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Induction of the unfolded protein response and cell death pathway in Alzheimer's disease, but not in aged Tg2576 mice

Jin Hwan Lee[1,2,3,4]*, Sun Mi Won[1,2,3]*, Jaehong Suh[1,2,3], Sun Joo Son[4], Gyeong Joon Moon[1,2,3,4], Ui-Jin Park[1,2,3,4] and Byoung Joo Gwag[1,2,3,4,5]

[1]Department of Neuroscience
[2]Department of Pharmacology
[3]Research Institute for Neural Science and Technology
Ajou University School of Medicine
[4]Department of Pharmacology
Neurotech Pharmaceuticals Co.
Suwon 442-749, Korea
[5]Corresponding author: Tel, 82-31-219-4221;
Fax, 82-31-219-5069; E-mail, bjgwag@ajou.ac.kr
*These authors contributed equally to this work.
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Abbreviations: AD, Alzheimer's disease; ATF6, activating transcription factor 6; CHOP, C/EBP homologous protein; ER, endoplasmic reticulum; GRP78, glucose-regulated protein 78; PDI, protein disulfide isomerase; p-PERK, RNA-dependent protein kinase-like endoplasmic reticulum kinase; UPR, unfolded protein response; XBP-1, X-box binding protein-1

Abstract

The endoplasmic reticulum (ER) stress results from disrupted protein folding triggered by protein mutation or oxidation, reduced proteasome activity, and altered Ca^{2+} homeostasis. ER stress is accompanied by activation of the unfolded protein response (UPR) and cell death pathway. We examined if the UPR and cell death pathway would be activated in Alzheimer's disease (AD). RT-PCR experiments revealed increased splicing of X-box binding protein-1 (XBP-1), an UPR transcription factor, in AD compared with age-matched control. Among target genes of XBP-1, expression of protein disulfide isomerase (PDI), but not glucose-regulated protein 78 (GRP78), was increased in AD, suggesting disturbed activation of the UPR in AD. C/EBP homologous protein (CHOP), caspase-3, caspase-4, and caspase-12, downstream mediators of cell death pathway, were activated in AD. Neither the UPR nor cell death pathway was induced in aged Tg2576 mice, a transgenic mouse model of Alzheimer's disease that reveals both plaque pathology and some cognitive deficits. The present study suggests that disturbed induction of the UPR and activation of the pro-apoptotic proteins contribute to neuropathological process in AD irrespective of amyloid β and senile plaque.

Keywords: Alzheimer disease; cell death; endoplasmic reticulum; protein disulfide-isomerases; unfolded protein response

Introduction

ER stress, accumulation of unfolded protein in the endoplasmic reticulum, can be provoked primarily by imbalance in homeostasis, proteasome activity during degeneration and differentiation (Kozutsumi *et al*., 1988; Wong *et al*., 1993; Friedlander *et al*., 2000; Cho *et al*., 2009). ER stress induces an adaptive signaling pathway called the UPR that involves activation of transcription factors, XBP-1 and activating transcription factor 6 (ATF6) (Yoshida *et al*., 2000; Lee *et al*., 2003). Activation of these transcription factors induces the UPR genes (e.g., GRP78, PDI). The coordinated activation of the UPR alleviates accumulation of unfolded proteins in the lumen of ER (Cox and Walter, 1996; Yoshida *et al*., 2001). However, prolonged activation of ER stress can turn on cell death pathway through activation of CHOP, caspase-4, and caspase-12 (Wang *et al*., 1996; Nakagawa *et al*., 2000; Hitomi *et al*., 2004).

ER stress has been implicated in abnormal protein processing and neuronal death in AD. Administration of amyloid β induced activation of ER stress in cultured neurons and in rabbit *in vivo* (Ghribi *et al*., 2001; Ferreiro *et al*., 2006). Caspase-4 and caspase-12 were shown to mediate ER stress-mediated apoptosis by amyloid β (Nakagawa *et al*., 2000; Kim *et al*., 2006). In a recent study, increased levels of GRP78 and p-PERK (RNA-dependent protein kinase-like endoplasmic reticulum kinase) were observed in the cortex and hippocampus from patients with AD, suggesting that ER stress induces activation of the UPR in AD (Hoozemans *et al*., 2005; Hoozemans, *et al*., 2009). Contrarily, Western blot analysis revealed

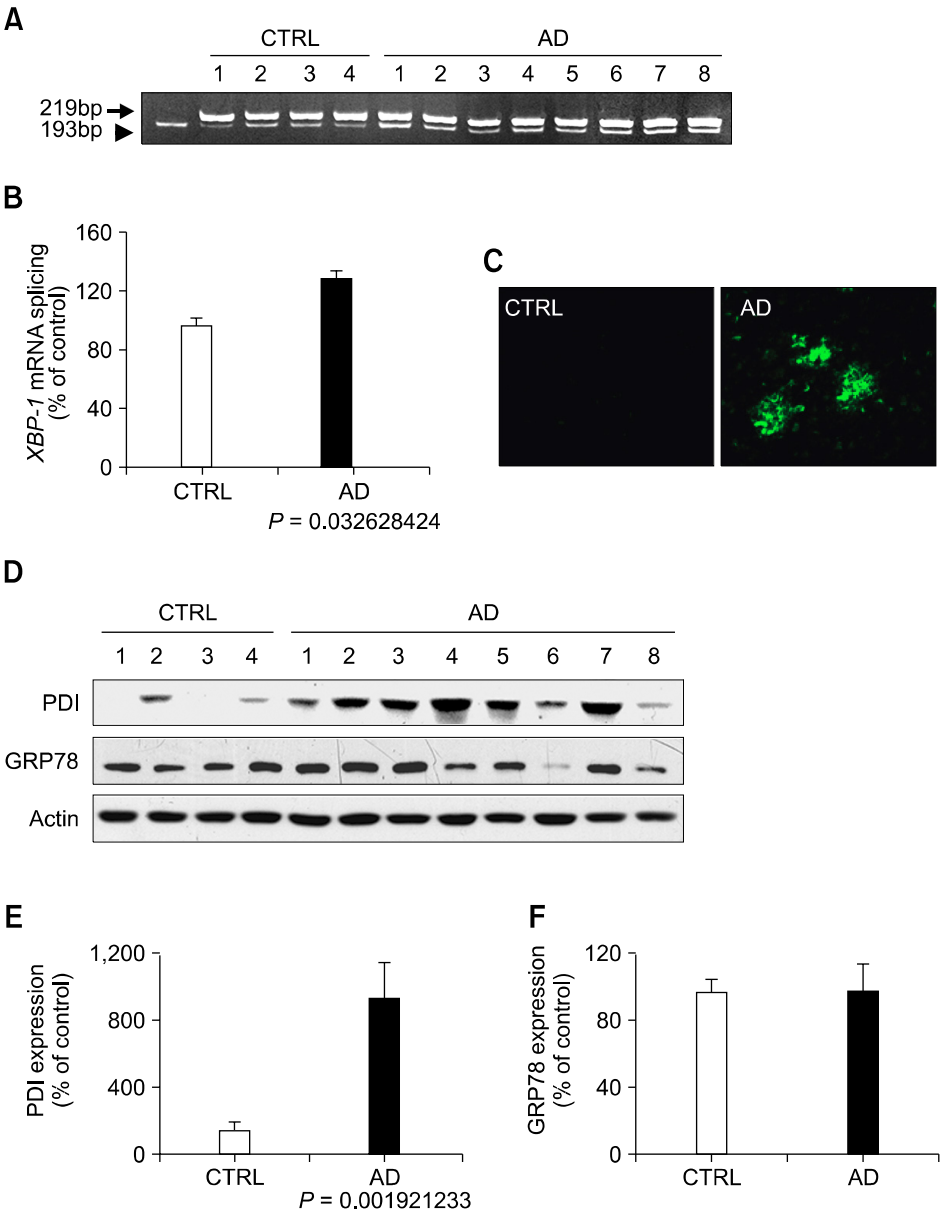

Figure 1. Induction of the unfolded protein response by ER stress in AD. (A, B) RT-PCR analysis of XBP-1 mRNA splicing in the temporal cortex of control (CTRL) and AD brain (A). XBP-1 mRNA splicing was analyzed by scaling intensity of splicing band (193bp) to total band (193 bp + 219 bp) (B). (C) Representative images of thioflavin S staining in the temporal cortex of control (CTRL) and AD brain. (D-F) Western blot analysis of PDI, GRP78, and actin (D). Levels of PDI (E) and GRP78 (F) were measured and normalized to the level of relevant actin (n = 4 for control and n = 8 for AD).

that expression of GRP78 was decreased or maintained in the cortical tissues of AD patients (Katayama *et al*., 1999; Sato *et al*., 2000). In the present study, activation of the UPR in AD was further examined by analyzing XBP-1 mRNA splicing and PDI expression as well as GRP78. In addition, activation of ER stress-induced cell death pathway in AD patients was investigated by analyzing activation of CHOP, caspase-3, caspase-4, and caspase-12. A putative role of amyloid β and plaques for ER stress was examined in aged Tg2576 transgenic mice that developed widespread plaque pathology and cognitive deficit.

Results

Induction of the unfolded protein response by ER stress in AD

We analyzed processing of mRNA encoding the transcription factor XBP-1 to determine if the UPR would be induced in the temporal cortex of AD patients. RT-PCR experiments showed overall increase in the spliced form (193 bp) of XBP-1 mRNA in the temporal cortex of AD brain (Figure 1A). The relative splicing ratio of XBP-1 mRNA was significantly increased compared to the age-matched control (Figure 1B). Extensive amyloid pla-

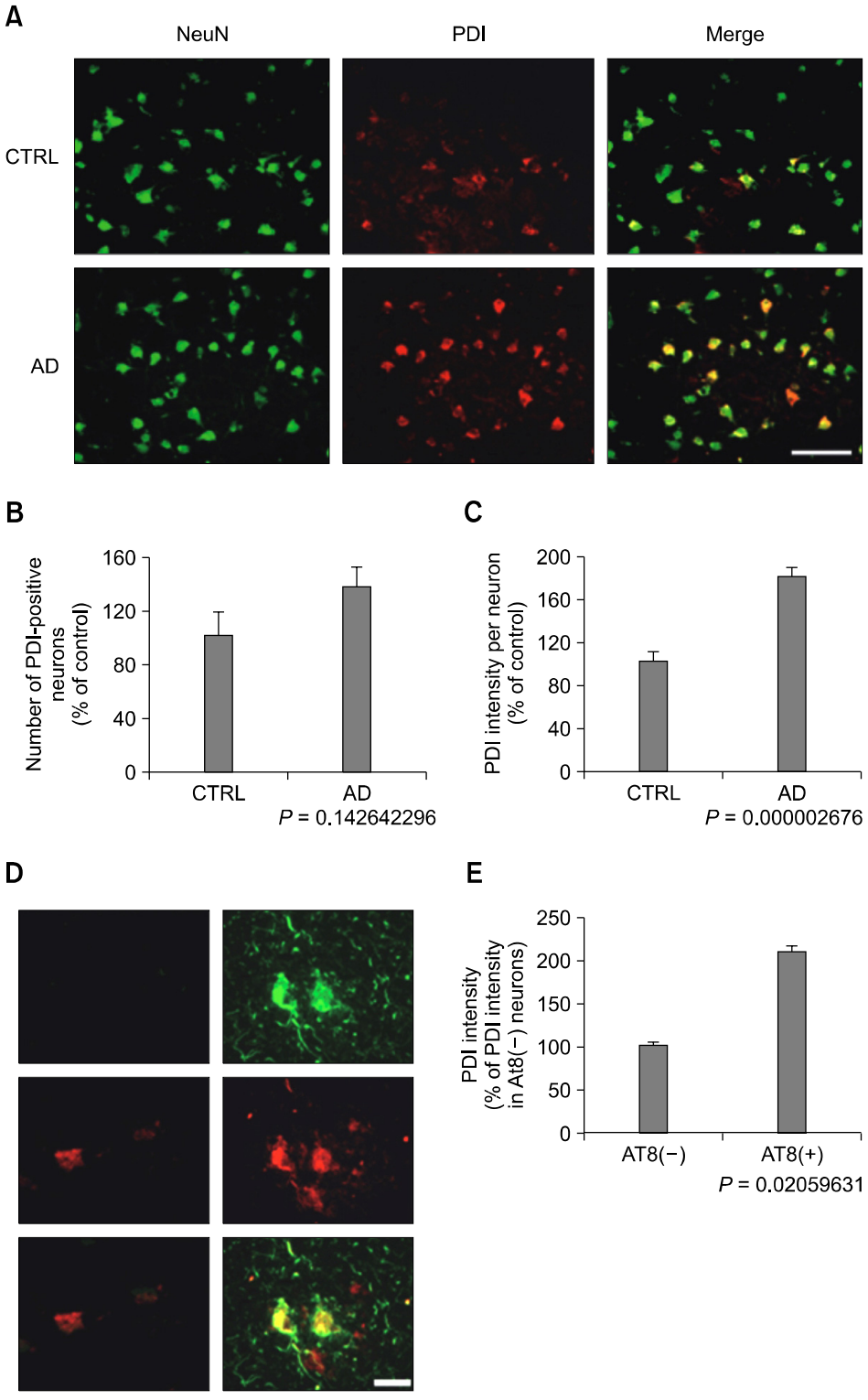

Figure 2. PDI is induced in tangle-bearing neurons in AD. (A-C) Fluorescence photomicrographs of cortical sections from control (CTRL) and AD immunolabeled with anti-NeuN antibody (left panel, green) and anti-PDI antibody (middle panel, red). Note that PDI expression is increased in neurons (right panel, yellow) (A). Analysis of PDI-positive neurons (B) and PDI intensity per neuron (C) collected from 400-450 neurons in the temporal cortex from 4 control and 8 AD patients. Bar, 100 μm. (D, E) Fluorescence photomicrographs of cortical neurons immunolabeled with anti-AT8 antibody (green) and anti-PDI antibody (red) in sections of AD and control (D). Note that AT8-positive neurons (AT8+) show higher levels of PDI than AT8-negative neurons (AT8-) (E). Analysis of PDI levels in AT8 (-) and (+) neurons collected from 250 to 320 neurons in the temporal cortex of AD. Bar, 20 μm.

ques were observed in the cortex of AD brain (Figure 1C).

We next examined the expression pattern of PDI and GRP78, the target UPR genes of XBP-1. Levels of PDI, a family of enzymes that catalyze disulfide bond formation, reduction, or isomerization of newly synthesized proteins in the lumen of the ER, were markedly increased up to 9.49 fold

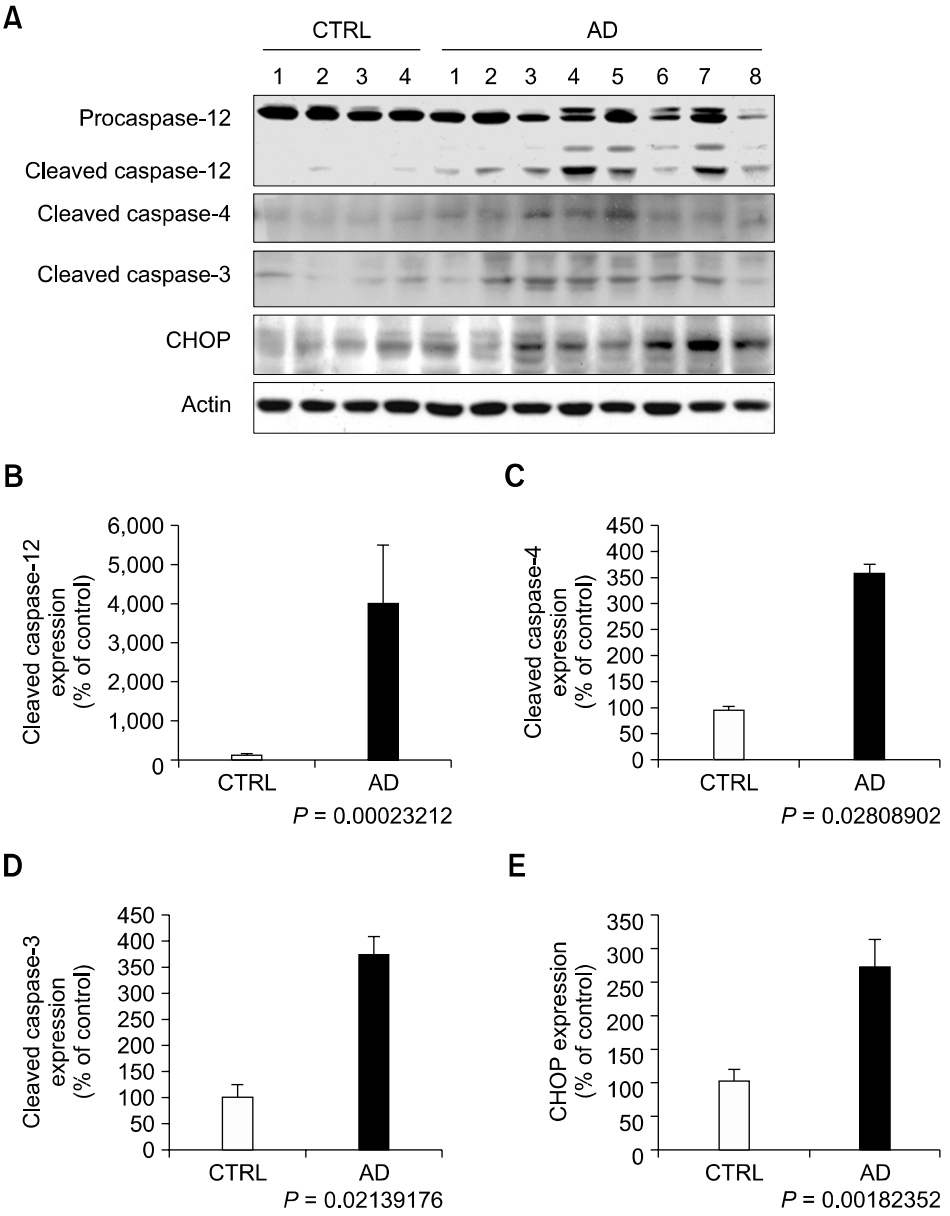

Figure 3. Activation of ER stress-induced cell death pathway in AD. Western blot analysis of caspase-12, caspase-4, caspase-3, CHOP, and actin in the temporal cortex of control (CTRL) and AD brain (A). Levels of cleaved caspase-12 (B), cleaved caspase-4 (C), cleaved caspase-3 (D), and CHOP (E) were measured and normalized to the level of relevant actin (n = 4 for control and n = 8 for AD).

in AD brain ($P < 0.01$) compared to the control (Figure 1). However, the expression of GRP78 that promotes proper folding of proteins in the ER was not increased in AD brain (Figure 1D).

PDI is induced in tangle-bearing neurons in AD

Immunohistochemistry revealed that PDI was expressed primarily in neurons in the temporal cortex from AD and the age-matched control brain. The number of neurons immunoreactive to PDI tended to be slightly increased in the temporal cortex of AD patients (P = 0.1426, Figure 2B). The expression of PDI was significantly increased in the cortical neurons from AD (Figure 2).

Additional experiments were performed to examine if induction of the UPR would be correlated with the neurofibrillary tangle. Several neurons immunoreactive to AT8 antibody recognizing the phosphorylated forms of tau at Ser202 residue were observed in the temporal cortex of AD patients, but not from the control group (Figure 2D). Interestingly, all AT8-positive neurons showed higher levels of PDI than AT8-negative neurons (Figure 2E). This implies that ER stress is induced primarily in the tangle-bearing cortical neurons from AD.

Activation of ER stress-induced cell death pathway in AD

ER stress-mediated cell death pathway was examined by Western blot analysis of caspase-3, caspase-4, caspase-12, and CHOP. Cleaved forms of caspase-3, caspase-4, and caspase-12 were barely detectable in the cortex of age-matched control group. The cleaved forms of caspase-3, caspase-4, and caspase-12 were significantly increased in AD brain (Figure 3). The expression of CHOP was also increased in the temporal cortex of AD brain compared to the control group.

Neither the UPR nor cell death pathway is induced in aged Tg2576 mice

We finally examined the possibility that the induction pattern of the UPR and cell death pathway by ER stress in AD brain would be related to amyloid burden. Levels of soluble or insoluble amyloid β were increased in the cortical areas of Tg2576 mice at the age of 17 months as previously reported (Holcomb *et al*., 1998; Yang *et al*., 2000; Kawarabayashi *et al*., 2001; Lim *et al*., 2001). Thioflaivin-S staining revealed widespread senile plaques in the cortical area of aged Tg2576 mice (Figure 4A). Levels of PDI and GRP78 were similar in the cortex of Tg2576 mice and wild type. Cleaved caspase-3, 9, 12, and CHOP expression were not altered in Tg2576 mice compared to the control (Figure 4B).

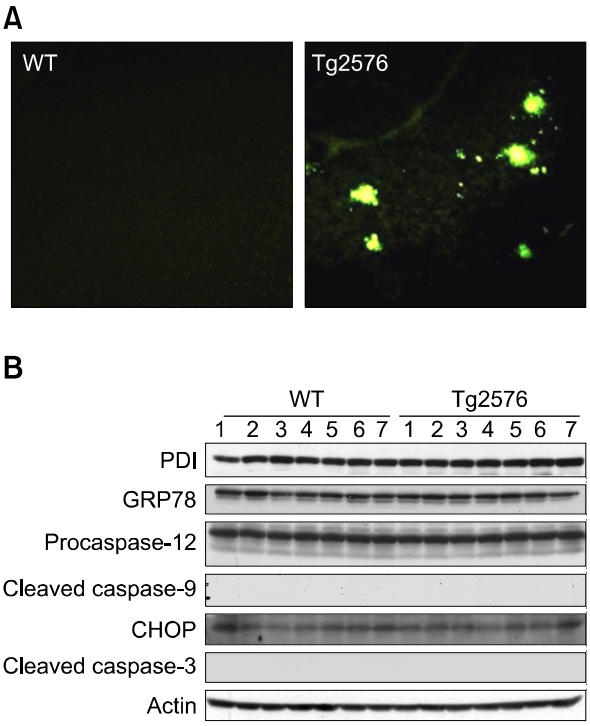

Figure 4. Neither the UPR nor cell death pathway is induced in aged Tg2576 mice. (A) Fluorescence photomicrographs of cortical sections from wild type (WT) and Tg2576 mice at the age of 17 months after thioflavin-S staining. (B) Western blot analysis of PDI, GRP78, cleaved caspase-3, 9, 12, CHOP, and actin in the cortex of wild type and Tg2576 mice.

Discussion

ER stress-induced UPR and cell death pathway appear to play a role in the pathogenesis of AD. Disturbed activation of the UPR is evident by splicing of XBP-1 mRNA and induction of PDI presumably in tangle-bearing neurons in the absence of up-regulation of GRP78. Cleavages of caspase-3, 4, 12 and upregulation of CHOP suggest activation of ER stress-induced proapoptotic pathway in AD. However, neither the UPR nor cell death pathway by ER stress is induced in aged Tg2576 mice loaded with amyloid β and plaques, raising a possibility that the ER stress-mediated events in AD can be induced irrespective of amyloid burden.

The UPR seems to be incompletely activated in the brain of AD patients. In particular, expression of GRP78, a key regulator of protein folding and assembly, is not induced but rather reduced although ER is under stressed condition in AD (Katayama *et al*., 1999; Sato,*et al*., 2000). The expression of GRP78 is also reduced by familial AD-linked presenilin-1 mutations that interfere with IRE1 function (Katayama *et al*., 1999). Hosoi *et al* (2010) found that systemic application of homocysteine increases XBP-1 splicing in various brain areas including hippocampus, hypothalamus and cortex in mice without inducing expression of GRP78 (Hosoi *et al*., 2010). Target genes of XBP-1 have been identified including $p58^{IPK}$, ERdj4, HEDJ, and protein disulfide isomerase PDI-P5 (Lee *et al*., 2003). In this study, GRP78 did not depend upon XBP-1s expression. The disturbed activation of the UPR likely renders neurons sensitive to apoptosis induced by ER stress or amyloid β (Guo *et al*., 1997; Yoneda *et al*., 2001). PDI is localized primarily in neurons and its expression is increased in AT8-positive neurons in AD, suggesting that ER stress is prominent in tangle-bearing neurons. Neurons lacking compensatory activation of the UPR are expected to undergo misfolding and abnormal aggregation and processing of various proteins such as tau, amyloid β-protein precursor, and neurofilament. It is conceivable to reason that the disturbed protein homeostasis may contribute to the formation of neurofibrillary tangle in AD.

Prolonged activation of the UPR can trigger apoptosis through activation of caspase-4, caspase-12, and CHOP, key mediators of ER stress-induced cell death pathway. We provide first evidence that cleavages of caspase-4 and caspase-12, and expression of CHOP are increased in the brain of AD patients. CHOP, a transcription factor induced by ATF6 or PERK, acts as a proapoptotic protein that suppresses transcription of Bcl-2 (Wang *et al*., 1996; McCullough *et al*., 2001). Moreover, ER stress reduces interactions of procaspase-12 and tumor necrosis factor receptor-associated factor 2, an adaptor protein linked to the cytoplasmic portion of IRE1 at ER membrane (Yoneda *et al*., 2001). Dissociated procaspase-12 is cleaved by the calcium-activated neutral protease calpain that is activated by ER stress (Siman *et al*., 2001) and in AD patients (Saito *et al*., 1993). Activated caspase-12 then induces cytochrome c-independent activation of caspase-9, which will lead to activation of caspase-3 in vulnerable neurons in the brain of AD patients (Morishima *et al*., 2002; Rohn *et al*., 2002; Kang *et al*., 2005). Taken together, ER stress-induced cell death pathway is activated and expected to participate in caspase-3-mediated neuronal loss in AD.

Mechanisms underlying induction of ER stress in AD remain largely unknown. Amyloid β has been proposed as a potential trigger of ER stress in AD based upon the findings that the UPR and cell death pathway are induced in cultured neurons exposed to amyloid β or over-expressing mutant PS1 (Yu *et al*., 1999; Nakagawa *et al*., 2000; Ferreiro *et al*., 2006; Seyb *et al*., 2006). However, none of ER stress markers have been detected in the cortex of aged Tg2576 mice undergoing extensive amyloid β burden and cognitive deterioration. The lack of the UPR and cell death pathway in aged Tg2576 indicates that amyloid β and plaques alone are not sufficient for induction of ER stress in aged AD patients, suggesting that neuronal injury and neurofribrillary tangles as well as amyloid plaques contribute to ER stress induced in AD. Moreover, other pathological events such as inflammation may influence induction of ER stress in AD which is accompanied by pneumonia or urosepsis.

Glutamate or free radicals, triggers of neuronal injury in AD (Smith *et al*., 2000; Li *et al*., 2004; Moreira *et al*., 2005; Cosman *et al*., 2007), may play a central role in inducing ER stress in AD. Activation of glutamate receptors sensitive to NMDA results in excess accumulation of Ca^{2+} in neurons that can induce free radical production and ER stress (Dugan *et al*., 1995; Reynolds and Hastings, 1995; Liu *et al*., 1998). It is of note that memantine, a partial NMDA receptor antagonist, is approved for treating moderate-to-severe AD patients (Li *et al*., 2004; Cosman *et al*., 2007).

ER stress induces activation of the UPR to reduce the accumulation of unfolded proteins. The UPR likely fails to remove unfolded proteins due to disturbed activation of the UPR and turns on ER stress-specific cell death pathway in the brain of AD patients. The pathological activation of the UPR is expected to contribute to accumulation and aggregation of misfolded proteins and neuronal death progressively evolving in AD and other neurodegenerative diseases including Parkinson's disease and amyotrophic lateral sclerosis (Bence *et al*., 2001; Forloni *et al*., 2002; Ryu *et al*., 2002; Soto, 2003; Paschen and Mengesdorf, 2005; Kikuchi *et al*., 2006; Kanekura *et al*., 2009). Further study will be needed to delineate a potential role of excitotoxicity, oxidative stress, and dysfunction of the ubiquitin proteasome system in inducing ER stress in neurodegenerative diseases.

Methods

Human brain tissue

Frozen tissues from 8 cases of AD and 4 cases of age-matched control were obtained from Boston University Alzheimer's Disease Center (Dr. Ann C. Mckee). AD was diagnosed according to clinical and neuropathological criteria (Supplemental Data Table S1).

Transgenic mice

Tg2576 mice and their corresponding wild type controls were purchased from Taconic Farms, Inc. (Germantown, NY). Tg2576 mice overexpress human APP695, containing the double-mutation Lys670-Asn and Met 671-Leu (K670N, M671L) (Hsiao *et al*., 1996). Animals were handled in accordance with a protocol approved by our institutional animal care committee. Tg2576 mice ($n = 7$) and their non-transgenic littermates ($n = 7$) at the age of 17 months were used in this study.

Isolation of total RNA and RT-PCR

Total RNA from tissues of AD human brain and transgenic mice was isolated according to manufacturer's instruction (iNtRON Bio technology co., Ltd). RNA integrity was confirmed by detection of 28S and 18S rRNA band. RNA was confirmed to be free of genomic DNA contamination by PCR in the absence of reverse transcriptase. The RNA samples were reverse transcribed in 20 μl of a reaction mixture containing 0.5 mM of mixed dNTP, 100 ng of oligo dT (5'-TTTTTTTTTTTTTTT-3'), 0.5 unit RNAsin, and 40 unit of MMLV reverse transcriptase at 42°C for 30 min. The samples were then incubated at 95°C for 5 min and

transferred to 4°C. 4 μl of RT product were subjected to PCR amplification with 20 pmole XBP-1 primer, 20 mM dNTP, and 1 unit Taq polymerase in 20 μl of 1 × reaction buffer (Promega). PCR primers were used as follows (5'-3'): for human XBP-1, GAAGCCAAGGGGAATGAAGT (forward) and GGGAAGGGCATTTGAAGAAC (reverse): for mouse XBP-1, CCATGGGAAGATGTTCTGGG (forward) and TCTGGTTGGCGGATCTACTC (reverse). PCR mixtures were heated to 94°C for 5 min and cycled 31 times for XBP-1 gene; 31 cycles consisted of 94°C for 15 s, 60°C for 30 s, and 72°C for 45 s. After additional incubation at 72°C for 10 min, PCR products were subjected to electrophoresis in 8% polyacrylamide gel and visualized with ethidium bromide staining. Relative intensity of PCR band was analyzed using Gel Doc 1000 video-imaging system (Bio-Rad, Hercules, CA).

Western blot analysis

Brain tissues were lysed in a lysis buffer containing 10 mM Tris-HCl pH 7.5, 50 mM NaCl, 1% Triton X-100, 30 mM sodium pyrophosphate, 50 mM NaF, 5 μM $ZnCl_2$, 2 mM PMSF, and 100 μg/ml leupeptin, 10 μg/ml pepstatin A, and 1 mM DTT. Lysates were centrifuged at 13,000 *g* for 10 min, the supernatants collected, subjected to electrophoresis on 12-15% SDS-polyacrylamide gel, and transferred to a nitrocellulose membrane. The blot was incubated in 5% nonfat dry milk, reacted with primary antibodies at 4°C for overnight. The primary antibodies used and the dilutions for each were rabbit polyclonal anti-caspase-12 antibody (New England Biolabs, Beverly, MA) recognizes both procaspase-12 of 54 kDa and active caspase-12 of 42 kDa at 1:2500, mouse polyclonal anti-PDI antibody (Pharmingen, San Diego, CA) at 1:2000, mouse polyclonal anti-GRP78 antibody (Pharmingen, San Diego, CA) at 1:5000, rabbit polyclonal anti-cleaved caspase-3 antibody (New England Biolabs, Beverly, MA), goat polyclonal anti-cleaved caspase-4 antibody (Santa Cruz Biotechnology), and rabbit polyclonal anti-CHOP antibody (Santa Cruz Biotechnology) at 1:2000. The blot was incubated with a horseradish peroxidase-conjugated anti-mouse or anti-rabbit secondary antibody (Cell Signaling, Beverly, MA). Target proteins were detected with enhanced chemiluminescence reagents (GE Healthcare, Little Chalfont, Buckinghamshire, UK) on X-ray film or with an LAS 1000 image analyzer (Fuji Photo Film Co.). The image was analyzed using the Image Gauge 3.12 (Fuji Photo Film Co.) according to parameters defined by the softerware algolithms for band and lane depiction. Net band intensities are defined as the sum of pixels within the area of the band limited by performed rectangular area after subtraction of the background pixels. To assess for protein degradation and to normalize protein load among samples, the membranes were incubated with antibody against β-actin after stripping the blots. No tissue extracts exhibited degradation of β-actin.

Immunohistochemistry

Sections at a thickness of 25 μm on a cryostat were incubated in 10% normal horse serum for 1 h, reacted with antibody recognizing NeuN (CHEMICON International, Inc., 1:200), rabbit polyclonal antibody recognizing PDI (Abcam Ltd. 332 Cambridge, UK, 1:50), mouse monoclonal antibody recognizing AT8 (Pierce Biotechnology, Inc., 1:200) overnight, and then reacted with FITC- or Texas-red-labeled secondary antibodies (Vector, Burlingame, CA, 1:200). In case of human, autofluorescence eliminator reagent (CHEMICON International, Inc.) was used to remove the autofluorescent pigment lipofusin. Finally, sections were washed with distilled water, air dried, and mounted with Vectashield (Vector, Burlingame, CA). All images were collected and analyzed with a fluorescence microscope (Zeiss, Germany) equipped with the REAL-14k precision digital camera (Apogee Instruments, Tucson, AZ) and Image Pro Plus Plug-in.

Thioflavin S staining

Brain sections (25 μm) were mounted on gelatin-coated slides. Slides were rinsed with distilled water and incubated with thioflavin-S (1% w/v, Sigma) for 10 min. Slides were incubated in 70% ethanol for 5 min and briefly washed in distilled water.

Statistical analysis

Data were analyzed with the SPSS software package (version 12.0 for Windows SPSS, Chicago, Ill.). Statistical analysis of the data was performed using an independent sample t test.

Supplemental data

Supplemental Data include a table and can be found with this article online at http://e-emm.or.kr/article/article_files/SP-42-5-08.pdf.

Acknowledgments

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