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Homocysteine potentiates β-amyloid neurotoxicity: role of oxidative stress

Pei I. Ho,*,† Scott C. Collins,*,‡ Sirikarnt Dhitavat,*,† Daniela Ortiz,* David Ashline,† Eugene Rogers*,†,§ and Thomas B. Shea*,†,‡

**Center for Cellular Neurobiology and Neurodegeneration Research and Departments of †Biochemistry, ‡Biological Sciences and §Health and Clinical Sciences, University of Massachusetts-Lowell, Lowell, Massachusetts, USA*

Abstract

The cause of neuronal degeneration in Alzheimer's disease (AD) has not been completely clarified, but has been variously attributed to increases in cytosolic calcium and increased generation of reactive oxygen species (ROS). The β-amyloid fragment (Aβ) of the amyloid precursor protein induces calcium influx, ROS and apoptosis. Homocysteine (HC), a neurotoxic amino acid that accumulates in neurological disorders including AD, also induces calcium influx and oxidative stress, which has been shown to enhance neuronal excitotoxicity, leading to apoptosis. We examined the possibility that HC may augment Aβ neurotoxicity. HC potentiated the Aβ-induced increase in cytosolic calcium and apoptosis in differentiated SH-SY-5Y human neuroblastoma cells. The antioxidant vitamin E and the glutathione precursor *N*-acetyl-L-cysteine blocked apoptosis following cotreatment with HC and Aβ, indicating that apoptosis is associated with oxidative stress. These findings underscore that moderate accumulation of excitotoxins at concentrations that alone do not appear to initiate adverse events may enhance the effects of other factors known to cause neurodegeneration such as Aβ.

Keywords: Alzheimer's disease, apoptosis, β-amyloid, calcium influx, homocysteine, oxidative stress.

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Homocysteine (HC), a non-protein amino acid reversibly formed and secreted during metabolism, is a potent neurotoxin (Sawada *et al*. 1982; Lee *et al*. 1988; Lipton *et al*. 1997; Kruman *et al*. 2000). Elevated levels of HC are correlated with multiple neurological disorders, including Alzheimer's disease (AD; Clarke *et al*. 1998; Gottfries *et al*. 1998; Miller 1999). Plasma HC has been reported to be elevated in some (Joosten *et al*. 1997; Clarke *et al*. 1998), but not all (Fekkes *et al*. 1998) confirmed cases of AD and represents an early marker of cognitive impairment in the elderly (Lehmann *et al*. 1999). HC is toxic to human and murine neuronal cells *in vitro* and in mouse brain (Lipton *et al*. 1997; Parsons *et al*. 1998; Kruman *et al*. 2000). HC is also an endogenous ligand for the NMDA receptor (Zeise *et al*. 1988), and a portion of its neurotoxicity in cortical neurons is achieved by overstimulation of NMDA receptors and resultant calcium influx (Lipton *et al*. 1997; Kim 1999; Kruman *et al*. 2000). HC is as least as excitotoxic as glutamate and enhances glutamate excitotoxicity (Sawada *et al*. 1982; Kruman *et al*. 2000). Moreover, while normal activation of NMDA receptors can provoke both excitation and inhibition, HC elicited only excitation of cerebellar neurons (Lee *et al*. 1988).

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The full range of causes of AD remains unclear, but has been considered to be multifactoral (Cotman *et al*. 1992; Cotman and Anderson 1995; Holscher 1998; Felician and Sandson 1999; Markesbery and Carney 1999; Drouet *et al*. 2000; Smith *et al*. 2000). One hallmark of AD is the accumulation of β-amyloid (Aβ; Haas *et al*. 1992; Seubert *et al*. 1992). Exposure of cultured neurons and neuronal cells to aggregated Aβ induces multiple neurodegenerative events, including accumulation of cytosolic calcium, generation of reactive oxygen species (ROS), hyperphosphorylation of tau protein and apoptosis; however, prevention of accumulation of calcium within the cytosol also prevents all of these other neurotoxic events (Ekinci *et al*. 1999, 2000;

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Address correspondence and reprint requests to P. I. Ho, Center for Cellular Neurobiology and Neurodegeneration Research, University of Massachusetts-Lowell, Lowell, MA 01854, USA.
E-mail: thomas_shea@uml.edu

Abbreviations used: AD, Alzheimer's disease; Aβ, β-amyloid; DMEM, Dulbecco's modified Eagle medium; HC, homocysteine; NAC, *N*-acetyl-L-cysteine; PS, phosphatidyl serine; ROS, reactive oxygen species

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Ekinci and Shea 2000 and refs cited therein), suggesting that calcium accumulation is an early and pivotal event in Aβ neurotoxicity. Since previous studies have indicated that glutamate, another NMDA receptor agonist, enhances Aβ toxicity (Mattson *et al*. 1992; Gray and Patel 1995), we examined whether or not HC also augments Aβ neurotoxicity in culture.

Materials and methods

Cell culture and treatment

SH-SY-5Y human neuroblastoma cells were cultured in Dulbecco's Q4 modified Eagle medium (DMEM; Cellgro) containing 10% fetal bovine serum in 5% CO_2. Cultures were differentiated for 7 days with 10 μM retinoic acid, during which time they elaborated extensive neurites that exhibit characteristics of axons. SH-SY-5Y cells differentiated in this manner have been useful for analysis of Aβ toxicity (e.g. Lambert *et al*. 1994; Shea *et al*. 1997; Ekinci *et al*. 1999). Cultures were then deprived of serum and treated for 2–4 h with 10–20 μM $A\beta_{25-35}$ and/or 125–250 μM HC. Treatment with each of these agents at certain concentrations results in complete destruction of cultures within a few hours, and induces multiple neurotoxic effects at sublethal concentrations (e.g. Ekinci *et al*. 1999; Kruman *et al*. 2000); we therefore utilized the lowest concentrations at which statistically significant increases in cytosolic calcium could be monitored. Additional cultures received 0.15 ng/mL vitamin E (as α-tocopherol) and 10 mM *N*-acetyl-L-cysteine (Olveri *et al*. 2001) which increases glutathione (e.g. Ou *et al*. 1999; Schulz *et al*. 2000). All supplies were from Sigma Chemical Co. (St Louis, MO, USA) unless otherwise specified.

Densitometric analyses

Intracellular calcium concentration was monitored as described previously (Ekinci *et al*. 1999) by incubation with Fluo-3 (acetoxymethyl ester; Molecular Probes, Eugene, OR, USA) for 30 min, followed by rinsing of cultures in serum-free medium and analysis of fluorescent intensity. Apoptotic degeneration was monitored by levels of externalized phosphatidyl serine (PS) as visualized by incubation for 5 min with 10 μL/mL meracyanine (Upstate Biotechnology, Lake Placid, NY, USA) followed by Q5 rinsing of cultures with serum-free medium (Ekinci and Shea 2000; Ekinci *et al*. 2000). At least 100 cells in randomly selected fields from duplicate cultures derived from two separate experiments were scored for fluorescent intensity using NIH Image analysis software. Images were captured under epifluorescent and phase-contrast illumination using a DAGE CCL-72 cooled CCD camera via a Scion LG-3 frame grabber operated by NIH IMAGE and stored as PICT files on a Macintosh Power PC 7100AV. Representative background areas devoid of cells were similarly analyzed and subtracted from cell values to yield net densitometric values. All fields for each individual assay were illuminated, captured and processed under identical settings. Corresponding phase-contrast images were obtained. Cell aggregates were excluded from the assays; only individual cells were quantified. Both of these methods were previously utilized to quantify Aβ-induced neurodegeneration (e.g. Ekinci *et al*. 1999, 2000). Values were exported to Excel for statistical analyses via Student's *t*-test and ANOVA.

Results

Treatment of differentiated SH-SY-5Y cells for 2 h with sublethal concentrations of Aβ and HC each induced a concentration-dependent increase in cytosolic calcium (Fig. 1). Aβ at 20 μM (e.g. Ekinci *et al*. 1999) and HC at 250 μM (e.g. Kruman *et al*. 2000) each resulted in statistically identical increases in cytosolic calcium over levels present in untreated control cells. Similarly, treatment with half of each of these respective concentrations (10 μM Aβ and 125 μM HC) induced marginal, but still identical, respective increases in cytosolic calcium. The marginal levels of cytosolic calcium induced by these lower concentrations facilitated subsequent examination for potential additive or synergistic effects on calcium influx among these agents. We reasoned that, should Aβ and HC have additive effects on cytosolic calcium, then treatment with 10 μM

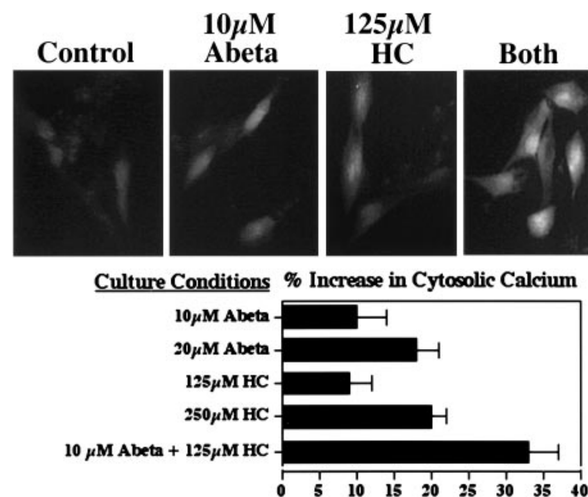

Fig. 1 Effect of Aβ and HC on cytosolic calcium levels in differentiated SH-SY-5Y cells. Cells differentiated for 7 days with 10 μM retinoic acid were treated for 2 h in the absence of serum with 10 or 20 μM Aβ, 125 or 250 μM HC, and 10 μM Aβ + 125 μM HC. Cytosolic calcium was then quantified via Fluo-3 intensity as described in Materials and methods. Micrographs present representative cells visualized under UV optics following treatment under the indicated conditions. The Fluo-3 signal is largely confined to the perikaryon and does not visualize neurites as clearly as meracyanine (e.g. Fig. 2). The accompanying graph presents densitometric analyses of ≥100 cells from two cultures each from two separate experiments. Values are presented as the percentage increase (mean ± SEM) in fluorescent intensity under each condition versus levels obtained in untreated control cultures. Levels of cytosolic calcium following all treatments were significantly greater than those in untreated controls ($p < 0.005$; Student's *t*-test). Cytosolic calcium levels following treatment with 10 μM Aβ + 125 μM HC were significantly greater than those obtained with either 20 μM Aβ or 250 μM HC alone ($p < 0.005$; Student's *t*-test). Two-way ANOVA indicated a positive interaction between the effects of 10 μM Aβ and 125 μM HC on cytosolic calcium levels versus either 10 μM Aβ or 125 μM HC alone ($F = 9.03$; $p = 0.0028$; degrees of freedom = 1).

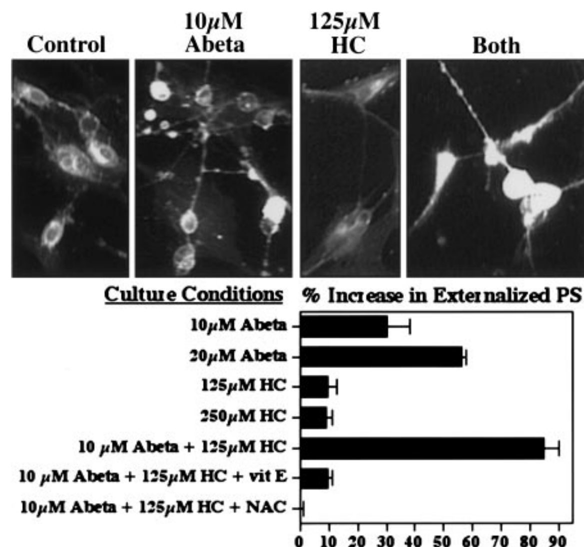

Fig. 2 Effect of Aβ and HC on externalized PS in differentiated SH-SY-5Y cells. Cells were differentiated and treated as described in the legend for Fig. 1, and externalized PS was then quantified via meracyanine intensity as described in Materials and methods. Micrographs present phase-contrast and corresponding UV images of representative cells. The accompanying graph presents densitometric analyses of ≥ 100 cells from two cultures each from two separate experiments. Values are presented as the percentage increase (mean ± SEM) in fluorescent intensity under each condition versus levels obtained in untreated control cultures. Levels of externalized PS following treatment with 10 or 20 μM Aβ were significantly greater than those in untreated controls ($p < 0.005$; Student's *t*-test). HC alone at either 125 or 250 μM increased externalized PS only marginally. Externalized PS levels following treatment with 10 μM Aβ + 125 μM HC were significantly greater than those obtained with either 20 μM Aβ or 250 μM HC alone ($p < 0.005$; Student's *t*-test). Co-treatment with 0.15 ng/mL vitamin E or 10 mM *N*-acetyl-L-cysteine prevented the increase in externalized PS induced by 10 μM Aβ + 125 μM HC.

Aβ + 125 μM HC should result in cytosolic calcium levels that equaled those obtained with either 20 μM Aβ or 250 μM HC alone; i.e. treatment with 0.5 × of each agent would have to increase cytosolic calcium to the level induced by 1 × of either agent alone to be considered additive. By contrast, should Aβ and HC instead have synergistic effects on cytosolic calcium, then treatment with 10 μM Aβ + 125 μM HC should result in cytosolic calcium levels that significantly exceed those obtained with either 20 μM Aβ or 250 μM HC alone. When this was carried out, we observed that treatment with 10 μM Aβ + 125 μM HC significantly exceeded those obtained with either 20 μM Aβ alone or 250 μM HC alone (Fig. 1), indicating that Aβ and HC exert an apparently synergistic effect on cytosolic calcium accumulation under these experimental conditions.

Since increased cytosolic calcium following Aβ treatment fosters neuronal apoptosis (e.g. Ekinci *et al*. 1999 and refs therein), we next examined whether or not HC under these conditions augmented Aβ-induced apoptosis. Both 125 μM and 250 μM HC alone induced only marginal increases in apoptosis within 2 h as evidenced by externalized PS (Fig. 2); treatment for ≥ 24 h with 250 μM HC induced significant apoptosis (not shown; e.g. see Kruman *et al*. 2000). As previously reported, 2 h-treatment with Aβ induced significant apoptosis (see also Ekinci *et al*. 1999). However, combined treatment with 10 μM Aβ + 125 μM HC increased in externalized PS significantly more than either agent alone and more than the sum of levels observed with each agent alone at each concentration, These data indicate that combined treatment with HC and Aβ has a synergistic effect on apoptotic neurodegeneration.

Prior studies have demonstrated that the Aβ-induced increase in cytosolic calcium increases ROS in these cells, and that these ROS, if not quenched, result in apoptosis (Ekinci *et al*. 1999, 2000). In order to ascertain whether or not the combined influence of HC and Aβ on apoptosis was also mediated by oxidative stress, we treated additional cultures with vitamin E, which prevents Aβ-induced apoptosis following calcium influx into these cultures (Ekinci *et al*. 1999, 2000) and with the glutathione precursor, *N*-acetyl-L-cysteine (NAC), which also protects SH-SY-5Y cells from oxidative stress (Hatanaka *et al*. 1996; Oliveri *et al*. 2001). Both of these agents prevented apoptosis during cotreatment of cultures with HC and Aβ (Fig. 2), confirming that oxidative stress plays a critical role in apoptosis induced by these agents.

Discussion

The findings of the present study demonstrate that HC potentiates the effects of Aβ on cytosolic calcium and neuronal apoptosis. A similar potentiation of Aβ-induced neurodegeneration has previously been proposed for glutamate (Mattson *et al*. 1992; Gray and Patel 1995). Prevention of apoptosis by antioxidants indicates that oxidative stress plays an essential role in the combined neurotoxic effects of HC and Aβ. These findings confirm and extend prior studies documenting neurotoxicity of HC in culture (Lipton *et al*. 1997; Parsons *et al*. 1998; Kruman *et al*. 2000). The synergistic influence of HC and Aβ on calcium influx is likely to be derived from stimulation of distinct calcium channels. The initial influx of calcium following exposure to Aβ occurs via the L voltage-sensitive calcium channel (Ueda *et al*. 1997; Ekinci *et al*. 1999; Silei *et al*. 1999; Ho *et al*. 2001), and the initial influx of calcium following exposure to HC occurs via NMDA channels (Lipton *et al*. 1997); resultant calcium influx via each channel fosters increased ROS (Lipton *et al*. 1997; Ekinci *et al*. 1999). We have not yet examined whether the synergistic increase in cytosolic calcium resulting from combined treatment with Aβ and HC is confined to influx

via these channels. Indeed, free radicals can stimulate calcium influx via additional channels including P/Q-type voltage-dependent channels (Ohkuma *et al*. 2001). It is also entirely possible that some of the increased cytosolic calcium observed following combined treatment with HC and Aβ is derived from internal sources, including mitochondria and the endoplasmic reticulum, and/or from additional plasma membrane channels and/or leakage across a compromised membrane. This possibility is underscored since leakage of calcium into the cytosol from all of these latter sources is induced by oxidative stress (Cotman *et al*. 1992; Lafon-Cazal *et al*. 1993; Dykens 1994; Zhou *et al*. 1996), and our data indicate that increased ROS mediates apoptosis following HC and Aβ treatment. In addition, HC may augment Aβ neurotoxicity by additional mechanisms independently of calcium influx (e.g. Zhou *et al*. 1996; Lipton *et al*. 1997; Kruman *et al*. 2000). It remains to be established whether or not the addition of these antioxidants reduces the extent of calcium influx, which would be expected to reduce apoptosis.

Clinical studies implicate homocysteinemia in neurodegeneration, including that related to AD (Joosten *et al*. 1997; Clarke *et al*. 1998; Fekkes *et al*. 1998; Gottfries 1998; Lehmann *et al*. 1999). The findings of the present study, taken together with the demonstration by Kruman *et al*. (2000) that HC enhances excitotoxicity and induces DNA damage leading to apoptosis, underscore that HC may augment multiple neurotoxic insults, and substantiate prior clinical findings demonstrating a correlation between HC and neurodegenerative conditions (Clarke *et al*. 1998; Miller 1999).

While this report was under review, similar findings were published from another laboratory (White *et al*. 2001), in which HC was demonstrated to potentiate Aβ toxicity in cultured neurons. In addition, these investigators demonstrated that HC also potentiated copper neurotoxicity. This latter finding further substantiates the likelihood of interplay of multiple neurotoxic agents in AD neuropathology, since copper has also been shown to potentiate Aβ neurotoxicity (Huang *et al*. 1999).

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Q2 **Gottfries 1998** has been changed to **Gottfries et al. (1998** so that this citation matches the list

Q3 **Kim 1999** has not been included in the list; is this actually Kim et al 1987?

Q4 Au: Please supply company details/address of Cellgro

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