Available online at www.sciencedirect.com
ScienceDirect
Journal of Nutritional Biochemistry 19 (2008) 754–761
Journal of Nutritional Biochemistry

Synergistic induction of ER stress by homocysteine and β-amyloid in SH-SY5Y cells☆

Hyun-Jung Kim[a], Hyang-Ki Cho[a], Young Hye Kwon[a,b,*]

[a]Department of Food and Nutrition, Seoul National University, Seoul 151-742, South Korea
[b]Research Institute of Human Ecology, Seoul National University, Seoul 151-742, South Korea

Received 13 February 2007; received in revised form 6 September 2007; accepted 25 September 2007

Abstract

Clinical studies have raised the possibility that elevated plasma levels of homocysteine increase the risk of atherosclerosis, stroke and possibly neurodegenerative diseases such as Alzheimer's disease (AD); however, the direct impact of homocysteine on neuron cells and the mechanism by which it could induce neurodegeneration have yet to be clearly demonstrated. Here, we investigated the effect of homocysteine on endoplasmic reticulum (ER) stress, the suggested mechanism of neurotoxicity, in human neuroblastoma SH-SY5Y cells. The effect of homocysteine on amyloid-β (Aβ)-induced neurotoxicity and the protective activity of folate were also investigated. Homocysteine led to increased expressions of the binding protein (BiP) and the spliced form of X-box-protein (XBP)-1 mRNAs, suggesting activation of the unfolded-protein response and an increase in apoptosis. When cells were cotreated with homocysteine and Aβ, caspase-3 activity was significantly increased, and expressions of BiP and the spliced form of XBP-1 mRNAs were significantly induced. The neurotoxicity of homocysteine was attenuated by the treatment of cells with folate, as determined by caspase-3 activity and apoptotic body staining. These findings indicate that homocysteine induces ER stress and, ultimately, apoptosis and sensitizes neurons to amyloid toxicity via the synergistic induction of ER stress. Furthermore, a neuroprotective effect of folate against homocysteine-induced toxicity was also observed. Therefore, the findings of our study suggest that ER stress-induced homocysteine toxicity may play an important physiological role in enhancing the pathogenesis of Aβ-induced neuronal degeneration.

Keywords: Homocysteine; ER stress; Apoptosis; Amyloid β-peptide; Folate

1. Introduction

Deficiency in folate has been shown to be related to neurodegeneration, thereby enhancing the influence of other risk factors for neurotoxicity; this is accomplished at least in part by increasing levels of the neurotoxin homocysteine. Homocysteine is a sulfur-containing amino acid formed in the course of the metabolism of methionine. High homocysteine levels contribute to cell injury in a range of diseases including arteriosclerosis, stroke and neurodegenerative diseases [1,2]. Varying factors have been shown to contribute to the development of hyperhomocysteinemia, including nutritional deficiencies in B vitamin cofactors, inherited genetic disorders and age [2]. An association between hyperhomocysteinemia and Alzheimer's disease (AD) has been well established [3,4]; furthermore, several follow-up studies have shown that high homocysteine concentration preceded the diagnosis of AD [5–7]. In previous studies using neuronal cells and animal brains [8], cytotoxicity caused by homocysteine was induced by the overstimulation of *N*-methyl-D-aspartate (NMDA) receptors and resultant calcium influx [9,10] as well as oxidative stress [11].

The endoplasmic reticulum (ER) is a subcellular compartment in which transmembrane, secretory and ER-resident proteins are folded and processed. Interference of the N-

☆ This work was supported by a grant from the Korea Research Foundation (KRF-2004-003-C00214). H-K Cho is supported by a second-stage BK21 Research Fellowship from the Ministry of Education and Human Resources Development.

* Corresponding author. Department of Food and Nutrition, Seoul National University, San 56-1 Sillim-Dong, Kwanak-Gu, Seoul 151-742, South Korea.
E-mail address: hye0414@snu.ac.kr (Y.H. Kwon).

0955-2863/$ – see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.jnutbio.2007.09.009

linked protein glycosylation, aberrant calcium homeostasis, viral infection and the reduction of disulfide bonds stimulate the accumulation of unfolded proteins in the ER [12]. In recent years, significant progress has been made in elucidating the molecular mechanisms of the mammalian unfolded protein response (UPR). The UPR represents a set of coordinated signaling cascades by which ER communicates with the nucleus to induce genes involved in adaptive pathways and apoptosis. These cascades are activated by an imbalance between the substrate protein burden on the ER and its protein folding capacity. The activation of UPR involves the concerted action of three proximal ER transmembrane proteins: RNA-dependent protein kinase (PKR)-like ER kinase, activating transcription factor 6 (ATF6) and inositol-requiring enzyme 1 (IRE1). The expression of chaperones such as the binding protein (BiP) is simultaneously increased to attenuate ER stress. On the other hand, excessive or prolonged ER stress results in irreversible cell injury and apoptotic cell death [13,14]. Homocysteine has been suggested to be one of several ER stress inducers on human endothelial cells [15,16], HepG2 cells [17] and primary neuronal cells [18], based on observations that it might cause the induction of ER chaperones.

AD, the most prevalent neurodegenerative disease in humans, is clinically characterized by a progressive loss of memory and cognitive impairment and pathologically characterized by the deposition of senile plaques composed of amyloid β-peptide (Aβ), the formation of intracellular neurofibrillary tangles due to an abnormal phosphorylation of tau proteins and neuronal loss due to neuronal death [19,20]. Several studies have suggested that neuronal death in AD could arise from ER stress due to the accumulation of unfolded proteins [19,21]. Homocysteine has also been shown to have an additive effect on the Aβ-induced oxidative stress and DNA impairment [1,22,23]; however, the specific intracellular signaling pathways leading to Aβ-triggered neuronal cell death have yet to be fully defined. Accordingly, in the present study, we investigated the mechanisms involved in homocysteine-induced cell death and the effect of homocysteine on neurotoxicity by Aβ, using human neuroblastoma SH-SY5Y cells. The possible neuroprotective effect of folate against homocysteine-induced toxicity was also investigated.

2. Materials and methods

2.1. Cell culture

Human neuroblastoma SH-SY5Y cells were cultured in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Cambrex, Walkersville, MD, USA), 100 U/ml penicillin, 100 μg/ml streptomycin at 37°C in a humidified 95% air and 5% CO_2 incubator. Before treatment, cells were washed with phosphate-buffered saline (PBS) and treated with Aβ 25-35 (Sigma, St. Louis, MO, USA), tunicamycin, homocysteine and/or folate under serum-free conditions for 4 h unless otherwise specified. The Aβ 25-35 fragment of Aβ has been shown to retain several toxicological properties of the native full-length peptide [24]. Tunicamycin (Sigma), which inhibits N-glycosylation of target asparagines residue in the luminal domains of proteins, was used as a positive control in inducing ER stress.

2.2. Determination of cell viability

Cell viability was assessed using the trypan blue exclusion test, which is based on the capability of viable cells to exclude the trypan blue dye. The effect of each treatment on cell viability was assessed as the percentage of the respective value obtained when control cells were taken as 100% viable.

2.3. Determination of reactive oxygen species generation

To monitor net intracellular accumulation of reactive oxygen species (ROS), the fluorescent probe 2′7′-dichlorofluorescein diacetate (DCF-DA) was used. Following treatment, cells were incubated with 10 μM DCF-DA in phenol-free DMEM medium for 30 min at 37°C. Cells were then harvested with PBS containing 0.5% Triton X-100. DCF fluorescence was measured using a fluorometer (Multilabel Plate Reader, Perkin Elmer, Waltham, MA, USA) with excitation at 490 nm and emission at 520 nm. Intensity of ROS was expressed as the relative value compared to control.

2.4. Determination of expression of ER stress genes using semiquantitative reverse transcriptase-polymerase chain reaction

Total cellular RNA was isolated using Trizol Reagent (Life Technologies, Rockville, MD, USA), and cDNA was synthesized using 2 μg of total RNA with the Superscript First-strand synthesis system (Life Technologies). For amplification of cDNA, primers for BiP (upstream, GGTACATTTGATCTGACTG; downstream, CACTTCCATAGAGTTTGCTG) and primers for XBP-1 (upstream, AAACAGAGTAGCAGCTCAGACTGC; downstream, TCCTTCTGGGTAGACCTCTGGGAG) were used. Expression of β-actin was examined as an internal control (upstream, GTTTGAGACCTTCAACACCCC; downstream, GTGGCCATCTCCTGCTCGAAGTC). Primers of XBP-1 were designed to generate cDNA product encompassing the IRE1 cleavage site as previously described [25]. The unspliced and spliced mRNAs generate 480- and 454-bp cDNA products, respectively. These fragments were further digested by PstI to check whether a PstI restriction site was lost after IRE1-mediated splicing of mRNA. cDNA products from the unspliced mRNA yielded two short fragments (289 and 191 bp) after digestion. For each combination of primers, the kinetics of polymerase chain reaction (PCR) amplification was studied, the number of cycles corresponding to plateau was determined, and PCR was performed within the exponential range. Amplified products were separated on an agarose gel and visualized with ethidium bromide staining.

2.5. Apoptosis analysis by 4′-6-diamidino-2-phenylindole staining

To detect apoptotic body, cells were plated at 4-well chamber slides, which were coated with poly-lysine. After treatment, medium was removed and cells were fixed with 4% paraformaldehyde and fixed cells were stored with 70% ethanol at −20°C until analysis. For 4′-6-diamidino-2-phenylindole (DAPI) staining, cells were incubated with 1 μg/ml DAPI for 10 min at room temperature.

2.6. Determination of caspase-3 activities

The activity of caspase-3 was measured by caspase-3 colorimetric assay kit (MBL, Woburn, MA, USA) as described by the manufacturer. Absorbance was measured using a multiwell reader (Bio-Rad, Tokyo, Japan) at 405 nm every 30 min to confirm the linearity of absorbance. Protein content was determined using the protein assay reagent (Bio-Rad, Hercules, CA, USA). Caspase-3 activity was first expressed as change in absorbance units per microgram of protein and per hour and then converted to the relative value compared to control.

2.7. Statistical analysis

The data were analyzed using SAS software. For all experiments, either Student's *t* test or one-way analysis of variance followed by Duncan's multiple range test were employed to assess the statistical significance. Data were expressed as mean±S.E.M., and statistically significant difference was considered to be present at $P<05$.

3. Results

3.1. Homocysteine-potentiated, Aβ-induced cytotoxicity in SH-SY5Y cells

We first determined the cytotoxic effect of homocysteine on SH-SY5Y cells by incubating cells in a medium supplemented with different concentrations of homocysteine. Progressive cell death was shown to be dose- and time-dependent (Fig. 1A); however, the treatment of cells with 5 mM cysteine did not induce any apparent cell toxicity (data not shown), suggesting that the effect was specific to homocysteine. Given that the level of plasma homocysteine is elevated in AD patients [5], we determined that homocysteine modifies neuronal vulnerability to Aβ (Fig. 1B). Exposure to 10 μM Aβ 25-35 caused the death of ~24.1% of the cells during a 4-h exposure period. When cells were exposed to both Aβ and homocysteine, the extent of cell death caused by combined treatment was significantly greater than that caused by homocysteine alone but not significantly greater than that caused by Aβ alone.

3.2. Homocysteine induced apoptosis in SH-SY5Y cells

Based on our observation that homocysteine acted to induce neurotoxicity, we found that SH-SY5Y cells died in an apoptotic manner, as deduced from the results of DAPI staining and caspase-3 activity. We were able to clearly observe the apoptotic bodies when cells were treated with 5 mM homocysteine for 4 h (Fig. 2B). Changes in cell morphology, such as cell shrinkage, were also observed in homocysteine-treated cells (data not shown). In contrast, very few condensed and fragmented nuclei were observed in the control cells (Fig. 2A). Caspase-3 activity was also significantly increased by 4 h of 5 mM homocysteine treatment (Fig. 2B). An increased number of apoptotic bodies were observed when cells were cotreated with 5 mM homocysteine and 10 μM Aβ for 4 h (Fig. 2A), indicating that homocysteine potentiates Aβ-induced neuronal toxicity. This result was also confirmed by a significant increase in caspase-3 activity when cells were cotreated with homocysteine and Aβ (Fig. 2B).

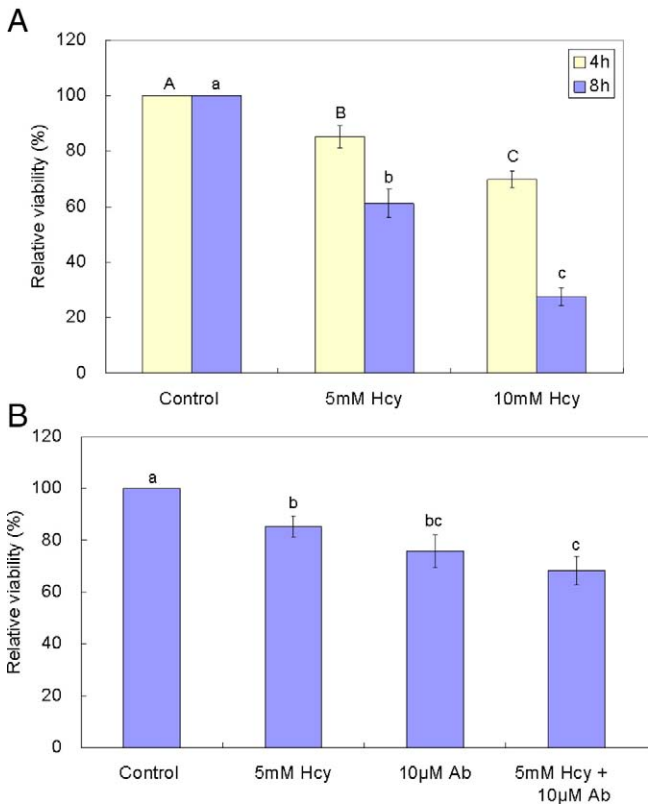

Fig. 1. The effect of homocysteine and Aβ on viability of SH-SY5Y cells. (A) Cells were treated with homocysteine (5 or 10 mM) for 4 or 8 h. Cell number was determined by trypan blue exclusion test (n=3 independent experiments). (B) Cells were treated with homocysteine and/or Aβ for 4 h. Cell number was determined by trypan blue exclusion test (n=3–5 independent experiments). Means not followed by the same superscript are significantly different at $P<05$.

3.3. Cytotoxic effect of homocystine did not involve oxidative stress in SH-SY5Y cells

To determine the intermediate accumulation of intracellular reactive oxygen, we used DCF-DA, which is freely permeable to cells and is converted to DCF in response to

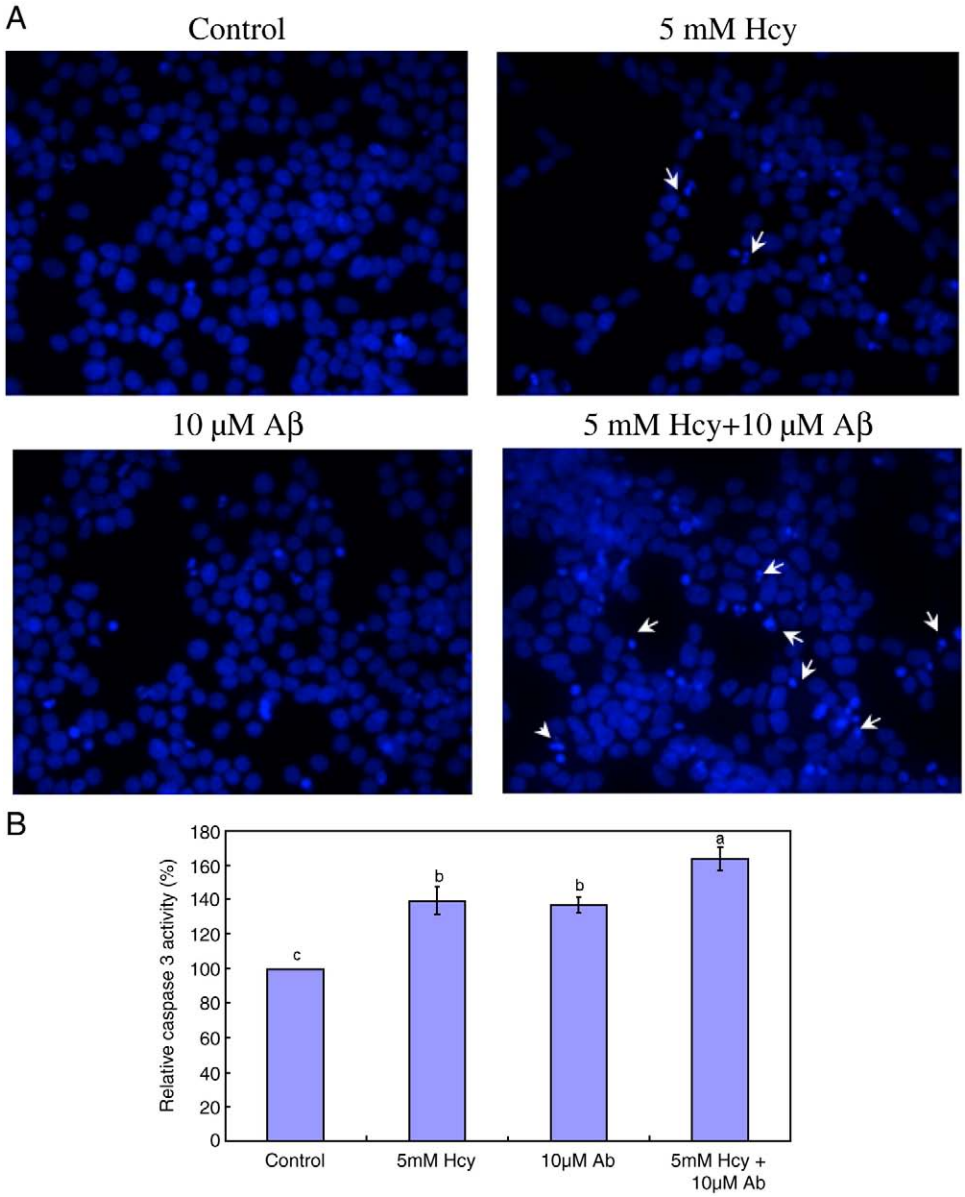

Fig. 2. The effect of homocysteine and Aβ on apoptosis of SH-SY5Y cells. (A) Cells were exposed to different treatments for 4 h, stained with DAPI and visualized under fluorescein UV optics (original magnification ×400). (B) The caspase-3 activity of cells after different treatments for 4 h (n=3 independent experiments). Means not followed by the same superscript are significantly different at P<05.

hydrogen peroxide (H_2O_2) or nitric oxide in cells. Exposure of cells to 5 mM homocysteine caused a significant decrease in ROS (80.1% of control; P<05), suggesting that homocysteine did not induce oxidative stress; this result was also supported by fluorescence microscopy (data not shown). To confirm the DCF-DA results, we pretreated cells with Vitamin C, Vitamin E or *N*-acetylcysteine (NAC) and measured the protective effect of antioxidants on homocysteine-induced cell death; in doing so, we failed to observe any inhibitory effects of these antioxidants (Table 1). In contrast, the antioxidants showed antioxidative effects in cells treated with H_2O_2. A 4-h treatment with Aβ (10 μM) also failed to significantly induce oxidative stress (103.5% of control). This result is consistent with the findings of a previous study that only a 24-h treatment significantly decreased GSH levels; no effect was observed at earlier time points (1 and 5 h) [26].

3.4. ER stress was involved in the cytotoxic effect of homocystine in SH-SY5Y cells

To confirm the observation that homocysteine could induce ER stress and subsequently ER stress-mediated apoptosis, we treated SH-SY5Y cells with 5, 10 and 20 mM homocysteine for 4 h and then measured the mRNA expression levels of ER stress response genes by reverse transcriptase-PCR (RT-PCR) (Fig. 3A). BiP has been shown to regulate the ER stress-sensing pathways, and XBP-1 mRNA splicing has been shown to be involved in the

Table 1
The effect of antioxidants on cell viability treated with H_2O_2 or homocysteine

Treatment	Relative viability (%)
Control	100
H_2O_2	46.2±0.9 *
H_2O_2 + Vit C	82.4±0.4 †
H_2O_2 + Vit E	70.9±1.5 †
H_2O_2 + NAC	81.4±1.8 †
Hcy	70.9±1.5 *
Hcy + Vit C	74.1±2.3
Hcy + Vit E	70.2±2.4
Hcy + NAC	73.5±1.5

Cells were pretreated with 100 μM Vit C, 100 μM Vit E or 10 mM NAC for 2 h and cotreated with 500 μM H_2O_2 or 5 mM Hcy (homocysteine) for 4h. Cell number was determined by trypan blue exclusion test (n=3 independent experiments).
Vit, vitamin.
* Significantly different with control at P<05.
† Significantly different with H_2O_2 at P<05.

transcriptional up-regulation of UPR-inducible genes [27]. Homocysteine greatly induced expressions of BiP and the spliced forms of XBP-1, especially the induction of BiP in a dose-dependent manner (Fig. 3B). The relative mRNA level of unspliced form of XBP-1 significantly (P<.05) decreased by homocysteine at concentration of up to 10 mM homocysteine (32.6±7.3% of control). Other transcription factors such as ATF4 and ATF6 also mediate the transcriptional regulation of UPR genes [13,14], which may explain differences in dose-responsiveness between BiP and XBP-1 mRNA levels.

In contrast, Aβ did not induce the expression of BiP or the spliced forms of XBP-1 (Fig. 3C); however, the cotreatment using both homocysteine and Aβ increased these expression levels, relative to those obtained using homocysteine alone, suggesting the synergistic effect of homocysteine and Aβ in inducing ER stress (Fig. 3D).

3.5. Folate protects homocysteine-induced apoptosis in SH-SY5Y cells

To investigate the protective activity of folate against homocysteine-induced toxicity, we observed the decrease in the number of apoptotic bodies when cells were treated with 5 mM homocysteine together with 10 μM folate for 4 h (Fig. 4A). This treatment also significantly decreased caspase-3 activity (Fig. 4B), suggesting the protective effect of folate against homocysteine-induced toxicity.

4. Discussion

Neurotoxicity caused by homocysteine has been implicated in the pathophysiology of neurodegenerative diseases such as AD; however, the direct impact of homocysteine on neuron cells and the mechanism by which it might induce neurodegeneration are yet to be established. Our study demonstrated dose- and time-dependent cytotoxicity and UPR induction by homocysteine; this finding suggests that ER stress, along with DNA damage [9] and overstimulation of calcium influx via NMDA receptors [11], results in neuron cell apoptosis. A previous study of primary neuronal cell culture also observed the induction of ER stress proteins, mostly chaperones, by 5 or 10 mM homocysteine for 24 h [18]. In addition to the induction of BiP, an ER-resident chaperone protein, we also observed the induction of the spliced form of XBP-1, the transcription factor that enhances transcription of chaperone proteins; this finding may explain the previously documented induction of chaperones.

Although the concentrations of homocysteine added to culture medium to induce UPR are very high (1-mM range) relative to that measured in a patient's serum, which rarely exceeds the 100-μM range [28], there were only 1.7- and 5.3-fold increases in intracellular homocysteine level recorded when HUVECs were treated with 1 and 5 mM exogenous

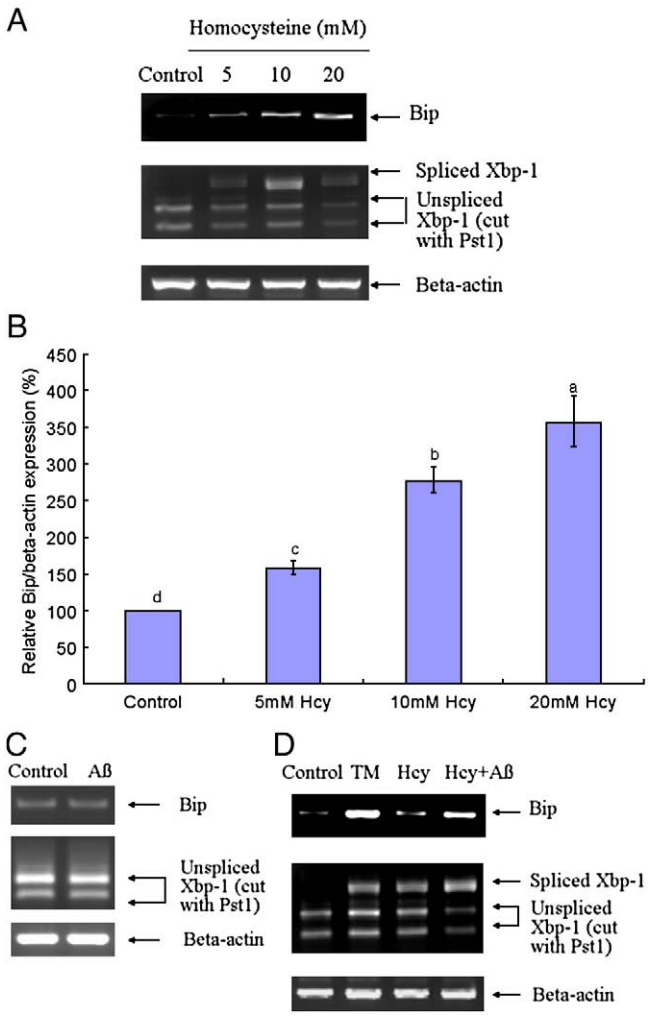

Fig. 3. The effect of homocysteine and Aβ on expressions of ER-stress response genes of SH-SY5Y cells. Total RNA was extracted from cells after incubation with each treatment for 4 h and RT-PCR was performed as described in methods. Levels of BiP mRNA were normalized to the control gene, β-actin. Means not followed by the same superscript are significantly different at P<05 (n=3 independent experiments).

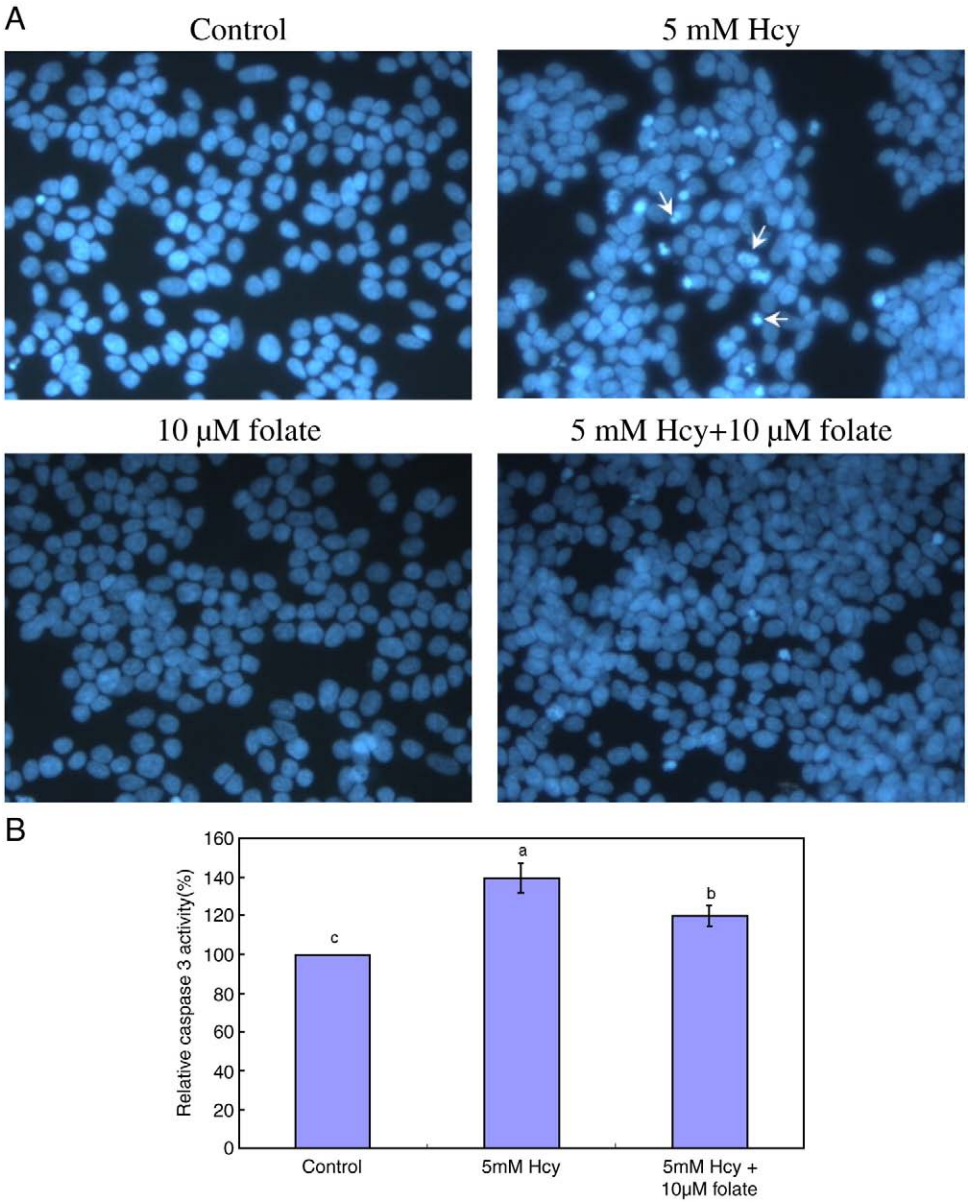

Fig. 4. The protective effect of folic acid on apoptosis of SH-SY5Y cells. (A) Cells were exposed to different treatments for 4 h, stained with DAPI and visualized under fluorescein UV optics (×400). (B) The caspase-3 activity of cells after different treatments for 4 h (n=3 independent experiments). Means not followed by the same superscript are significantly different at P<05.

homocysteine for 4 h, respectively [15]. In a previous study using HepG2 cells, intracellular homocysteine levels changed by factors of between 2 and 6 with 1–5 mM homocysteine treatment for 4 h [17]. In addition, the hyperhomocyteinemic diet (high-methione/low-folate diet) caused a marked elevation of C/EBP-homologous protein expression in the livers of mice, along with an approximately 25-fold increase in plasma homocysteine levels [17]. This finding indicates that the culture model employed in the present study may be useful in identifying the involved neuropathogenic mechanisms associated with homocysteine elevations; however, the overall cytotoxicity by homocysteine in the present study was less than that reported in the previous study using primary cultured hippocampal neurons [23], in which the treatment of cells with 250 μM homocysteine for 48 h decreased cell viability to ~50% of control.

Lentz and Sadler [29] suggested that homocysteine disrupts the formation of disulfide bonds in molecules because of its reactive sulfhydryl residue, thereby resulting in disturbances in ER function; however, cysteine, the normal plasma concentration of which is 25–30 times higher than that of homocysteine, is not considered to be a significant risk factor for cardiovascular diseases [30]. Our study also showed that cysteine does not induce cell toxicity, in agreement with the findings of previous studies that

cysteine did not cause ER stress in endothelial cells [15] and epithelial cells [31]; together, these results indicate that homocysteine-induced ER stress is specific. Agnati et al. [32] reported that Aβ peptides directly interacted with homocysteine due to electrostatic interactions between the Arg guanidinium group and the sulfur of homocysteine. Following binding to homocysteine, Aβ peptides favor a β-sheet conformation, which may result in the development of neurotoxicity. Therefore, further study is required on the increased formation of β-fibrils due to binding between other thiol-containing compounds and Aβ.

In the present study, homocysteine treatment did not induce oxidative stress, which is consistent with the results of previous studies [15,16,33]; however, the present study does not disregard the importance of oxidative stress, as intracellular levels of H_2O_2 were significantly increased in the presence of homocysteine and Cu^{2+} [33]. Homocysteine also suppressed the expression of antioxidant enzymes such as glutathione peroxidase and superoxide dismutase in vascular endothelial cells [34], suggesting that homocysteine may enhance the cytotoxic effects of agents or conditions that elicit oxidative damage.

Degenerative neurons in AD exhibit increased oxidative damage, impaired energy metabolism and perturbed cellular Ca^{2+} homeostasis, and Aβ appears to play an important role in these abnormalities [20]. Although the disturbance of cellular Ca^{2+} homeostasis is one of the suggested toxic mechanisms exerted by Aβ [21], several studies report inconsistent results in this regard [35], and the question of whether the modulation of ER Ca^{2+} release can make neurons vulnerable to Aβ toxicity has yet to be clarified. In our study, Aβ did not induce expressions of BiP and the spliced form of XBP-1 mRNA; this is consistent with the findings of a study using primary cell culture of cortical neurons incubated with 25 μM Aβ for up to 24 h [36]. In contrast, a study by Suen et al. [37] showed an increase in BiP protein levels in neurons treated with 25 μM Aβ for 16 h; however, their study also showed that the modulation of calcium release from ER can only provide partial neuroprotection against Aβ peptide toxicity, suggesting in turn that multiple pathways are involved in Aβ-induced neuronal toxicity.

The ability of elevated homocysteine levels to increase the vulnerability of cultured neuroblastoma cells to Aβ-induced death suggests a mechanism whereby individuals with low folate intake and elevated homocysteine levels might be at increased risk of AD. In the present study, we observed an increased expression of ER stress response genes when cells were cotreated with homocysteine and Aβ. To the best of our knowledge, this represents the first report of such an observation. These data suggest that elevated neurotoxicity may be due to the potentiation of ER stress by cotreatment of homocysteine and Aβ. In previous studies, Aβ has been shown to induce oxidative stress and DNA damage in cultured neurons [38,39] and in the brains of AD patients [26]. It has also been suggested that by impairing the DNA repair capacity in neurons, folate deficiency and elevated homocysteine levels may lower the threshold level of DNA damage required to trigger neuronal death [23]. According to this view, folate deficiency and elevated homocysteine levels may induce ER stress and the accumulation of DNA damage that may already have been promoted by an age-related increase in oxidative stress and the accumulation of Aβ.

In the present study, we found that folate attenuated the apoptosis caused by homocysteine. This result suggests that an increase in folate intake may prevent the progression of AD by antagonizing the neuronal toxicity due to high levels of homocysteine. Plasma homocysteine concentrations in excess of 14 μM, a cutoff for hyperhomocysteinemia, doubled the risk of AD in the Framingham study [5]. The association between plasma homocysteine level and risk of dementia was not significantly changed by adjustment for the plasma levels of folate, Vitamin B_6 or Vitamin B_{12} [5]. In contrast, a prospective population-based study by Ravaglia et al. [6] showed that low serum folate concentration was associated with an increased risk of both dementia and AD, independent of hyperhomocysteinemia. Although the present study did not use a folate-deficient medium, which acts to induce impaired methylation reactions in the central nervous system, increased concentration of homocysteine is associated with an increased ratio of *S*-adenosyl homocysteine/*S*-adenosyl methionine (SAM) [8], raising the possibility of impaired methylation reaction by homocysteine. Methyl groups are required for the synthesis of myelin, neurotransmitters, membrane phospholipids and DNA in the brain [40]; therefore, further studies are needed to confirm the role of decreased SAM levels in homocysteine-induced cytotoxicity in the brain.

In summary, we demonstrated that homocysteine induces UPR and ultimately apoptosis, as well as sensitizing neurons to amyloid toxicity. We also observed a neuroprotective effect of folate against homocysteine-induced toxicity. Therefore, the present results suggest that ER stress-induced homocysteine toxicity plays a direct role in neuronal health; this should be considered a separate issue from its effect on vascular integrity and is an important step in the pathogenesis of AD especially when homocysteine is combined with Aβ.

Acknowledgment

We thank Yu-mi Jang for technical assistance.

References

[1] Mattson MP, Shea TB. Folate and homocysteine metabolism in neural plasticity and neurodegenerative disorders. Trends Neurosci 2003;26:137–46.
[2] Luchsinger JA, Mayeux R. Dietary factors and Alzheimer's disease. Lancet Neurol 2004;3:579–87.
[3] Morris MS. Homocysteine and Alzheimer's disease. Lancet Neurol 2003;2:425–8.

[4] Clarke R, Smith AD, Jobst KA, Refsum H, Sutton L, Ueland PM. Folate, Vitamin B_{12}, and serum total homocysteine levels in confirmed Alzheimer disease. Arch Neurol 1998;55:1449–55.
[5] Seshadri S, Beiser A, Selhub J, Jacques PF, Rosenberg IH, D'Agostino RB, et al. Plasma homocysteine as a risk factor for dementia and Alzheimer's disease. N Engl J Med 2002;14:476–83.
[6] Ravaglia G, Forti P, Maioli F, Martelli M, Servadei L, Brunetti N, et al. Homocysteine and folate as risk factors for dementia and Alzheimer disease. Am J Clin Nutr 2005;82:636–43.
[7] Tucker KL, Qiao N, Scott T, Rosenberg I, Spiro Ar. High homocysteine and low B vitamins predict cognitive decline in aging men: the Veterans Affairs Normative Aging Study. Am J Clin Nutr 2005;82:627–35.
[8] Obeid R, Herrmann W. Mechanisms of homocysteine neurotoxicity in neurodegenerative diseases with special reference to dementia. FEBS Lett 2006;580:2994–3005.
[9] Kruman II, Culmsee C, Chan SL, Kruman Y, Guo Z, Penix L, et al. Homocysteine elicits a DNA damage response in neurons that promotes apoptosis and hypersensitivity to excitotoxicity. J Neurosci 2000;20:6920–6.
[10] Ho PI, Ortiz D, Rogers E, Shea TB. Multiple aspects of homocysteine neurotoxicity: glutamate excitotoxicity, kinase hyperactivation and DNA damage. J Neurosci Res 2002;70:694–702.
[11] Ho PI, Ashline D, Dhitavat S, Ortiz D, Collins SC, Shea TB, et al. Folate deprivation induces neurodegeneration: roles of oxidative stress and increased homocysteine. Neurobiol Dis 2003;14:32–42.
[12] Kaufman RJ. Orchestrating the unfolded protein response in health and disease. J Clin Invest 2002;110:1389–98.
[13] Boyce M, Yuan J. Cellular response to endoplasmic reticulum stress: a matter of life or death. Cell Death Differ 2006;13:363–73.
[14] Xu C, Bailly-Maitre B, Reed JC. Endoplasmic reticulum stress: cell life and death decisions. J Clin Invest 2005;115:2656–64.
[15] Outinen PA, Sood SK, Liaw PC, Sarge KD, Maeda N, Hirsh J, et al. Characterization of the stress-inducing effects of homocysteine. Biochem J 1998;15:213–21.
[16] Zhang C, Cai Y, Adachi MT, Oshiro S, Aso T, Kaufman RJ, et al. Homocysteine induces programmed cell death in human vascular endothelial cells through activation of the unfolded protein response. J Biol Chem 2001;276:35867–74.
[17] Werstuck GH, Lentz SR, Dayal S, Hossain GS, Sood SK, Shi YY, et al. Homocysteine-induced endoplasmic reticulum stress causes dysregulation of the cholesterol and triglyceride biosynthetic pathways. J Clin Invest 2001;107:1263–73.
[18] Althausen S, Paschen W. Homocysteine-induced changes in mRNA levels of genes coding for cytoplasmic- and endoplasmic reticulum-resident stress proteins in neuronal cell cultures. Brain Res Mol Brain Res 2000;84:32–40.
[19] Katayama T, Imaizumi K, Manabe T, Hitomi J, Kudo T, Tohyama M. Induction of neuronal death by ER stress in Alzheimer's disease. J Chem Neuroanat 2004;28:67–78.
[20] Mattson MP. Pathways towards and away from Alzheimer's disease. Nature 2004;430:631–9.
[21] Mattson MP, Chan SL. Dysregulation of cellular calcium homeostasis in Alzheimer's disease: bad genes and bad habits. J Mol Neurosci 2001;17:205–24.
[22] Ho PI, Collins SC, Dhitavat S, Ortiz D, Ashline D, Rogers E, et al. Homocysteine potentiates beta-amyloid neurotoxicity: role of oxidative stress. J Neurochem 2001;78:249–53.
[23] Kruman II, Kumaravel TS, Lohani A, Pedersen WA, Cutler RG, Kruman Y, et al. Folic acid deficiency and homocysteine impair DNA repair in hippocampal neurons and sensitize them to amyloid toxicity in experimental models of Alzheimer's disease. J Neurosci 2002;22: 1752–62.
[24] Pike C, Walencewicz-Wasserman A, Kosmoski J, Cribbs D, Glabe C, Cotman C. Structure-activity analyses of beta-amyloid peptides: contributions of the beta 25-35 region to aggregation and neurotoxicity. J Neurochem 1995;64:253–65.
[25] Calfon M, Zeng H, Urano F, Till JH, Hubbard SR, Harding HP, et al. IRE1 couples endoplasmic reticulum load to secretory capacity by processing the XBP-1 mRNA. Nature 2002;415:92–6.
[26] Akterin S, Cowburn RF, Miranda-Vizuete A, Jimenez A, Bogdanovic N, Winblad B, et al. Involvement of glutaredoxin-1 and thioredoxin-1 in beta-amyloid toxicity and Alzheimer's disease. Cell Death Differ 2006;13:1454–65.
[27] Forman MS, Lee VM, Trojanowski JQ. 'Unfolding' pathways in neurodegenerative disease. Trends Neurosci 2003;26:407–10.
[28] Ron D. Hyperhomocysteinemia and function of the endoplasmic reticulum. J Clin Invest 2001;107:1221–2.
[29] Lentz SR, Sadler JE. Homocysteine inhibits von Willebrand factor processing and secretion by preventing transport from the endoplasmic reticulum. Blood 1993;81:683–9.
[30] Jacobsen DW. Hyperhomocysteinemia and oxidative stress: time for a reality check? Arterioscler Thromb Vasc Biol 2000;20:1182–4.
[31] Roybal CN, Yang S, Sun C, Hurtado D, Vander Jagt DL, Townes TM, et al. Homocysteine increases the expression of vascular endothelial growth factor by a mechanism involving endoplasmic reticulum stress and transcription factor ATF4. J Biol Chem 2004;279:14844–52.
[32] Agnati L, Genedani S, Leo G, Forni A, Woods A, Filaferro M, et al. Abeta peptides as one of the crucial volume transmission signals in the trophic units and their interactions with homocysteine. Physiological implications and relevance for Alzheimer's disease. J Neural Transm 2007;11444:21–31.
[33] Austin RC, Sood SK, Dorward AM, Singh G, Shaughnessy SG, Pamidi S, et al. Homocysteine-dependent alterations in mitochondrial gene expression, function and structure. Homocysteine and H_2O_2 act synergistically to enhance mitochondrial damage. J Biol Chem 1998; 273:30808–17.
[34] Outinen PA, Sood SK, Pfeifer SI, Pamidi S, Podor TJ, Li J, et al. Homocysteine-induced endoplasmic reticulum stress and growth arrest leads to specific changes in gene expression in human vascular endothelial cells. Blood 1999;94:959–67.
[35] Canevari L, Abramov AY, Duchen MR. Toxicity of amyloid beta peptide: tales of calcium, mitochondria, and oxidative stress. Neurochem Res 2004;29:637–50.
[36] Yu MS, Suen KC, Kwok NS, So KF, Hugon J, Chang RCC. Beta-amyloid peptides induces neuronal apoptosis via a mechanism independent of unfolded protein responses. Apoptosis 2006;11: 687–700.
[37] Suen KC, Lin KF, Elyaman W, So KF, Chang RC, Hugon J. Reduction of calcium release from the endoplasmic reticulum could only provide partial neuroprotection against beta-amyloid peptide toxicity. J Neurochem 2003;87:1413–26.
[38] Loo DT, Copani A, Pike CJ, Whittemore ER, Walencewicz AJ, Cotman CW. Apoptosis is induced by beta-amyloid in cultured central nervous system neurons. Proc Natl Acad Sci U S A 1993;90: 7951–5.
[39] Kadowaki H, Nishitoh H, Urano F, Sadamitsu C, Matsuzawa A, Takeda K, et al. Amyloid beta induces neuronal cell death through ROS-mediated ASK1 activation. Cell Death Differ 2005;12:19–24.
[40] Selhub J, Bagley LC, Miller J, Rosenberg IH. B vitamins, homocysteine, and neurocognitive function in the elderly. Am J Clin Nutr 2000;71:614S–20S.