The glial glutamate transporter, GLT-1, is oxidatively modified by 4-hydroxy-2-nonenal in the Alzheimer’s disease brain: the role of Aβ1–42

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Abstract
Glutamate transporters are involved in the maintenance of synaptic glutamate concentrations. Because of its potential neurotoxicity, clearance of glutamate from the synaptic cleft may be critical for neuronal survival. Inhibition of glutamate uptake from the synapse has been implicated in several neurodegenerative disorders. In particular, glutamate uptake is inhibited in Alzheimer’s disease (AD); however, the mechanism of decreased transporter activity is unknown. Oxidative damage in brain is implicated in models of neurodegeneration, as well as in AD. Glutamate transporters are inhibited by oxidative damage from reactive oxygen species and lipid peroxidation products such as 4-hydroxy-2-nonenal (HNE). Therefore, we have investigated a possible connection between the oxidative damage and the decreased glutamate uptake known to occur in AD brain. Western blots of immunoprecipitated HNE-immunoreactive proteins from the inferior parietal lobule of AD and control brains suggest that HNE is conjugated to GLT-1 to a greater extent in the AD brain. A similar analysis of beta amyloid (Aβ)-treated synaptosomes shows for the first time that Aβ1–42 also increases HNE conjugation to the glutamate transporter. Together, our data provide a possible link between the oxidative damage and neurodegeneration in AD, and supports the role of excitotoxicity in the pathogenesis of this disorder. Furthermore, our data suggests that Aβ may be a possible causative agent in this cascade.

Keywords: Alzheimer’s disease, beta amyloid, excitotoxicity, GLT-1, 4-hydroxy-2-nonenal, oxidative stress.


As the major excitatory neurotransmitter in the mammalian CNS, glutamate is essential for synaptic transmission; however, excessive neuronal stimulation by glutamate (known as excitotoxicity) can induce neuronal damage and even death (Maragakis and Rothstein 2001). Therefore, as a potential neurotoxin, clearance of glutamate from the synapse may be critical for neuronal survival. Cellular uptake by glutamate transport (GT) is the primary mechanism in maintaining synaptic glutamate concentrations (Anderson and Swanson 2000), which is primarily mediated by astrocytes (Rothstein et al. 1996). Pharmacological inhibition of GT leads to neurodegeneration and death (Robinson et al. 1993; Rothstein et al. 1993; Lievens et al. 2000), consistent with a role for impaired GT in excitotoxicity.

Excitotoxic mechanisms of cell death have been implicated in several neurodegenerative disorders, such as stroke, amyotrophic lateral sclerosis (ALS) and Alzheimer’s disease (AD), although evidence is lacking in many instances (Maragakis and Rothstein 2001). Glutamate transport is inhibited in AD brain (Masliah et al. 1996) through an as yet unknown mechanism. Oxidative damage is elevated in the AD brain (Markesbery 1997), whereas oxidation of proteins (e.g. transporters) is known to decrease their activity (Volterra et al. 1994; Keller et al. 1997a,b; Blanc et al. 1998). The correlation of these two pathological features could possibly provide an understanding of the mechanisms underlying neurodegeneration in AD.

Alzheimer’s disease is characterized by the deposition of the beta-amyloid peptide (Aβ), that may play a role in the pathogenesis of the disorder (Selkoe 1996). Aβ1–42 induces oxidative damage to neural proteins and lipids in vitro, which can be inhibited by antioxidants (Yatin et al. 2000), and is thought to mediate the oxidative damage present in AD brain (Varadarajan et al. 2000; Varadarajan et al. 2001). Lipid oxidation by Aβ (Butterfield et al. 1994) can result in the formation of 4-hydroxy-2-nonenal (HNE), which is suggested to play a major role in the toxicity of Aβ (Mark et al. 1997). In particular, the addition of either Aβ or HNE to cells or synaptosomes results in the inhibition of GT (Harris et al. 1996; Keller et al. 1997a,b). Thus, we hypothesized that decreased GT in the brain in AD may result from covalent modification by HNE of the glial glutamate transporter GLT-1 (EAAT2). Furthermore, we

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Abbreviations used: Aβ, beta amyloid; AD, Alzheimer’s disease; APP, amyloid precursor protein; GT, glutamate transport; HNE, 4-hydroxy-2-nonenal; IP, immunoprecipitation; IPL, inferior parietal lobule; PBS, phosphate-buffered saline; PMIs, post mortem intervals.
investigated whether the addition of Aβ1–42, the predominant form of this peptide in AD, to synaptosomes would lead to modification of GLT-1 by HNE. Both studies lead to results that may explain the inhibition of GT in AD and may provide some evidence for excitotoxic neurodegeneration in AD.

### Experimental procedures

#### Materials

For the treatment of synaptosomes, HNE was purchased from Cayman Chemical and Aβ1–42 was purchased from AnaSpec. Nonidet P-40 was purchased from Roche Diagnostics and protease inhibitors were from ICN. The anti-HNE antibody was a generous gift from Dr. Szweda and was purchased from Roche Diagnostics and protease inhibitors were from ICN.

#### Brain tissue sampling

Inferior parietal lobule (IPL) tissue samples used for analyses were taken at the normal range. Neuropathologic evaluation of control brains revealed no different from AD patients with or without diffuse Lewy bodies. Control AD patients were also diagnosed with diffuse Lewy bodies, and results were obtained from Pharmacia. All other reagents were purchased from Sigma.

#### Synaptosomal preparation and treatment

Rat cortical synaptosomes were prepared by ultracentrifugation on liquid nitrogen, and stored at ~80°C. The Rapid Autopsy Program of the University of Kentucky Alzheimer’s Disease Research Center resulted in extremely short post mortem intervals (PMIs). Demographic data for the subjects are presented in Table 1. All AD subjects displayed progressive intellectual decline and met NINCDS-ADRDA Group criteria for the clinical diagnosis of probable AD (McKhann et al. 1984). All AD subjects met accepted guidelines for the histopathologic diagnosis of AD (National Institute on Aging and the Reagan Institute Working Group 1997). Hematoxylin-eosin and modified Bielschowsky staining and the 10-D-5 immunohistochemical reaction were used on multiple neocortical, hippocampal, amygdala, brainstem and cerebellum sections for diagnosis. Some AD patients were also diagnosed with diffuse Lewy bodies, and results were different from AD patients with or without diffuse Lewy bodies. Control subjects underwent annual neuropsychological testing as a part of our normal volunteer longitudinal aging study, and did not have a history of dementia or other neurological disorders. All control subjects had test scores in the normal range. Neuropathologic evaluation of control brains revealed only age-associated gross and histopathologic alterations.

#### Immunoprecipitation and western blotting

Immunoprecipitation (IP) was performed essentially as described previously (Keller et al. 1997a). Synaptosomes or tissue samples from the IPL of AD or control brains were briefly sonicated in a modified RIPA buffer (1% Nonidet P-40, 50 mM Tris, 150 mM NaCl, 2 mM EDTA, 5 μg/mL leupeptin, 5 μg/mL aprotinin, 2 μg/mL pepstatin, 0.25 mM phenylmethylsulfonyl fluoride, 10% glycerol, pH 7.6), and solubilized for 4 h at 4°C. Lysates were centrifuged, protein concentrations determined on the soluble fraction, and 500 μg total soluble protein aliquoted for each sample. A rabbit polyclonal antibody (5 μL) raised against covalent HNE-protein Michael adducts (Uchida et al. 1993) was added directly to the lysates, forming complexes with all HNE-immunoreactive proteins. Antibody/lysate solutions were incubated on a rotary mixer overnight at 4°C. HNE-protein antibody complexes were precipitated with protein A-conjugated acrylic beads. Protein A beads were added in 50 μL aliquots from a stock of 300 mg/mL in PBS and mixed on a rotary mixer for 6 h at 4°C. Beads were then centrifuged and washed three times with the modified RIPA buffer and immunoprecipitates were resuspended in Laemmli sample buffer.

The GLT-1 immunoreactivity among HNE-immunoprecipitated proteins was analyzed by western blotting on a 15% polyacrylamide gel. After transfer to nitrocellulose, blots were blocked with 3% bovine serum albumin, probed with anti-GLT-1 (1 : 3000; overnight at 4°C), and developed with a horseradish peroxidase-conjugated secondary antibody and a chemiluminescent substrate. The anti-GLT-1 was raised against a peptide corresponding to its 20 C-terminal amino acids, which are of identical sequence in rat and human. Untreated lysates were run on western blots as a positive control for GLT-1 immunoreactivity.

### Results and Discussion

Glutamate transporters function by removing synaptically released glutamate, a potential neurotoxin. Inhibition of this function results in neurodegeneration and death (Robinson et al. 1993; Rothstein et al. 1993; Lievens et al. 2000). Because GT is inhibited in cells and synaptosomes after treatment with Aβ, HNE or other reagents that can induce lipid oxidation (Volterra et al. 1994; Harris et al. 1996; Trotti et al. 1996; Keller et al. 1997a,b; Blanc et al., 1998), the role of HNE in this process was investigated. Also, because Aβ induces lipid peroxidation (Butterfield et al. 1994) and HNE formation (Mark et al. 1997), we analyzed a possible mechanism by which Aβ1–42 could decrease GT. Synaptosomes were treated with 10 μM of either HNE or Aβ for 4 h at 37°C or remained untreated. Following IP of HNE-modified proteins from the synaptosomes, western blots probed with anti-GLT-1 indicated increased immunoreactivity in samples treated with both HNE and Aβ (Fig. 1b). In comparison with untreated control samples, GLT-1 is modified by HNE to a greater extent in treated synaptosomes (i.e. greater HNE immunoreactivity), and is therefore immunoprecipitated to a greater extent from treated synaptosomes (i.e. greater GLT-1 immunoreactivity on western blots). Interestingly, equimolar concentrations of HNE and Aβ were incubated with synaptosomes; however, addition of HNE does not modify GLT-1 to the extent that Aβ does. This might indicate a catalytic effect by Aβ in oxidizing lipids, or simply that exogenously added HNE might react with a number of aqueous soluble proteins before diffusing to the membrane-bound glutamate transporter. After insertion into the membrane bilayer, Aβ oxidizes lipids (Butterfield et al., 1994), and HNE thus generated could react more rapidly with membrane-bound proteins as the transmembrane protein, GLT-1.

Our data are consistent with previous reports of HNE modification of GLT-1. The HNE binds to an immunoprecipitated GLT-1 in solution as well as in cultured cortical astrocytes and synaptosomes (Keller et al. 1997a; Blanc et al. 1998). As an electrophilic aldehyde, HNE reacts covalently with cysteine, lysine and histidine residues of proteins, disrupts their structure, and alters their function (Esterbauer et al. 1991; Subramaniam et al. 1997). The activities of both neuronal and glial GT proteins (e.g. GLT-1) are regulated by a thiol-based redox mechanism (Trotti et al. 1997), suggesting that HNE binding may play a role in disrupting the function of these proteins.
Fig. 1 HNE modifies GLT-1 in synaptosomes after treatment with Aβ1–42 and in the IPL region of the AD brain. HNE-immunoreactive proteins were immunoprecipitated with an anti-HNE antibody and immunoprecipitates were analyzed for GLT-1 immunoreactivity by western blotting. (a) A representative blot indicates that GLT-1 is present among HNE immunoprecipitated proteins. Lysates (L) were run on western blots as a positive control for GLT-1 immunoreactivity, which is indicated by the arrow. C, control; H, HNE-treated synaptosomes; Aβ, Aβ1–42-treated synaptosomes. Molecular weight markers are indicated in kDa. (b) Relative to control (C), GLT-1 is modified by HNE in three separate preparations of synaptosomes treated with 10 μl of either HNE (H) or Aβ1–42 (Aβ) for 4 h. (c) Relative to control (C), GLT-1 is modified by HNE in IPL tissue from Alzheimer’s disease (AD) brains (n = 7 AD, 4 control). Data are the mean ± SEM. (*p < 0.03 vs. respective control; ANOVA).

We demonstrate that GLT-1 is modified by HNE in synaptosomes treated with Aβ1–42 and in the IPL region of the AD brain. The ability of Aβ1–42 treatment to oxidatively modify GLT-1 is particularly relevant to AD, where the 42-amino acid variant of Aβ is hypothesized to play a causative role (Selkoe 1996; Varadarajan et al. 2001). Although it is known that GT is inhibited in the AD brain (Masliah et al. 1996), the mechanism of this inhibition remains unknown. Levels of both free and protein-bound HNE are increased in AD (Markesbery and Lovell 1998; Montine et al. 1998), and may be related to both Aβ overproduction and GT inhibition. Accordingly, we analyzed HNE modifications to GLT-1 from the IPL of AD and control brains, and therefore a possible mechanism by which GT might be inhibited in the AD brain. The IPL region displays severe histopathologic alterations in AD that correlate to oxidative damage (Hensley et al. 1995) including elevated lipid peroxidation (Palmer and Burns 1994). Western blot analysis of HNE-modified proteins from IPL lysates results in increased GLT-1 immunoreactivity (Fig. 1a), suggesting that GLT-1 is covalently modified by HNE in this region of the AD brain. Based upon HNE-mediated inhibition of GT in models of AD, this suggests that HNE modification of GLT-1 may contribute to decreased GT in the AD brain.

Much experimental evidence indicates the importance of glial GT proteins, especially GLT-1 (Anderson et al. 2000; Maragakis and Rothstein 2001). Decreasing GT by knock-out or knock-down of GLT-1 protein, removal of astrocytes (and glial transporter such as GLT-1), or by using competitive inhibitors of GLT-1 increases neuronal sensitivity to glutamate and induces neurodegeneration and death (Rosenburg and Aizenman 1989; Robinson et al. 1993; Rothstein et al. 1993; Rothstein et al. 1996; Tanaka et al. 1997). Similarly, HNE inhibition of GT, especially GLT-1, may induce excitotoxicity and neurodegeneration. Similar mechanisms have been explored that link oxidative damage to excitotoxicity in neurodegeneration (Trotti et al. 1998), and existing evidence for such a mechanism is strongest in ALS (Ludolph et al. 2000). Studies reveal decreased total GT-1 protein and GT, and increased GLT-1 modification by HNE in spinal cords from ALS patients (Bristol and Rothstein 1996; Pedersen et al. 1998). Further, riluzole, currently approved for use in ALS, is thought to prevent glutamate release (Maragakis and Rothstein 2001). The current study, coupled with previous results implicating altered GT, suggests that similar mechanisms may also play a role in AD.

We demonstrate that GLT-1 is modified by HNE in synaptosomes...


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