Endoplasmic Reticulum Stress Responses in Mouse Models of Alzheimer’s Disease: Overexpression Paradigm versus Knock-in Paradigm

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Running title: ER stress in AD mouse models

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ABSTRACT
Endoplasmic reticulum (ER) stress is believed to play an important role in the etiology of Alzheimer’s disease (AD). The accumulation of misfolded proteins and perturbation of intracellular calcium homeostasis are thought to underlie the induction of ER stress, resulting in neuronal dysfunction and cell death. Several reports have shown an increased ER stress response in amyloid precursor protein (APP) and presenilin1 (PS1) double transgenic (Tg) AD mouse models. However, it remains unclear whether the ER stress observed in these mouse models is actually caused by AD pathology. APP and PS1 contain one and nine transmembrane domains, respectively, for which it has been postulated that overexpressed membrane proteins can become wedged in a misfolded configuration in ER membranes, thereby inducing non-specific ER stress. Here, we used an App-knockin (KI) AD mouse model that accumulates Aβ without overexpressing APP.
to investigate whether the ER stress response is heightened because of amyloid β peptide (Aβ) pathology. Thorough examinations indicated that no ER stress responses arose in App-KI or single APP-Tg mice. These results suggest that PS1 overexpression or mutation induced a non-specific ER stress response that was independent of Aβ pathology in the double Tg mice. Moreover, we observed no ER stress in a mouse model of tauopathy (P301S Tau-Tg mice) at various ages, suggesting that ER stress is also not essential in tau pathology-induced neurodegeneration. We conclude that the role of ER stress in AD pathogenesis needs to be carefully addressed in future studies.

INTRODUCTION
Alzheimer’s disease (AD) is the most common neurodegenerative disease and the main cause of dementia. The neuropathological hallmarks of AD include extracellular deposits of amyloid-β (Aβ) as the major component of senile plaques, and neurofibrillary tangles composed of hyperphosphorylated tau protein (1). Aβ is generated from amyloid precursor protein (APP), a type I membrane protein, through sequential proteolytic cleavages mediated by the β- and γ-secretases. γ-Secretase is a membrane-associated complex consisting of four different proteins: presenilin1/2 (PS1/2), nicastrin, Aph1, and Pen2. PS1/2 is a catalytic subunit (1).

For around two decades, APP and/or PS1-overexpressing (transgenic, Tg) mice have been used widely as AD mouse models for basic and clinical studies. However, the underlying processes of Aβ overproduction in conventional mouse models differ greatly from that in AD patients. APP overexpression in animal models overproduces in an unphysiological manner fragments other than Aβ, such as soluble amyloid precursor protein (sAPP), C- terminus fragment of APP (CTF) and APP intracellular domain (AICD). Moreover, APP and/or PS1 overexpression can induce an artificial endoplasmic reticulum (ER) stress response due to increased cytoplasmic calcium concentrations (2). To overcome these drawbacks of the overexpression paradigm, we recently developed mouse models utilizing an App-knockin (KI) strategy. The App-KI mice, which express humanized Aβ with familial AD mutations at endogenous levels, exhibit AD-associated pathologies including pronounced Aβ amyloidosis and gliosis (3) (4). In contrast, App-KI failed to reproduce some of the observations made using conventional mouse models (3-5). For example, the early lethality of Calpastatin-knockout (KO) X APP23 mice, which contradicted the chronic nature of AD, was not reproduced in Calpastatin-KO X App-KI (3,6). Moreover, with App-KI mice, we detected no calpain-dependent conversion of p35 to p25, which upregulates CDK5 activity. Although calpain activation is generally considered to play an important role in AD progression due to its involvement in caspase-dependent neuronal cell death and

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CDK5-mediated hyper-phosphorylation of tau, our observations indicate that the role of calpain may have been overestimated.

In this study, we focused our attention on ER stress. The accumulation of unfolded/misfolded proteins within the ER lumen along with the disruption of calcium homeostasis leads to ER dysfunction, known as ER stress. Under ER stress conditions, cells escape from serious damage by activating adaptive response pathways known as the unfolded protein response (UPR). UPR restores proteostasis in the ER by arresting protein synthesis, degrading unfolded/misfolded proteins, and increasing molecular chaperone concentrations. On the other hand, UPR induces cell death signaling upon prolonged stress or serious damage. Several reports have suggested that ER stress induced by Aβ accumulation is involved in neurodegeneration in AD (7-9). To this end, exposure of hippocampal brain slices, primary neurons, or cell lines to oligomerized or fibrilized Aβ has been shown to induce ER stress (10,11). Moreover, UPR upregulation has been detected in several AD mouse models such as APP/PS1, 5XFAD, and 3XTg (10-12). However, to the present time it has been difficult to clarify whether ER stress is triggered by Aβ pathology in vivo. To answer this important question, i.e. which abnormally overexpressed membrane proteins or Aβ deposition triggers ER stress, we evaluated the ER stress response in several AD mouse models, including App-KI.

RESULTS

UPR regulates three key pathways via three ER binding proteins (13): pancreatic ER kinase (PERK), activating transcription factor-6 (ATF-6), and inositol-requiring enzyme-1 (IRE-1). The first pathway, triggered by PERK phosphorylation, arrests protein synthesis via abrogating the activity of eukaryotic translation initiation factor 2α (eIF2α) by phosphorylation, and activates ATF4-mediated gene expression of ER chaperones. The second pathway, initiated by ATF-6, induces the expression of ER molecular chaperones such as GRP78/BiP and GRP94, and protein-folding enzymes such as protein disulfide isomerases (PDIs), to prevent protein misfolding. In the third pathway, phosphorylated IRE-1 induces the expression of genes related to protein folding, autophagy, and apoptosis (such as C/EBP homologous protein (CHOP)) by activating transcription factor XBP1. Under normal conditions, PERK, ATF6, and IRE1 remain in an inactive state due to GRP78 binding. In response to ER stress, however, misfolded proteins interrupt GRP78 and sensor protein interactions, thereby initiating UPR signaling.

Several reports have described activation of the ER stress response in AD mouse models. For instance, in the 5XFAD model, which overexpresses familial AD-linked APP and PS1 mutants, phosphorylated eIF2α and XBP-1 mRNA levels are elevated (14,15). The APP/PS1 mouse shows age-dependent increases of
GRP78, phospho-PERK, phospho-eIF2α, and CHOP (12). Moreover, increased GRP78 is also detected in the 3XTg mouse, which expresses mutant APP, PS1, and tau (16). However, in contrast to these findings, Lee et al. (17) observed no UPR signals in Tg2576 mice. Accordingly, it remains controversial whether Aβ pathology is an essential trigger of ER stress.

GRP78 acts as an important sensor of ER stress, and its expression is upregulated by UPR to prevent protein misfolding. GRP78 also appears to be the most sensitive and earliest ER stress marker in APP/PS1-Tg mice (13). Based on this evidence, we first analyzed GRP78 as an ER stress marker. We also examined levels of several ER stress markers: CHOP, PDI, phosphorylated-eIF2α (p-eIF2α) and spliced-Xbp1. To examine whether Aβ deposition induces ER stress, we quantified levels of ER stress markers in the cortices of young and older AppNL-G-F mice (Fig.1a, b, c). In AppNL-G-F mice, Aβ accumulation begins at 2 months (M), and occupies the entire cortex and hippocampus by around 9M (3). Western blot analysis showed no significant upregulation in any of the ER stress markers tested at 6M and 14M in AppNL-G-F mice compared to wild-type (WT), suggesting that increased Aβ deposition is not correlated with the ER stress response (Fig.1a, c). Given that we detected elevation of ER stress markers except for PDI in thapsigargin-treated primary cultured cortical neuron or Neuro2A cells (Fig. 1a, b), our observations are not due to failure to specifically detect ER stress markers. PDI was increased in primary cortical neuron cells even under more severe conditions (data not shown).

To compare ER stress response between APPNL-G-F and APP-Tg mice, we analyzed ER stress markers using cortical and hippocampal samples (Fig.1a, b, d, e). As APP is a membrane binding protein, we expected that APP overexpression would induce chronic ER stress. However, we observed no significant increase of ER stress markers in two APP-overexpressing mouse models: APP23 and Tg2576 (Fig.1d, e). These results indicate that neither Aβ deposition nor APP overexpression induces detectable ER stress. As such, our observations contradict previous reports describing the ER stress induced in double transgenic mice overexpressing mutant APP and PS1 (10-12). Consistently with previous reports, we detected activation of ER stress in APP/PS1 and 3XTg-AD mice. In contrast to APPNL-G-F and single APP-Tg mice, APP/PS1 mice showed an increased ER stress marker, p-eIF2α in hippocampus (6 and 14M) and cortex (6M), and 3XTg-AD showed higher levels of GRP78 and CHOP in hippocampus compared to age-matched wild-type controls (Fig1a). These results indicate that this effect could be a non-specific artifact caused by the genetic modification of PS1 or double modifications of APP and PS1. We confirmed overexpression of PS1(ΔE9) in APP/PS1 mice using antibodies and protocols that had
been fully validated. (18,19). We must, however, indicate that we did not detect other ER stress markers in the APP/PS1 mouse brains, in a manner distinct from the previous report (12), due presumably to the reasons described in DISCUSSION.

Under prolonged ER stress conditions, cells cease to protect themselves, and turn on cell death signals. In AD and other neurodegenerative diseases, tau pathology correlates well with neurodegeneration (20). We therefore hypothesized that ER stress might mediate tau-induced neuronal cell death. To investigate this further, we analyzed ER stress markers in cortices (3-15 months (M)) and hippocampi (12M) of P301S-Tau-Tg mice on a C57BL/6 background (Fig.2). In these mice, brain atrophy associated with neuronal cell death starts from around 9-12M (unpublished data); however, we observed no changes in all stress markers between 3M and 15M (Fig.2a, b). These results suggest that tau pathology does not accompany ER stress, and that the ER stress response is unrelated to tau-induced neurodegeneration.

DISCUSSION

In the present study, we found an absence of ER stress responses in App-KI and single APP-overexpressing mice. We thus conclude that neither Aβ nor APP overproduction triggers ER stress. Lee et al.(17) have consistently shown that ER stress does not occur in Tg2576 mice. The elevated UPR detected in several lines of APP and PS1 double transgenic mouse is thus likely to be a non-specific artifact. As presenilins are polytopic membrane proteins containing nine transmembrane domains, we suggest that mutant PS1 overexpression impacts specifically on ER membranes in which presenilins are enriched (21,22).

A number of studies have reported that PS1 plays a role in the regulation of ER calcium homeostasis (reviewed in Honarnejad et al. 2012, and Zhang et al. 2010) (23,24). PS1 modulates not only the function of sarco/endoplasmic reticulum Ca2+-ATPase (SERCA), which transfers calcium from the cytosol to the lumen, but also of ER-associated calcium channels such as the inositol trisphosphate receptor and ryanodine receptor (25-29). In addition, familial AD (FAD)-linked mutations of PS1 alter its activity in calcium transfer (reviewed in Honarnejad et al. 2012, and Zhang et al. 2010) (23,24). Alteration of the ER cytosolic calcium concentration is a strong inducer of ER stress, as seen in cells treated with the SERCA inhibitor thapsigargin (Fig.1)(30). Based on these findings, the genetic modification of PS1 is very likely to affect the ER stress response. Indeed, FAD-linked PS1 mutation results in the delayed activation of UPR in fibroblasts and primary cultured neurons of mutant PS1-KI mice (31,32). Moreover, deletion or overexpression of PS1 in primary neurons also alters the ER stress response (33,34). Taken together, the ER stress responses observed in APP/PS1 double mutant mice are not causally associated with
AD etiology. Artificial ER stress responses induce artificial cellular responses and cell death. We therefore suggest that the results obtained with APP/PS1 double mutant mice should be further validated.

In this study, however, we did not detect marked activation of ER stress in APP/PS1 mice even though we utilized the strain identical to the one used by Barbero-Camps et al., (12,35). We presume that partial reproducibility was due to reduced expression levels of APP and PS1 in the APP/PS1 mice (Fig. 1a) after a number of passages.

In addition to the above, we detected no ER stress response in a mouse model of tauopathy, suggesting that ER stress does not contribute to tau-induced neurodegeneration. It is plausible that tau overexpression will not induce ER stress because tau is basically a cytosolic protein.

Several groups have reported that the ER stress response is upregulated in postmortem human AD brains (14,36,37). In contrast, Katayama et al. (31) showed a significant decrease of GRP78 in the brains of AD patients. The postmortem degradation of mRNA and protein may be different between control and AD patients because neurons in AD brain had undergone degeneration, which would accompany destruction of lysosomes and mitochondria, before sampling. We thus need to be careful when we analyze and discuss mRNA and protein levels in postmortem samples. In addition, since calcium concentrations and calcium-related responses might be altered by postmortem conditions, ER stresses in postmortem samples require careful interpretation. To this end, we have shown an unphysiological activation of the calcium-dependent protease calpain in postmortem mouse brains (5).

Our observations raise serious concerns surrounding efforts to translate basic findings obtained using APP/PS1-gene modified mice to clinical applications. If pharmacological candidates that improve the pathological and neurological parameters of the APP/PS1-gene modified mice exert their effects via the modification of non-specific ER stress, then these candidates may not be effective in a preclinical setting or in clinically defined AD patients. Choosing appropriate models is thus extremely important if the mechanisms underlying AD are to be fully elucidated (4).

EXPERIMENTAL PROCEDURES

Animals

All animal experiments were carried out in accordance with RIKEN Brain Science Institute guidelines. We previously produced AppNL-G-F/NL-G-F-knock-in (AppNL-G-F) mice using genomic DNA containing introns 15 to 17 of mouse App with the humanized Aβ sequence into which KM670/671NL (Swedish), I716F (Iberian) or E693G (Arctic) mutations (3) had been introduced. APP23 mice (38), which overexpress Swedish mutation-containing APP751, were maintained on a C57BL/6J background. Tg2576 mice (39), which overexpress
Swedish mutation-containing APP695, were maintained on a mixed B6-SJL background. APP/PS1 (APPswe/PSEN1dE9) mice, which overexpress APP695 (Swedish) and PS1 (deltaE9), and 3XTg-AD mice, which are APP695 (Swedish)-transgenic/ Tau (P301L)-transgenic/ PS1 (M146V)-knockin were maintained on a C57BL/6J background. Tau P301S transgenic (Line PS19) mice, were created on a B6C3H/F1 background (40). PS19 mice were back-crossed onto a C57BL/6 background.

Cell culture
Primary cultured cells were prepared as below. Cortices and hippocampi were separated from E16-18 embryos of WT mice and move to Neurobasal medium (Thermo Fisher Scientific, Waltham, MA USA). Tissues were chopped by scalpels and treated with 5mL of 0.25% trypsin at 37°C for 15min with rotation. Then, 0.125mL of 1% DNaseI was added and mixed by pipetting. After centrifuge of the tissues at 1500rpm for 3min, 5mL of HBSS containing 0.125mL of 1% DNaseI was added to the pellet, and incubated at 37 °C for 5min moving slightly in water bath. Tissues were again centrifuged at 1500rpm for 3 min, and resulting pellet were suspended in 15mL Neurobasal medium containing 2% B27 and 0.5mM glutamate. Cells were filtrated by Falcon 2360 Cell Strainer 100μm Nylon, and seeded in cell culture plates with Neurobasal medium containing B27 and glutamate. Prepared cells at DIV7 were used for experiments. Neuro2A cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum and incubated at 37 °C in a humidified atmosphere of 5% CO2. To induce ER stress, cells were treated with thapsigargin (final 2 μM for 8 hours for primaty cells, final 5μM for 18 hours for Neuro2A cells).

Western Blotting
Extripated brains were immediately frozen in liquid nitrogen, and stored at -80°C. The cortices were homogenized in 400 μL of Tris-HCl (50 mM Tris-HCl pH7.5, 150 mM NaCl, and 1% Triton-X 100) containing a protease inhibitor cocktail and a phosphatase inhibitor cocktail (Sigma-Aldrich, St Louis, MO, USA). The homogenates were centrifuged at 15,000 x g for 20 min at 4 °C. Resulting supernatants were used for subsequent analyses. Protein concentrations were determined using a BCA protein assay kit (Pierce, Rockford, IL). An equivalent amount of protein from each animal was mixed with 4x sample buffer with 2-mercaptoethanol, separated by SDS-polyacrylamide gel electrophoresis, and transferred electrophoretically to a 0.22 μm PVDF membrane (Merck Millipore, MA, USA). The membrane was treated with the ECL Prime blocking agent (GE Healthcare, Little Chalfont, UK), and reacted with each primary antibody (Table I) diluted in Tris-buffered saline containing 0.05% Tween20 (TBST), pH7.5, overnight at 4°C. The membrane was washed three times in
TBST for 5 min, and treated with horse radish peroxidase-conjugated anti-rabbit or mouse IgG (GE Healthcare) for 1 hour. Immunoreactive bands on the membrane were visualized with ECL Select (GE Healthcare) and scanned with a LAS-3000mini LuminoImage analyzer (Fuji Film, Japan). The Neuro2A lysates were analyzed in a similar manner at an identical protein concentration.

RNA Isolation and Polymerase Chain Reactions (PCR)
The cortex samples were homogenized in 1 mL of RNAiso Plus total RNA extraction reagent (Takara). Neuro2A cells and primary cultured cortical neuron cells (1 X 10⁷ cells/1 sample) were dissolved in 500 µM of RNAiso Plus. Total RNA from each samples were isolated according to the manufacture’s instruction. To obtain complementary DNA, a reaction mixture containing 2 µg of RNA and Primerscript reverse transcriptase (Takara) was reacted according to the manufacturer’s directions as follows: 60 min at 42 °C, then 10 min at 70 °C to stop the reaction. The semi-quantified polymerase chain reaction (PCR) was performed using KOD fx neo (Toyobo, Osaka, Japan) for Xbp1, or Takara Ex-Taq (Takara) for CHOP and β-actin. PCR was conducted at; 94 °C for 2 min, and then 40 cycles of 98 °C for 10 sec, 50 °C for 30 sec and 68°C for 1 min, using primers 5’-agaggagccagggcaaagagttcaacg-3’ (sense) and 5’-tcggagacagacaggatgtgcca-3’ (antisense) for Xbp1; 95 °C for 2 min, and then 40 cycles of 95 °C for 30 sec, 50 °C for 30 sec and 72°C for 1 min, using primers 5’-gggtcagaaggattctgtg-3’ (sense) and 5’-ggctcaacatgtctggg-3’ (antisense) for β-actin.

Statistical Analysis
All data are shown as means ± SEM. Differences between groups were examined for statistical significance with one-way or two-way ANOVA.

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CONFLICTS OF INTEREST
SH, TS and TCS serve as a member, an advisor and a CEO, respectively, for RIKEN BIO Co. Ltd.

AUTHOR CONTRIBUTIONS
SH, AI, TS and TCS made research plans. SH, AI, NK and NW performed experiments. SH and TCS wrote the manuscript. SH, TO, MY and TCS supervised the entire research.
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FIGURE LEGENDS

Figure 1. Expression of ER stress markers in $App^{NL-G-F}$ and APP-Tg mice.
(a) Representative western blot shows expression levels of ER stress markers in the cortices and hippocampi of 6-month-old (M) or 14M WT, $App^{NL-G-F}$, APP23 and Tg2576 mice. The expressions in 6 and 15M APP/PS1, and 23M 3XTg-AD mice were also determined. Values shown in figures are band intensity of each band which is divided by intensity of $\beta$-actin (for GRP78, CHOP, and PDI) or total eIF2$\alpha$ (for p-eIF2$\alpha$). As a positive control, ER stress markers in thapsigargin-treated primary cultured cortical neuronal cells or Neuro2a cells were confirmed. Arrowhead shows bands of CHOP or p-eIF2$\alpha$, and asterisk shows nonspecific bands. (b) mRNA levels of unspliced/spliced Xbp1 and CHOP were determined. XBP1 mRNA was detected by semi-quantified reverse transcription-PCR. Unspliced/spliced-XBP1 was observed as a 152/126-bp band respectively. (c-e) Expression levels of ER stress markers in cortices (c,d) or hippocampi (e) were normalized to that of $\beta$-actin (for GRP78, CHOP, and PDI) or total level of eIF2$\alpha$ (for p-eIF2$\alpha$), and reported as relative levels compared to expression in 6M WT mice. Expression level of spliced-Xbp1 mRNA was divided by that of unspliced-Xbp1 mRNA. Positive control is thapsigargin-treated primary cultured cells. Data are shown as means ± SEM (n=3). Differences between groups were examined for statistical significance with one-way ANOVA. n.s.: no significant difference.

Figure 2. Expression of ER stress markers in P301S-Tau-Tg mice.
(a) Expression levels of ER stress markers in the cortices (3-15M) and hippocampi (12M) of WT and P301S-Tau-Tg mice were determined. Arrowhead shows bands of CHOP or p-eIF2$\alpha$ and asterisks show nonspecific bands. (b) XBp1 mRNA was detected by semi-quantified reverse transcription-PCR. (c, d) Shown are mean levels ± SEM of relative expression of ER stress markers (n=3). Differences between groups were examined for statistical significance via two-way ANOVA. n.s.: no significant difference.
Table 1. Antibodies used for western blot analyses. The following antibodies were used at the indicated dilutions to detect ER stress markers.

| Protein | Antibody | Dilution |
|---------|----------|----------|
| APP     | Merck Millipore #MAB348 (clone 22c11) | 1:2500 |
| Tau     | Thermo #AHB0042 (Tau5) | 1:2500 |
| GRP78   | abcam #ab21685 | 1:5000 |
| CHOP    | abcam #ab11419 | 1:2500 |
| Phospho-eIF2α | Cell Signaling #3398 | 1:1000 |
| eIF2α   | Cell Signaling #9722 | 1:1000 |
| PDI     | Hiroi et al, Endocrinol., 2006 | 1:5000 |
| PS1     | Tomita et al, J. Neurosci., 1999 | 1:5000 |
|         | Sato et al, J. Neurosci., 2008 | 1:5000 |
| β-actin | SIGMA #A5441 | 1:5000 |
|          | Cortex | Hippocampus |
|----------|--------|-------------|
|          | 6M     | 14M         | 6M | 6M | 15M | 23M |
| WT       |        |             | WT | APP | PS1 |     |
| App EGF  |        |             |    |     |     |     |
| App23    |        |             |    |     |     |     |
| Tg2576   |        |             |    |     |     |     |
| APP      |        |             | 1.00 | 1.16 | 1.17 |     |
| GRP78    |        |             | 1.00 | 1.04 | 0.70 |     |
| CHOP     |        |             | 1.00 | 1.24 | 1.35 |     |
| PDI      |        |             | 1.00 | 1.24 | 1.35 |     |
| p-eIF2α  |        |             | 1.00 | 1.44 | 0.70 |     |
| eIF2α    |        |             |     |     |     |     |
| β-actin  |        |             |     |     |     |     |

**Fig. 1**
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Endoplasmic reticulum stress responses in mouse models of Alzheimer disease: overexpression paradigm versus knock-in paradigm
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