivity by using a beta liquid scintillation counter (Packard, 1600TR) and for assaying of total cell protein (Bio-Rad protein assay kit). Glutamate uptake was expressed as pmoles of glutamate/mg of total cellular protein. Glutamate uptake is defined as the amount of radioactivity derived from [\textsuperscript{3}H]glutamate retained in cells at specified times. The experiment was performed in triplicate and repeated through at least 3 different culture sessions.

A separate experiment was performed to assess the effect of scrambled peptide on glutamate uptake by comparing uptake by astrocytes plated directly onto culture wells and by those plated onto scrambled peptide. In this experiment, [\textsuperscript{3}H]glutamate uptake in astrocytes also was quantified at 1, 5, 10, 15, 20, and 60 min following the addition of [\textsuperscript{3}H] glutamate (46 Ci/mmol) to culture media at a final concentration of 5nM. Cultures treated with 1 mM TBA were used to determine background glutamate uptake. Glutamate uptake and total cell proteins were assayed in triplicate and were replicated at least 4 times. Glutamate uptake was expressed as pmol of glutamate/mg of cellular protein.

The effect of nitric oxide on glutamate uptake by astrocytes was studied by exposing cultured astrocytes to sodium nitroprusside (SNP), which is a spontaneous generator of nitric
oxide in aqueous solution (Southam et al., 1991). Sodium nitroprusside (5 mM) was added to cultures 15 min before the addition of [3H]glutamate at a final concentration of 5nM. Glutamate uptake was measured at 5 and 15 min incubation times. Cultures treated with 1 mM TBA were used to determine background glutamate uptake. Glutamate uptake and total cell protein were assayed as described before in triplicate and were replicated at least 4 times. Glutamate uptake was expressed as pmol of glutamate/mg of cellular protein.

2.4. Glucose uptake

Glucose uptake by astrocytes was assessed in cultured astrocytes treated with β25-35 peptide or scrambled control peptide. Astrocyte cultures treated with glucose uptake blocker, phloretin, were used as a positive control in each experiment, and those astrocytes plated directly onto culture wells were used as the negative control. At 7 days postplating, culture media were replaced with MEM supplemented with 10 mM HEPES buffer, and D-[U-14C]glucose (313 and 336 mCi/mmol, ICN) was added to the culture wells at final media concentrations of 600 nM and 640 nM (0.1 μCi/500μl media in well). The positive control cultures received 10 mM phloretin 15 min before addition of [14C]glucose. Glucose uptake was stopped after 0 and 60 min of incubation by 3 washes with ice-cold PBS. Cells were lysed in 400 μl of 0.1N NaOH overnight. Cell lysates were used for determination of radioactivity and total cell protein. Time 0 was used to determine the background uptake of [14C]glucose. Glucose uptake was expressed as nmol of glucose/mg of cellular protein. Glucose uptake is defined as the amount of radioactivity derived from [14C]glucose retained in cells at specified times. All experiments were conducted in triplicate and repeated in at least 4 separate cultures.
2.5. Inhibitory effect of phloretin on glutamate uptake

The role of glucose on glutamate uptake was studied by exposing cultures to 10 mM phloretin. At 7 days postplating, culture media were replaced with MEM supplemented with 10 mM HEPES buffer, and [³H] glutamate (46 Ci/mmol) was added to culture wells at a final concentration of 5nM. Phloretin was added 15 min before addition of [³H] glutamate. Glutamate uptake was stopped at 0 and 60 min incubation periods by 3 washes with ice-cold PBS. Cultures treated with 1 mM TBA were used to determine background glutamate uptake. Glutamate uptake and total cell protein were assayed in triplicate and were replicated at least 3 times. Glutamate uptake was expressed as pmol of glutamate/mg of cellular protein.

2.6. Total cell protein assay

Total cell protein in each culture well was determined by using the Bio-Rad Protein Assay kit. Protein concentrations were calculated from the absorbency values. The standard curve was obtained by using bovine gamma globulin as a standard at serial dilutions in the linear range of the assay. Absorptions were read on an Elisa reader at 595 nm (Molecular Devices).

2.7 Statistical analysis

All data were analyzed for treatment differences by using analysis of variance (ANOVA). Intertreatment differences were determined by subsequent posthoc test (Tukey’s B).
3. Results

3.1. Effect of β25-35 on astrocytic glutamate uptake

To examine whether β-amyloid has a time-dependent effect on glutamate uptake into cultured astrocytes, glutamate uptake was studied at 1, 5, 10, 15, 20, and 60 min following the addition of [³H]glutamate in culture media (Fig. 1).

Fig. 1. Effect of β25-35 on astrocytic glutamate uptake. Astrocytes were exposed to β25-35 peptide for 7 days after plating on day 0. The accumulation of [³H]glutamate in the control astrocytes was significantly greater than that in those groups exposed to β25-35 at all assay times [P<0.0001]. This greater level of glutamate uptake in control astrocytes also increased linearly with time (r²=0.87). As indicated by the absence of interaction between the sampling time and the experimental condition (control and β-amyloid-treated), results indicate the inhibitory action of β-amyloid on glutamate uptake by astrocytes. Values represent mean + S.E.M. (n=12).
Astrocytes plated directly onto culture wells served as the control. The accumulation of
[^H]glutamate in the control astrocytes was significantly greater than that in those groups
exposed to β25-35 at all assay times \(F(1,143)=54.29, P<0.0001\). This greater level of
 glutamate uptake in control astrocytes also increased linearly with time \(r^2=0.87\). In contrast,
astrocytes exposed to β25-35 showed significantly lower glutamate uptake as compared with
that of control astrocytes at all sampling times \(F(1,143)=54.29, P<0.0001\). As indicated by
the absence of interaction between the sampling time and the experimental condition
(control and β-amyloid-treated), the results clearly indicate the inhibitory action of β-amyloid
on glutamate uptake by astrocytes.

Since our initial experiment did not include scrambled peptide as the control, a
separate experiment was performed to assess the effect of scrambled peptide on glutamate
uptake. Glutamate uptake by astrocytes was studied in the same manner as in the previous
experiment. There was no difference in glutamate uptake between astrocytes plated directly
onto culture wells and those plated onto the scrambled control peptide (data not shown).
Both groups of astrocytes showed a time-dependent linear increase in glutamate uptake
\(r^2=0.87\). These results suggest that glutamate uptake is not affected by the scrambled
control peptide, but is inhibited by β25-35.

3.2. Effect of β25-35 and nitric oxide on astrocytic glutamate uptake

Sodium nitroprusside (SNP) was used to study the effect of nitric oxide on astrocytic
 glutamate uptake at 5 and 15 min following exposure to[^H]glutamate (Fig. 2). Sodium
nitroprusside (0.5 mM) did not affect glutamate uptake into astrocytes as the uptake by
SNP-treated cultures was not different from that by control cultures.
Fig. 2. Effect of β25-35 and nitric oxide on astrocytic glutamate uptake. Astrocytes were exposed to β25-35 peptide for 7 days after plating on day 0. At 7 days postplating, the effect of sodium nitroprusside (SNP) on glutamate uptake was measured at 5 and 15 min incubation times. SNP (0.5 mM) did not affect astrocytic glutamate uptake. Accumulation of glutamate in control astrocytes was significantly higher at 15 min sampling time than at 5 min [P=0.001]. However, astrocytes exposed to β25-35 showed significantly lower glutamate levels than the control astrocytes did at both sampling times [P=0.0016]. A combination of SNP and β25-35 did not induce any more significant inhibition than β25-35 alone did at either 5 or 15 min sampling times, confirming that SNP does not inhibit glutamate uptake into astrocytes. Values represent mean ± S.E.M. (n=12).

However, astrocytes exposed to β25-35 showed significantly lower glutamate uptake than the control astrocytes did at both sampling times [F(1,88)=10.65, P=0.0016]. A combination of SNP and β25-35 did not cause any more significant inhibition than β25-35 alone did at either 5 or 15 min sampling times, confirming that SNP does not inhibit glutamate uptake into astrocytes.
3.3. Effect of β25-35 on astrocytic glucose uptake

To assess astrocytic glucose uptake, astrocytes treated with β25-35 or scrambled control peptide were incubated with $[^{14}\text{C}]$glucose for 60 min. Phloretin-treated cultures were used as a positive control in each experiment and those astrocytes plated directly onto culture wells were used as the negative control. Glucose uptake was significantly lower in the astrocytes exposed to β25-35 (P<0.01) or phloretin (P<0.01) as compared with that of the control groups $[F(3,47)=6.189, \text{P}=0.0013]$ (Fig. 3).

![Graph showing glucose uptake](image)

**Fig. 3.** Effect of β25-35 on astrocytic glucose uptake was assessed by exposing astrocytes to β25-35 or scrambled control peptide for 7 days, followed by incubating them with $[^{14}\text{C}]$glucose for 60 min. Phloretin-treated cultures (15 min prior to addition of $[^{14}\text{C}]$glucose) were used as a positive control in each experiment, and those astrocytes plated directly onto culture wells were used as the negative control. Glucose uptake was significantly affected by treatments (P=0.0013). The groups of astrocytes exposed to β25-35 (P<0.01) or phloretin (P<0.01) showed significantly lower glucose uptake as compared with the control groups. Values represent mean ± S.E.M. (n=12).
Astrocytes plated onto scrambled control peptide did not differ in glucose uptake from those plated directly onto culture wells, suggesting a specific inhibitory action of B25-35 on glucose uptake by astrocytes.

3.4. Effect of phloretin on glutamate uptake into astrocytes

To test the hypothesis that glutamate uptake into astrocytes is dependent upon glucose uptake, glutamate uptake into astrocytes was assessed following exposure to phloretin, a glucose uptake blocker. Astrocytes exposed to phloretin absorbed significantly less glutamate than those in the control culture \([F(1,17)=14.08, P=0.0017]\) (Fig. 4).

**Fig. 4.** Effect of phloretin on glutamate uptake into astrocytes. At 7 days post-plating, \([^3H]glutamate uptake into astrocytes was assessed following 15 min exposure to 10 mM phloretin, a glucose uptake blocker. Astrocytes exposed to phloretin were found to take up significantly less glutamate than those in the control culture \([P=0.0017]\). Values represent mean ± S.E.M. (n=9).
4. Discussion

In our present study, glutamate and glucose uptakes were examined in cultured astrocytes following 7 days of exposure to β25-35. The cellular effects of β-amyloid were studied by using a synthetic β-amyloid peptide (β25-35) that corresponds to amino acids 25-35 of the full-length peptide. Astrocytes were exposed to β25-35 coated onto the surface of culture wells (Goodwin et al., 1995). This exposure method differs from that of previous studies that have sought to determine the effects of β-amyloid by solubilizing it or suspending it in culture medium despite the fact that the β-amyloid found in the AD neuritic plaques is immobile and insoluble. Coating β25-35 onto wells of a culture dish not only ensures that most cells are exposed to β25-35, but it also prevents any possible physical cell damage by aggregated, mobile β25-35 in culture medium.

The present study on glutamate uptake into astrocytes supports the previous studies (Harris et al., 1995; Harris et al., 1996; Parpura-Gill et al., 1995). Our initial study demonstrated that β25-35 inhibits glutamate uptake by cultured hippocampal astrocytes (Parpura-Gill et al., 1995). To further investigate the inhibitory effects of β-amyloid on glutamate uptake by astrocytes, the present study examined glutamate uptake by using postnatal (P2-5) rat hippocampus and exposing the cultured astrocytes to β25-35 for 7 days. This method differs from previous studies by Harris et al. (1995, 1996) in which fetal (E18) astrocyte cultures were used in a short exposure time (30 min) to an aqueous solution of β25-35. The importance of chronic exposure in inducing significant neuronal responses, e.g., apoptosis (Forloni et al., 1993) and oxidative stress (Cafe et al., 1996) has been shown. Therefore, our present study was to examine the long-term (7 days) effect of β-amyloid on
astrocytic glutamate and glucose uptake in vitro. Glutamate uptake by control astrocytes was shown to be time-dependent, as had been reported previously (Flott and Seifert, 1991; Hara et al., 1989; Piani et al., 1993). However, those astrocytes plated onto B25-35 for 7 days showed significantly lower glutamate uptakes regardless of the sampling times (1 min to 1 hr). This inhibitory effect is specific to B25-35, because the scrambled control peptide did not affect the astrocytic glutamate uptake.

The inhibitory effect of β-amyloid on glutamate uptake by astrocytes suggests the possibility of increased neuronal vulnerability in the brain of AD patients. Both neurons and astrocytes possess a high affinity glutamate uptake system (Kanai et al., 1993); however, astrocytes have been demonstrated to take up most of the extracellular glutamate (McLennan, 1976). Since glutamine synthetase is found in astrocytes (Norenberg and Martinez-Hernandez, 1979), glutamate in culture media should be taken up rapidly by astrocytes, where it is converted chiefly to glutamine by the glutamine synthetase (Farinelli and Nicklas, 1992). Therefore, the decreased capacity of astrocytes to uptake glutamate may have significant effects on neuronal function and survival.

Glutamate-mediated neuronal cell injury has been studied extensively (Choi, 1988, Meldrum and Garthwaite, 1990). An excessive Ca\(^{2+}\) influx through NMDA glutamate receptors and prolonged elevations in intracellular Ca\(^{2+}\) levels lead to cell death (Meldrum and Garthwaite, 1990; Siesjo, 1989). This Ca\(^{2+}\) influx mediated by glutamate activates Ca\(^{2+}\)-dependent nitric oxide synthase in cultured cortical neurons, and this process can be prevented by nitric oxide synthase inhibitor (Vige et al., 1993) or competitively reversed by L-arginine (Dawson et al., 1991). Nitric oxide at high levels is neurotoxic (Chao et al., 1992).
It reacts with superoxide anions and forms peroxinitrite anions that decompose to yield highly damaging hydroxyl free radicals and nitrogen dioxide (Beckman et al., 1990). Furthermore, nitric oxide inhibits glutamate transport in hippocampal synaptosomes from rat brain (Pogun et al., 1994). These observations suggest nitric oxide as one mediator of the glutamate neurotoxicity. However, when SNP was used as a nitric oxide generator (Southam and Garthwaite, 1991), we did not detect any effect of nitric oxide on glutamate uptake by astrocytes. Neuronal and glial glutamate carriers are structurally different and respond differently to glutamate uptake blocker (Kanai et al., 1993). Therefore, it is likely that SNP affects glutamate uptake differently in astrocytes and synaptosomes.

The mechanism involved in β-amyloid-mediated inhibition of glutamate uptake is not known. β-amyloid generates oxygen-dependent free radicals in aqueous solution (Hensley et al., 1994), and it was suggested that these oxygen-dependent free radicals are responsible for inhibition of glutamate uptake into astrocytes (Harris et al., 1995; Harris et al., 1996). Moreover, the scrambled control peptide also was shown to induce significant levels of free radicals in aqueous solution (Hensley et al., 1994). Because our present study and that of Harris et al. (1996) showed that scrambled β25-35 has no inhibitory effect on astrocytic glutamate uptake, it is possible that β-amyloid may interfere with astrocytic glutamate uptake by some free radical-independent mechanisms.

In light of evidence showing that glutamate uptake is dependent mainly on glucose availability rather than oxygen (Swanson, 1992; Swanson et al., 1994) and that inhibition of glycolysis causes failure of glutamate uptake (Ogata et al., 1992; Swanson, 1992), we studied the effect of β25-35 on glucose uptake by astrocytes. The β25-35 exerted significant
inhibition on astrocytic glucose uptake, suggesting that the suppressive effect of β-amyloid on astrocytic glucose uptake affects glutamate uptake. To further support our hypothesis, we studied astrocytic glutamate uptake in the presence of phloretin, a glucose uptake blocker. Phloretin induced approximately a fourfold decrease in glutamate uptake, which is comparable to the fourfold decrease in the glutamate uptake (measured at 60 min sampling time) by β25-35. Our finding that phloretin also inhibits glutamate uptake may explain the previous studies that demonstrated glucose dependency of glutamate uptake in situ (Swanson et al., 1994). Since glial cells may respond differently to β25-35 and β1-40 (Goodwin et al., 1995), confirmation of the present study using full length β-amyloid peptide needs to be done in future studies.

Ample evidence has been provided to show that glucose metabolism may have changed in the brains of AD patients (Hoyer, 1991). Studies by positron emission tomography (PET) scans detected a decrease in glucose uptake and metabolism in the cerebral cortex (Duara et al., 1986; Jagus et al., 1991; Rapoport et al., 1991; Riege et al., 1988) and immunohistochemistry revealed a decrease in GLUT1 and GLUT3 activities in several regions of the brain including the hippocampus (Harr et al., 1995; Simpson et al., 1994). These studies suggested that the decrease in cerebral glucose uptake seen in PET studies may reflect, in part, decreased glucose transport and utilization at the cellular level. This change in glucose metabolism may result in the lower glutamate uptake found in the brain of AD patients (Kanai et al., 1993). Although our present study based on cultured rat astrocytes may not accurately represent an in situ environment for astrocytes, previous in vitro experiments (Araujo and Cotman, 1992) have often proved their usefulness in
simulating cellular responses in situ (Gomez-Pinilla et al., 1990; Griffin et al., 1989; Haga et al., 1989). Thus, our present data predicts that β-amyloid may cause a decrease in astrocyte glucose uptake in the human brain. The hallmark of the Alzheimer's disease is the neuritic plaque that consists of a β-amyloid protein core surrounded by dystrophic neurites and reactive astrocytes (Hardy and Allsop, 1991). The functional role played by these reactive astrocytes is not known, but it is possible that neuronal degeneration associated with β-amyloid plaque is induced in part by reactive astrocytes that failed to maintain normal glucose metabolism. We propose that β-amyloid inhibition of astrocytic glucose uptake limits glutamate uptake in the brain of AD patients. This condition potentiates a decrease in glucose incorporation into glycogen (Swanson et al., 1990), which could potentially lead to glycogen depletion in astrocytes. Since glycogen in the brain is stored primarily in astrocytes, the neurotoxic action of β-amyloid may involve limiting the availability of glucose in the brain of AD patients by limiting glucose uptake and subsequent depletion of glycogen in astrocytes. Thus, our present study suggests the possible link between two proposed mechanisms of pathogenesis of the Alzheimer's disease: glutamate neurotoxicity (Choi, 1988; Meldrum and Garthwaite, 1990) and global defect in cerebral energy metabolism (Marcus et al., 1989).

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The present studies suggested the effect of β-amyloid on astrocytes in the pathogenesis and pathophysiology of AD as related to the astrocytes' functions of: 1) regulation of cerebral blood flow by their release of nitric oxide, and 2) regulation of brain microenvironment by maintenance of low extracellular levels of glutamate through a glutamate uptake process and adequate glucose supply as a main glycogen reserve in the brain.

**The Effects of Cytokines on Astrocytic Nitric Oxide Release**

In the first study of this dissertation, the effects of β-amyloid and cytokines (IFN-γ, IL-1β, and TNF-α) on nitric oxide release by astrocytes were examined. Previous studies have shown that astrocytes are capable of releasing nitric oxide (Simmons and Murphy, 1992), and that the combination of cytokines (IFN-γ and IL-1β) induced iNOS and subsequent nitric oxide release by astrocytes (Murphy et al., 1993). The present dissertation confirmed the previous finding that exposure of astrocytes to individual cytokines (IL-1β, TNF-α and IFN-γ) is not sufficient for the induction of the nitric oxide release by astrocytes, whereas, IFN-γ (100U/ml) in combination with IL-1β (100U/ml) is a potent inducer of astroglial release of nitric oxide. Furthermore, the present study revealed yet another inducer of astrocytic nitric oxide release, a combination of cytokines IFN-γ and TNF-α. Notably, the combination of IL-1β and TNF-α did not cause the induction of astrocytic nitric oxide release. The present study also demonstrated that IL-1β is a more potent IFN-γ costimulatory factor than TNF-α in inducing nitric oxide release from hippocampal...
astrocytes. This finding of obligatory synergistic action of IFN-γ with IL-1β, or TNF-α in inducing astroglial nitric oxide release is particularly important in light of evidence for the temporal relationship between the severity of AD and levels of cytokines (IL-1, IFN-γ and TNF-α) (Aisen and Davis, 1994; Huberman et al., 1994; Sheng et al., 1997).

Previous studies have implicated IL-1 as a driving force in the progression of AD pathology because it induces amyloid precursor protein mRNA and thus favoring β-amyloid production (Aisen and Davis, 1994; Sheng et al., 1997). It also appears that IL-1 is the only cytokine reported in the brain during early stages of AD (Griffin et al., 1995). A correlation between IFN-γ production and the severity of AD was also demonstrated. Increased IFN-γ production by mononuclear cells was detected in patients with the advanced stage of AD, while no change in IFN-γ production was detected in mild AD cases (Huberman et al., 1994). Additionally, T cells (IFN-γ producers) have been co-localized with most of mature neuritic plaques (Rogers et al., 1998).

While other studies have shown that TNF-α alone can induce iNOS in C6 glioma cells (Murphy et al., 1993; Rossi and Bianchini, 1996), in the present study TNF-α alone did not enhance the release of nitric oxide from hippocampal astrocytes. Similarly, hippocampal microglial cells were also reported to be unresponsive to TNF-α alone (Goodwin et al., 1995). It appears that TNF-α levels in AD patients reflect the disease stage as previously noted for IFN-γ levels. In mild cases of AD TNF-α production by mononuclear cells was decreased (Huberman et al., 1994), but an increased TNF-α serum level was detected in the overall AD patient population (Fillit et al., 1991). The relationship between the disease stage
and the TNF-α levels is also suggested in the hypothesis stating that changes in TNF-α levels in AD result from either local cerebral or systemic inflammatory reaction associated with AD (Fillit et al., 1991). Although, IL-1 appears to be the first cytokine detected in the brain tissue, during the progression of AD, the present results showed no astrocytic nitric oxide release upon stimulation with IL-1β. Therefore, astrocytic nitric oxide release should not play a significant role in the pathology of early AD. However, with the further progression of AD, an increase in level of IFN-γ could act as a costimulatory factor needed for the induction of astrocytic nitric oxide release. Additionally, as IFN-γ levels increase in latter stages of the disease, so do TNF-α levels, and thus another stimulatory combination of cytokines could induce nitric oxide release from astrocytes.

Gradual changes of cytokine production (Androsova et al., 1995; Rogers et al., 1998) in AD patients from slight to marked production of IFN-γ and TNF-α and the correlation with the severity of the disease (Androsova et al., 1995; Shalit et al., 1995) further support the temporal dependency of cytokine effects and their role in the pathogenesis of AD.

**β-Amyloid and Nitric Oxide Release**

β25-35 did not stimulate the astrocytic release of nitric oxide alone or in the presence of either individual cytokines (IFN-γ, TNF-α, or IL-1β) or cytokine combinations (IFN-γ and TNF-α, or IFN-γ and IL-1β). Conversely, β-amyloid significantly reduced the astrocytic release of nitric oxide induced by IFN-γ combination with IL-1β or TNF-α. Given the role of nitric oxide in regulation of cerebral blood flow (Faraci and Brian, 1994), the present finding is important because it implicates β-amyloid as a modulator of cerebrovascular function in
AD. To further clarify the importance of the current findings, one needs to review the regulation of cerebral blood flow.

Cerebral circulation is regulated mainly by 1) neurotransmitter release from perivascular neurons and 2) endothelial factors (Whal and Schilling, 1993). Coupling between cerebral function and blood flow is one of the fundamental characteristics of cerebral circulation and is mostly mediated by nitric oxide (Whal and Schilling, 1993; Bruhwylé et al., 1993). Neurotransmitter release (e.g., Acetylcholine) from perivascular neurons causes endothelium dependent vasodilatation as the neurotransmitter activates endothelial NOS which, in turn, produces nitric oxide, relaxing the vascular muscle cells. Neuronal nitric oxide has been shown to mediate glutamate-induced dilation of cerebral arterioles (Farachi and Breese, 1993; Farachi and Brian, 1994) most likely through activation of soluble guanyl cyclase in vascular smooth muscle or pericytes. Furthermore, inhibitors of nitric oxide synthase block increases in blood flow in neuronally active regions of the brain (Arisztid et al., 1992). Cytokines induced astroglial release of nitric oxide has been proposed to dilate cerebral vessels and alter cerebral blood flow (Kovach et al., 1992; ladecola, 1992; Murphy et al., 1993; Wang et al., 1992).

It was demonstrated that aging has an inhibitory effect on endothelium-dependent cerebral vasodilatation (Faraci and Brian, 1994). Additionally, a recent in vitro study revealed that β-amyloid induces necrotic endothelial cell death and inhibits the production of nitric oxide (Sutton et al., 1997). Since the neuronal loss is one of the hallmarks of AD and vasoactive endothelial function is significantly impaired (Faraci and Brian, 1994; Sutton et al., 1997), it may be that the local cerebral blood flow is almost entirely dependent on
astrocytic regulation. Under these circumstances, β-amyloid mediated inhibition of the astrocytic nitric oxide release would have a down regulatory effect on cerebral blood flow in AD patients. Indeed, a reduction in cerebral blood flow in AD patients has been already reported (Bonte et al., 1990; Bonte et al., 1993; Holman et al., 1992).

Similar to the present study findings based on rat astrocytes cultures, human astrocytes release nitric oxide upon stimulation with a combination of IL-1β and IFN-γ (Lee et al., 1993), and increased levels of cytokines (IL-1, TNF-α, IFN-γ) have been detected in the brains of AD patients (Dickson et al., 1993; Griffin et al., 1989; Haga et al., 1989; Huberman et al., 1994). It is, therefore, reasonable to assume that β-amyloid exerts a similar suppressive effect on human astrocytes' nitric oxide release. The underlying mechanism of how the modulatory action of β-amyloid on astrocyte-generated nitric oxide induced by cytokines may affect vascular functions and local cerebral blood flow in situ is yet to be determined.

**The Effect of β-Amyloid on Astrocytic Glutamate Uptake.**

The present study examined the long-term (7 days) effect of β-amyloid on astrocytic glutamate uptake. Glutamate uptake by control astrocytes (not exposed to amyloid) was shown to be time-dependent, as reported previously (Flott and Seifert, 1991; Hara et al., 1989; Piani et al., 1993). However, astrocytes plated onto β25-35 for 7 days showed significantly lower glutamate uptakes regardless of the sampling times (1 min to 1 hr). This inhibitory effect is specific to β25-35, because the scrambled control peptide did not affect the astrocytic glutamate uptake. The significance of this finding is related to glutamate toxicity implicated in AD pathogenesis. Neurotoxic properties of glutamate have been well
documented as a cause of neuronal death in AD (Maragos et al., 1987). The inhibitory effect of β-amyloid on astrocytic glutamate uptake suggests an accumulation of extracellular glutamate and prolonged neuronal exposure to elevated levels of glutamate. Glutamate-mediated neuronal cell injury has been studied extensively (Choi, 1988; Meldrum and Garthwaite, 1990). An excessive Ca\(^{2+}\) influx through NMDA glutamate receptors and prolonged elevations in intracellular Ca\(^{2+}\) levels lead to cell death (Meldrum and Garthwaite, 1990; Siesjo, 1989). Glutamate toxicity has been proposed as a pathogenic factor in AD. The finding in the present study supports that hypothesis and, in addition offers a possible explanation of the mechanism responsible for elevation of glutamate levels.

Both neurons and astrocytes possess a high affinity glutamate uptake system (Kanai et al., 1993) which maintains low extracellular glutamate levels. However, astrocytes appear to play a key role in maintenance of low glutamate levels because they take up most of the extracellular glutamate (McLennan, 1976). Therefore, the decreased capacity of astrocytes to uptake glutamate may have significant effects on neuronal function and survival because elevated glutamate levels could induce toxic effects. If one takes into consideration that β-amyloid itself increases neuronal susceptibility to glutamate toxicity (Koh et al., 1990), β-amyloid inhibition of astrocytic glutamate uptake and subsequent elevation of extracellular glutamate may be sufficient to cause neuronal death. The mechanism involved in β-amyloid-mediated inhibition of glutamate uptake is not known and some of the possible factors (e.g. nitric oxide, glucose) that may affect astrocytic glutamate uptake were investigated in this dissertation.
Astrocytic Glutamate Uptake and Nitric Oxide

The possible effect of nitric oxide on astrocytic glutamate uptake was examined using sodium nitroprusside (SNP), a spontaneous nitric oxide generator (Southam and Garthwaite, 1991). Nitric oxide has been suggested as a mediator of the glutamate neurotoxicity as the Ca^{2+} influx mediated by glutamate activates Ca^{2+}-dependent nitric oxide synthase (Vige et al., 1993). At high levels, nitric oxide is neurotoxic (Chao et al., 1992) because it reacts with superoxide anions and forms peroxinitrite anions that decompose to yield highly damaging hydroxyl free radicals and nitrogen dioxide (Beckman et al., 1990). Furthermore, nitric oxide inhibits glutamate transport in hippocampal synaptosomes from rat brain (Pogun et al., 1994). These observations suggest nitric oxide as a factor that may mediate glutamate neurotoxicity. However, the present study showed that SNP did not affect glutamate uptake by astrocytes. A combination of SNP and B25-35 did not cause any more significant inhibition than that caused by B25-35 alone, confirming that SNP does not affect astrocytic glutamate uptake. These results are significant because they are suggestive of two different glutamate uptake processes in neurons and astrocytes.

The finding that astrocytic glutamate uptake is not affected by SNP suggests that nitric oxide mediation of glutamate neurotoxicity is not related to changes in astrocytic glutamate uptake. Considering that nitric oxide does inhibit glutamate transport in hippocampal synaptosomes from the rat brain (Pogun et al., 1994), whereas astrocytic uptake is not affected by nitric oxide, it is likely that the glutamate carrier in synaptosomes has a different susceptibility to nitric oxide than the astrocytic carrier as demonstrated by an opposite effect of SNP on astrocytic and neuronal glutamate uptakes.
Astrocytic Glutamate Uptake and Oxygen Dependent Free Radicals

β-amyloid generates oxygen-dependent free radicals in an aqueous solution (Hensley et al., 1994), and it has been suggested that these oxygen-dependent free radicals are responsible for the inhibition of glutamate uptake into astrocytes (Harris et al., 1995; Harris et al., 1996). Moreover, the scrambled control peptide was also shown to induce significant levels of free radicals in an aqueous solution (Hensley et al., 1994). Since the present study also showed that scrambled β25-35 has no inhibitory effect on astrocytic glutamate uptake, it is likely that β-amyloid may interfere with astrocytic glutamate uptake by a free radical-independent mechanisms (e.g. glucose uptake).

Astrocytic Glucose Uptake and β-Amyloid as Related to Glutamate Uptake

This study tested the effect of β25-35 on glucose uptake by astrocytes. It has been shown that astrocytic glutamate uptake depends mainly on glucose availability (Swanson, 1992; Swanson et al., 1994) and that inhibition of glycolysis causes failure of glutamate uptake (Ogata et al., 1992; Swanson, 1992). In the present study, β25-35 exerted significant inhibition on astrocytic glucose uptake, suggesting that the suppressive effect of β-amyloid on astrocytic glucose uptake may affect glutamate uptake. To further support this hypothesis, astrocytic glutamate uptake was studied in the presence of phloretin, a glucose uptake blocker. Phloretin induced approximately a fourfold decrease in glutamate uptake, which is comparable to the fourfold decrease in the glutamate uptake induced by β25-35 (measured at 60 min sampling time). The demonstrated phloretin effect on astrocytic glutamate uptake suggests that the suppressive effect of β25-35 on glucose uptake, in turn, affects glutamate uptake.
Ample evidence has been provided to show that glucose metabolism may have changed in the brains of AD patients (Hoyer, 1991). Studies using positron emission tomography (PET) scans detected a decrease in glucose uptake and metabolism in the cerebral cortex (Duara et al., 1986; Jagus et al., 1991; Rapoport et al., 1991; Riege and Metter, 1988), and immunohistochemistry revealed a decrease in GLUT1 and GLUT3 activities in several regions of the brain, including the hippocampus (Harr et al., 1995; Simpson et al., 1994). These studies suggest that the decrease in cerebral glucose uptake seen in PET studies may reflect, in part, decreased glucose transport and utilization at the cellular level. Additionally, it appears that this change in glucose metabolism may result in the lower glutamate uptake also found in the brain of AD patients (Kanai et al., 1993). Although the present study, based on cultured rat astrocytes, may not accurately represent an in situ environment for astrocytes, previous in vitro experiments (Araujo and Cotman, 1992) have often proved their usefulness in simulating cellular responses in situ (Gomez-Pinilla et al., 1990; Griffin et al., 1989; Haga et al., 1989). Thus, the present dissertation findings support the hypothesis that β-amyloid may cause a decrease in astrocyte glucose uptake in the human brain.

**Concluding Remarks**

The hallmark of Alzheimer's disease is the neuritic plaque that consists of a β-amyloid protein core surrounded by dystrophic neurites and reactive astrocytes (Hardy and Allsop, 1991). Even though Alzheimer's disease has not been considered an "inflammatory disease", evidence in recent years suggests that the degenerative processes in AD are accompanied by activation of immune mechanisms (Aisen and Davis, 1994).
It has been demonstrated that IL-1 causes the induction of amyloid precursor protein mRNA and thus creates an environment favoring β-amyloid production (Sheng et al., 1996). Since β-amyloid deposition is considered a central event in pathogenesis of AD, the effect of IL-1 has been proposed as one of the first and perpetuating factors in amyloidogenesis and AD pathogenesis (Aisen and Davis, 1994) (Chart 1). Astrocytes proliferate in response to IL-1β, and they also release TNF-α (Aisen and Davis, 1994). As IL-1 induces amyloidogenesis and β-amyloid deposition, astrocytes change their morphology and release additional IL-1 (Araujo and Cotman, 1992), thus perpetuating a vicious circle.

With the progression of the pathologic processes in AD, additional cytokines (TNF-α and IFN-γ) are produced (Huberman et al., 1994; Fillit et al., 1991; Araujo and Cotman, 1992). As they diffuse into the brain parenchyma, the combinations of these cytokines can induce astrocytes to release nitric oxide, which could potentially be neurotoxic in AD brains. However, β-amyloid suppresses this effect leading to a decrease in astrocytic nitric oxide release as demonstrated in the present study. It is through release of nitric oxide that astrocytic endfeet processes mediate dilation of cerebral vasculature (Montgomery, 1994; Faraci and Brian, 1994). Therefore, in AD brains, the presence of β-amyloid (e.g., perivascular deposits) would lead to impaired vascular dilation resulting in reduced cerebral blood flow (Chart 1).

Additionally, it is also possible that neuronal degeneration associated with β-amyloid plaque is induced in part by reactive astrocytes that failed to maintain normal glucose metabolism (Chart 1). Besides a reduced glucose supply caused by reduced cerebral blood flow, additional β-amyloid inhibition of astrocytic glucose uptake would limit glutamate
Chart 1: Putative role of astrocytes in pathogenesis of AD
uptake in the brain of AD patients. This condition potentiates a decrease in glucose incorporation into glycogen (Swanson et al., 1990), which could potentially lead to glycogen depletion in astrocytes. Since glycogen in the brain is stored primarily in astrocytes, the neurotoxic action of β-amyloid may involve limiting the availability of glucose in the brain of AD patients by limiting glucose uptake and subsequent depletion of glycogen in astrocytes. Thus, our present study suggests the possible link between two proposed mechanisms of pathogenesis of the Alzheimer's disease: glutamate neurotoxicity (Choi, 1988; Meldrum and Garthwaite, 1990) and global defect in cerebral energy metabolism (Marcus et al., 1989).
REFERENCES


