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Original Contribution

ACROLEIN, A PRODUCT OF LIPID PEROXIDATION, INHIBITS GLUCOSE AND GLUTAMATE UPTAKE IN PRIMARY NEURONAL CULTURES

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Abstract—Oxidative stress has been implicated in the pathogenesis of several neurodegenerative disorders including Alzheimer's disease (AD). Increased lipid peroxidation, decreased levels of polyunsaturated fatty acids, and increased levels of 4-hydroxynonenal (HNE), F_2-isoprostanes, and F_4-neuroprostanes are present in the brain in patients with AD. Acrolein, an α,β-unsaturated aldehydic product of lipid peroxidation has been demonstrated to be approximately 100 times more reactive than HNE and is present in neurofibrillary tangles in the brain in AD. We recently demonstrated statistically significant elevated concentrations of extractable acrolein in the hippocampus/parahippocampal gyrus and amygdala in AD compared with age-matched control subjects. Concentrations of acrolein were two to five times those of HNE in the same samples. Treatment of hippocampal cultures with acrolein led to a time- and concentration-dependent decrease in cell survival as well as a concentration-dependent increase in intracellular calcium. In cortical neuron cultures, we now report that acrolein causes a concentration-dependent impairment of glutamate uptake and glucose transport in cortical neuron cultures. Treatment of cortical astrocyte cultures with acrolein led to the same pattern of impairment of glutamate uptake as observed in cortical neuron cultures. Collectively, these data demonstrate neurotoxicity mechanisms of arolein that might be important in the pathogenesis of neuron degeneration in AD. © 2000 Elsevier Science Inc.

Keywords—Free radicals, Lipid peroxidation, Alzheimer's disease, Neurotoxin, Neuronal cultures

INTRODUCTION

Increasing evidence supports the potential role of oxidative stress in neuronal degeneration in Alzheimer's disease (AD). Recent studies show an increase in the redox active metal iron [1–3] as well as increased levels of lipid peroxidation, [4] a decline in membrane polyunsaturated fatty acids, [5,6] and increased protein oxidation [7,8] and DNA oxidation [9–11] in the brain in patients with AD. Other studies from our laboratory demonstrate increased levels of oxidatively modified DNA in AD ventricular cerebrospinal fluid (CSF) concomitant with decreased levels of the free repair product, [12] and increased levels of 4-hydroxynonenal (HNE), a neurotoxic marker of lipid peroxidation, in AD brain [13] and ventricular CSF [14]. Elevated levels of F_2-isoprostanes and F_4-neuroprostanes, markers of lipid peroxidation, are found in AD CSF, [15,16] and increased levels of F_2-isoprostanes are present in the AD brain [17]. Markers of oxidative stress in neurofibrillary tangles (NFT) and senile plaques (SP) are present in the brain in AD [18–23]. Peroxidation of lipids leads to the formation of a number of aldehydic by-products, including malondialdehyde, C_3-C_{10} straight chain aldehydes, and α,β-unsaturated aldehydes such as HNE and acrolein [18,24,25].

Acrolein (CH_2=CH–CHO) occurs in the environment as a ubiquitous pollutant that is generated as a by-product of overheated organic materials. In vivo, acrolein is formed in the metal-catalyzed oxidation of polyunsaturated fatty acids, including arachidonic acid [26]. Acrolein is the strongest electrophile among the unsaturated aldehydes and shows the highest reactivity with nucleophiles, including sulfhydryl groups of cysteine, histidine, and lysine.[24] Acrolein formed in vivo, through iron-catalyzed oxidation of arachidonic and docosahexenoic acids, exhibits facile reactivity with various biomol-

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ecules, including proteins and phospholipids, has the potential to inhibit many enzymes, and quickly depletes cellular glutathione levels [27]. It is postulated that acrolein may inactivate the reductase responsible for the reduction of vitamin E radicals [27] and, coupled with the depletion of glutathione, leads to further lipid peroxidation. Acrolein is capable of modifying DNA bases with the formation of exocyclic adducts [28,29]. Studies of Uchida et al. [26,30] and Esterbauer et al. [24] demonstrated that acrolein is rapidly incorporated into proteins and generates carbonyl derivatives. More recently, Uchida et al. [26] demonstrated that acrolein preferentially reacts with lysine residues that are prominent components of tau, and Calingasan et al. [31] described the presence of acrolein adducts in NFT and dystrophic neurites surrounding SP. We recently demonstrated statistically significant elevations of extractable acrolein in AD amygdala (2.5 $\pm$ 0.9 nmol/mg of protein) compared with control amygdala (0.3 $\pm$ 0.5 nmol/mg of protein), and AD hippocampus and parahippocampal gyrus (5.0 $\pm$ 1.6 nmol/mg of protein) compared with age-matched control AD hippocampus and parahippocampal gyrus (0.7 $\pm$ 0.1 nmol/mg of protein) [32]. Our study demonstrated a significant time- and concentration-dependent decrease in cell survival in hippocampal neuron cultures treated with acrolein. Acrolein also led to a concentration-dependent increase in intracellular calcium concentration in hippocampal cultures.

The present report demonstrates that acrolein toxicity may be mediated through the inhibition of glucose transport and glutamate uptake.

MATERIALS AND METHODS

Neuron cultures

Primary cortical cultures were established from gestation day 18 rat embryos as described by Mattson et al. [33] as modified by Xie et al. [34]. Cortical neurons were plated at a density of 100/mm^2 in polyethyleneimine-coated plastic 60 mm dishes. Cultures were maintained in Eagle's minimum essential medium (MEM; Gibco BRL, Grand Island, NY, USA) supplemented with 10% (v/v) qualified bovine serum containing 20 mM KCl and 1 mM pyruvate in an environment of 94% air/6% CO_2 at 37°C. Cortical neuron cultures contained ~98% pure neurons as previously described [34]. Pure primary astrocyte cultures were established from cerebral hemispheres of 2 day old Sprague-Dawley rats as described by Keller et al. [35]. Astrocytes were plated in uncoated 100 mm dishes and maintained in MEM supplemented with 26 mM sodium bicarbonate, 10 mM glutamine, and 10% (v/v) fetal bovine serum. Astrocyte cultures prepared using this method are >98% type I astrocytes as assessed by anti-glial fibrillary acid protein staining [35]. For treatment, neurons 7 days in culture or astrocytes 2 weeks in culture were switched to serum-free Locke's solution consisting of 154 mM NaCl, 5.6 mM KCl, 2.3 mM $CaCl_2$, 1.0 mM $MgCl_2$, 3.6 mM $NaHCO_3$, 10 mM glucose, 5 mM HEPES (pH 7.2) with 10 mg/l gentamicin sulfate. Cortical neuron survival was assessed by photographing and counting the number of intact neurons in premarked microscopic fields before treatment and at each indicated time point (3, 6, 12, and 24 h). Viability of cortical neurons, treated for glucose and glutamate uptake, and astrocyte cultures, treated for glutamate uptake, was determined by measuring lactate dehydrogenase (LDH) release as described by Koh and Choi [36]. LDH results for each culture were normalized against total LDH release measured when all cells were lysed. Results of survival studies are for a mean of six to nine dishes per each concentration over three experiments. For survival studies, cultures were switched to Locke's medium and acrolein was added at 0.5, 1, 10, and 25 μM (final concentration). For glucose transport, 1 nM, 5 nM, 10 nM, 25 nM, 100 nM, and 1 μM acrolein were added for 4 h. Glutamate uptake in cortical neurons was measured in the presence of 250 nM, 500 nM, 750 nM, 1 μM, and 10 μM acrolein for 4 h. Glutamate uptake in astrocyte cultures was measured in the presence of 0.5, 1, 10, 15, and 25 μM acrolein for 4 h. Acrolein was purchased from Aldrich Chemical Co. (Milwaukee, WI, USA). It was stored at 4°C and was stable for approximately 2 months. For treatment, acrolein was diluted into Locke's medium fresh each day and used immediately.

Glucose transport assays

Glucose transport was assayed in cultured cortical neurons based on the method of Horner et al. [37] as modified by Mark et al., [38] who demonstrated that glucose uptake in cultured cortical neurons is comparable to that of cultured hippocampal neurons. After 4 h treatment with acrolein, Locke's medium with glucose was removed and the cells washed three times with glucose-free Locke's. For glucose uptake assays, glucose-free Locke's medium was added to the dishes along with 1.5 μCi of 2-[^{3}H]deoxyglucose (NEN Life Science Products, Boston, MA, USA). After a 5-min incubation, the medium was quickly aspirated and the cells washed four times in phosphate-buffered saline. Nonlinearity of uptake over time and saturation effects were minimized by measuring uptake over a short time period (5 min). Cells were then lysed in 200 μl of 0.5 M NaOH/0.5% sodium dodecyl sulfate and scraped. Aliquots (160 μl) of lysate were mixed with 5 ml of Scintiverse E liquid scintillation cocktail (Fisher Scientific, Pittsburgh, PA, USA) and counted for 5 min using a Packard 2500TR liquid scintillation counter. Blanks for the assay consisted of cultures pretreated (1 h) with 5 μM phloretin

(Sigma Chemical Co., St Louis, MO, USA), which significantly impairs glucose uptake in neuronal cultures [39]. Protein content was determined using the Pierce BCA (Pierce, Rockford, IL, USA) method, and uptake was normalized to protein content for each individual sample. Results are expressed as mean ± SEM% control and represent the mean of 6 to 18 dishes per each concentration used over three separate experiments.

Glutamate uptake

Glutamate uptake was assayed using the method of Swanson [39] with modification. Cortical cultures essentially free of astrocytes (<2%) or pure astrocyte cultures (>98%) were switched to Locke's medium and treated with acrolein for 4 h as described above. Cortical cultures were treated with 250 nM, 500 nM, 1 μM, and 10 μM acrolein. After treatment, 1.0 μCi of ^{3}H-labeled glutamate (3,4-[^{3}H]glutamic acid, 44.0 Ci/nmol) (NEN Life Science Products, Boston, MA, USA) was added for 5 min. After exposure to glutamate, the medium was quickly aspirated, and the cells washed three times with PBS. Cells were then lysed in 200 μl of 0.5 M NaOH/0.5% SDS and scraped. Aliquots (20 μl) were taken for protein measurement using the Pierce BCA method. Aliquots (160 μl) were mixed with 5 ml of liquid scintillation cocktail and counted for 5 min using a Packard 2500 liquid scintillation counter. Because of higher densities of the astrocyte cultures, they were treated with 0.5, 1, 10, 15, and 25 μM acrolein for 4 h, and glutamate uptake was assayed as described above. Blanks for the assay consisted of cultures exposed to 200 μM nonlabeled glutamate concomitant with labeled glutamate. As was the case for glucose uptake, short treatment time (5 min) limited saturation effects. Because the assay measures glutamate taken up by the cells, it should include glutamate that has been metabolized. Results are expressed as mean ± SEM% of control and are the mean of 5 to 16 dishes per each concentration used over three experiments for cortical neurons and 4 to 6 dishes per each treatment over two experiments for astrocyte cultures.

Statistical analyses

Statistical analyses were carried out for cell survival and for glucose and glutamate uptake using analysis of variance with Dunnett's post hoc test for individual differences and the commercially available ABSTAT software.

RESULTS

As shown in Fig. 1, acrolein led to a time- and concentration-dependent decreased survival of cortical neuron cultures. Within 6 h of treatment, 10 μM acrolein led to an approximately 20% decrease in neuron survival and all cells were dead at a concentration of 25 μM. Figure 2 demonstrates the neurite fragmentation and cell body vacuolization of cortical neurons treated with 10 μM acrolein for 6 h. Results of cortical neuron viability at 4 h measured by LDH release yielded similar results to those obtained using cell counts. Treatment of cortical cultures with acrolein led to an LDH release of 5% total LDH for 1 μM acrolein and 14% of total LDH release for treatment with 25 μM for 4 h.

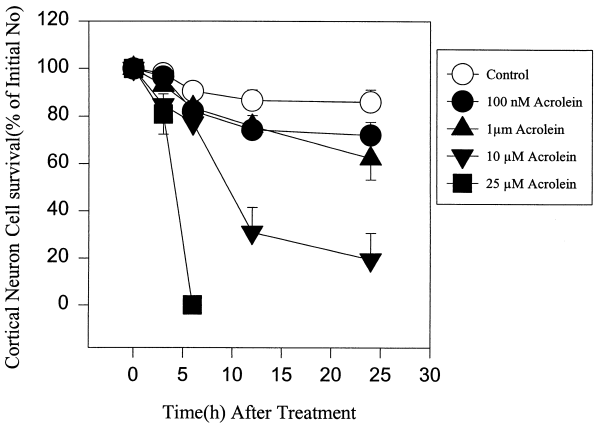

Fig. 1. Response of cultured rat cortical neurons to acrolein. Acrolein led to a time- and concentration-dependent decrease in cell viability.

The effects of acrolein on glucose uptake are shown in Fig. 3. Acrolein led to severe concentration-dependent decrease in glucose transport with 1 nM acrolein leading to a 21% decrease in levels of glucose transport, whereas 5 nM acrolein led to a statistically significant 43% decrease in enzyme activity. Acrolein (10 nM) led to a 50% decrease, whereas 25 nM led to a 57% decrease and 100 nM a 63% decrease in glucose uptake. Higher concentrations led to almost complete inhibition of glucose uptake. All concentrations of acrolein that were >5 nM led to statistically significant ($p < .05$) decreases in glucose transport, which were comparable to values obtained for cultures treated with phloretin. In contrast, cortical neuron cultures ($n = 6$) treated with 1 μM HNE led to only a 10% decrease in glucose uptake compared with the 75% decrease observed for cultures treated with 1 μM acrolein. The impairment of glucose uptake is far in excess of the decrease in cell viability shown in Fig. 1. These results suggest that cell death does not account for the impairment of enzyme activities observed.

Figure 4A demonstrates that acrolein led to a concentration-dependent decrease in glutamate uptake in cortical neurons. All concentrations of acrolein ≥750 nM led to statistically significant ($p < .05$) decreases in glutamate uptake, which approached values observed for blank samples. In comparison, treatment of cortical cul-

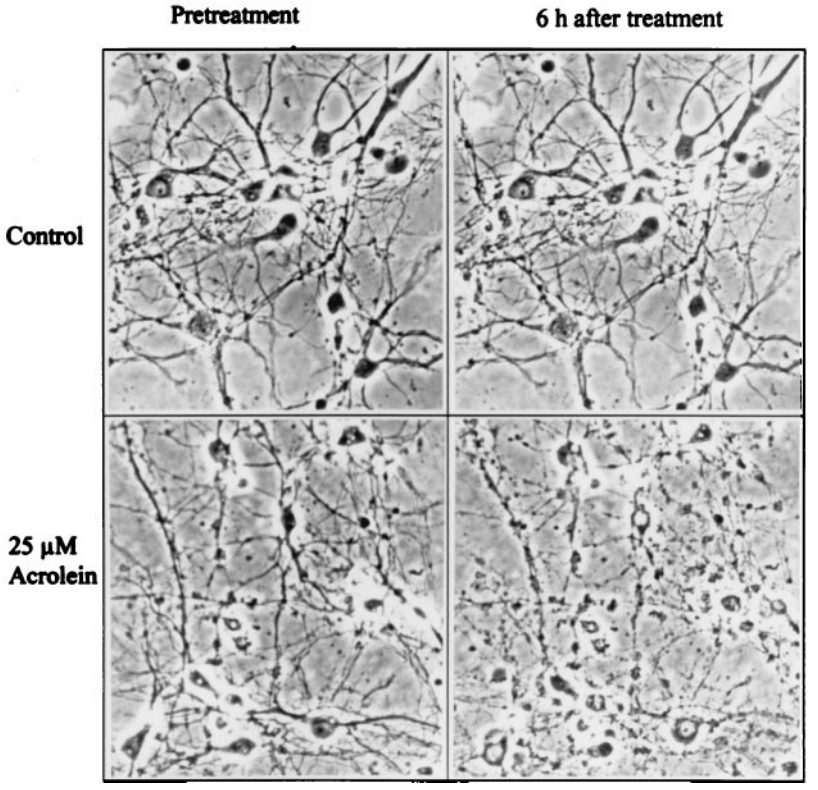

Fig. 2. Micrographs of primary cortical neurons (400×) before and after 6 h treatment with acrolein. The top panel shows control cells in serum-free Locke's medium alone. In the bottom panel, the micrograph on the left is before treatment and the right is cells treated with 25 μM acrolein for 6 h. Cell death is indicated by vacuolization of cell bodies and extensive neurite fragmentation.

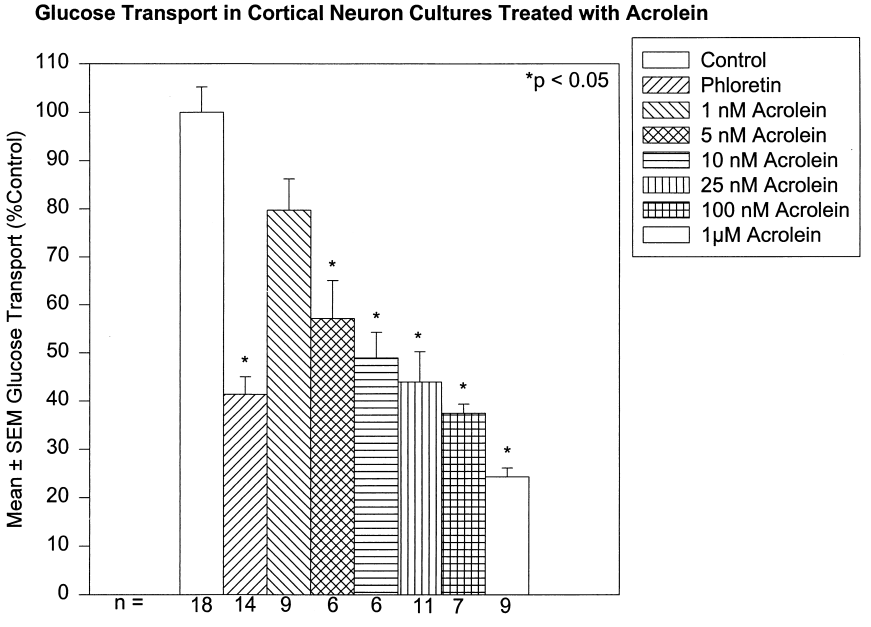

Fig. 3. Glucose transport in cortical cultures treated with acrolein for 4 h. Acrolein led to a significant concentration-dependent decrease in glucose transport. All concentrations greater than 1 nM led to statistically significant ($p < .05$) decreases in glucose uptake. Results are expressed as mean ± SEM% of controls.

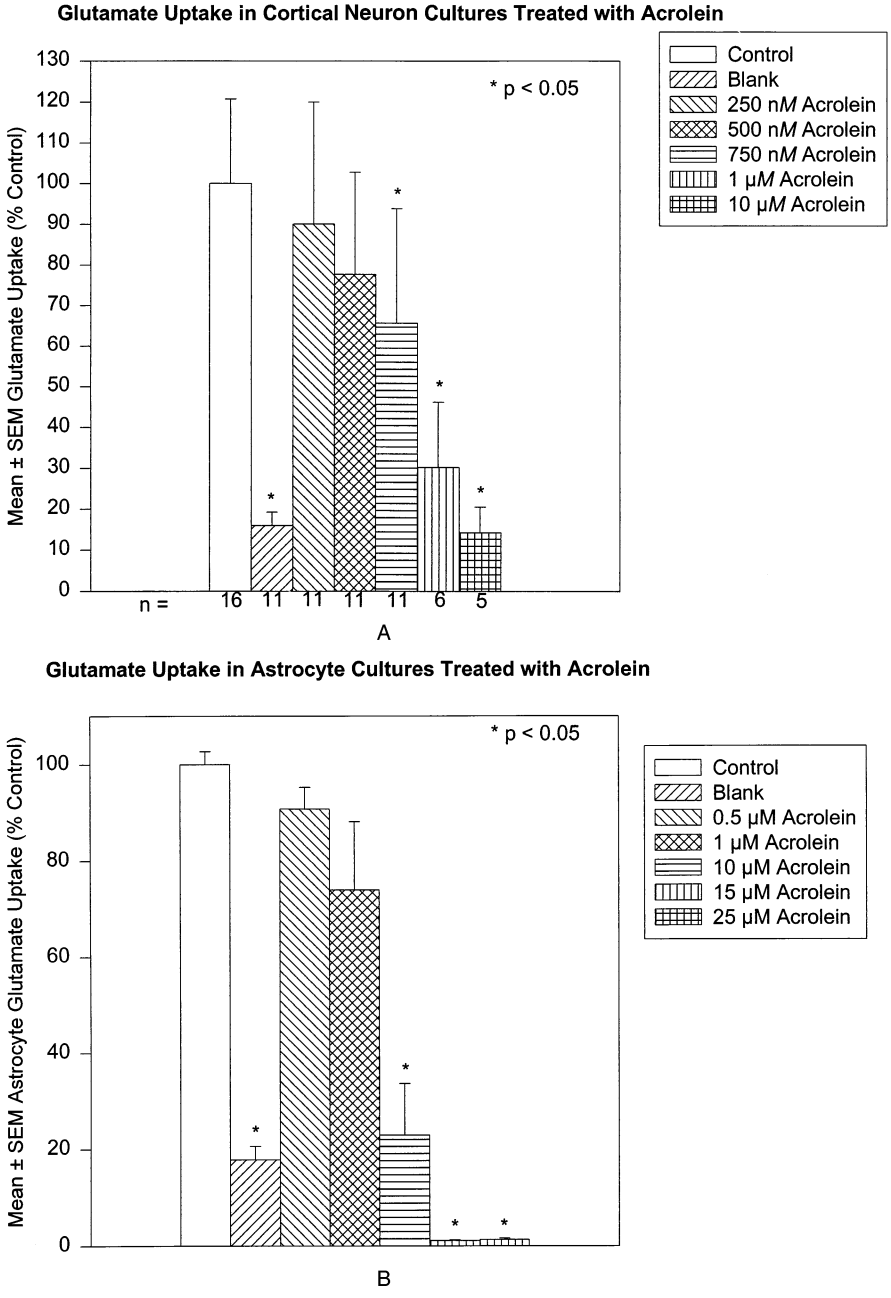

Fig. 4A. Glutamate uptake is significantly ($p < .05$) depleted in cortical neurons treated with acrolein. Results are expressed as mean ± SEM% of controls. (B) Glutamate uptake is significantly depleted ($p < .05$) in primary astrocyte cultures treated with acrolein. Results are expressed as mean ± SEM% of controls.

tures ($n = 6$) with 10 μM HNE led to a 37% decrease in glutamate uptake compared with the 86% decrease in glutamate uptake observed for cultures treated with 10 μM acrolein. Figure 4B demonstrates that treatment of essentially pure astrocyte cultures with acrolein leads to a similar pattern of concentration-dependent decreased glutamate uptake with concentrations greater than 10 μM leading to a statistically significant ($p < .05$) decrease in glutamate uptake. The impairment of glutamate uptake observed is well in excess of levels of decreased cell viability, indicating that the decrease in activity is not simply due to cell death (23.8% total LDH) for 25 μM treatment.

DISCUSSION

This is the first study to investigate the effects of acrolein on neuronal function. Previous studies of Cal-

ingasan et al. [31] using immunostaining showed that acrolein was present in greater than 50% of AD NFT and in dystrophic neurites surrounding SP. Our previous study demonstrated statistically significant elevations of acrolein in AD amygdala and hippocampus and parahippocampal gyrus using high-performance liquid chromatography with fluorescence detection [32]. Our study also demonstrated that acrolein leads to a time- and concentration-dependent decrease of cell viability in hippocampal neuron cultures and a corresponding increase in intracellular calcium concentrations.

To determine the effects of acrolein on cortical neuron survival and function, we exposed primary rat cortical neurons to increasing concentrations of acrolein. For comparison to levels of acrolein observed in tissue, concentrations used in culture systems expressed in units of nmol/mg of protein ranged from 0.04 nmol/mg of protein (1 nM) to 1.1 μmol/mg of protein (25 μM). In survival studies, acrolein led to a time- and concentration-dependent decrease in cell viability. Compared with HNE, which is toxic to a number of cell culture systems, [38,40–43] acrolein is considerably more toxic. The mechanisms of toxicity of acrolein have been suggested to be due to further lipid peroxidation [44] or to depletion of glutathione [27]. Our study demonstrates that the mechanism of acrolein neurotoxicity involves impairment of glucose transport and glutamate uptake.

This study demonstrates a concentration-dependent decrease in glucose transport with concentrations as low as 5 nM leading to a 45% decrease in transport of glucose. Treatment of cortical neurons with 100 nM acrolein led to a 62% decrease in glucose transport. In comparison, HNE at a concentration of 1 μM led to only a 10% reduction of glucose. These data suggest that acrolein is far more potent than HNE in the impairment of glucose uptake. These data also demonstrate a concentration-dependent inhibition of glutamate uptake, with acrolein concentrations as low as 500 nM leading to a 25% decrease in uptake in cortical neurons. Treatment with 1 μM acrolein (43 nmol/mg of protein) led to a 70% decrease in glutamate uptake, whereas 1 μM HNE led to a 20% decrease in glutamate uptake. Treatment of astrocyte cultures with 1 μM acrolein led to a 26% decrease in glutamate uptake, whereas 10 μM acrolein led to an 80% decrease and higher concentrations led to nearly complete inhibition ($\sim$98%). Glutamate is the major excitatory neurotransmitter in mammalian brain and, in excess, can disrupt calcium homeostasis leading to neuron death [45]. Previous studies demonstrate that glutamate uptake is impaired in acute neuron injuries such as hypoxia [39] and in chronic neurodegenerative disorders, including AD, [43,46] and that oxidative stress, [40,47–51] exposure to amyloid β-peptide, [52] and the aging process [53] lead to impaired glutamate uptake in brain cells.

In summary, our data provide further evidence for the role of oxidative stress and markers of lipid peroxidation in the pathogenesis of neuron degeneration in AD. This study demonstrated that acrolein, as a product of lipid peroxidation, can severely impair cellular glucose transport and glutamate uptake in cultured neurons and astrocytes leading to cell death. In light of acrolein's ability to impair enzymatic function critical to cell survival and its relatively high brain levels in AD, it is possible that this neurotoxic product of lipid peroxidation functions as a significant factor in the pathogenesis of neuron degeneration in AD and other neurodegenerative disorders.

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