Journal of Alzheimer's Disease 20 (2010) 261–273
DOI 10.3233/JAD-2010-1360
IOS Press

Presenilin-1 Holoprotein is an Interacting Partner of Sarco Endoplasmic Reticulum Calcium-ATPase and Confers Resistance to Endoplasmic Reticulum Stress

Haifeng Jin[a], Nobuo Sanjo[a,*], Toshiki Uchihara[b], Kazuhiko Watabe[c], Peter St. George-Hyslop[d,e], Paul E. Fraser[d] and Hidehiro Mizusawa[a]

[a]*Department of Neurology and Neurological Science, Graduate School of Medical and Dental Science, Tokyo Medical and Dental University, Bunkyo-ku, Tokyo, Japan*
[b]*Department of Neurology, Tokyo Metropolitan Institute for Neuroscience, Fuchu, Tokyo, Japan*
[c]*Molecular Neuropathology, Tokyo Metropolitan Institute for Neuroscience, Fuchu, Tokyo, Japan*
[d]*Centre for Research in Neurodegenerative Diseases, Departments of Medical Biophysics and Medicine* (*Neurology*)*, University of Toronto, Toronto, Ontario, Canada*
[e]*Department of Clinical Neuroscience, University of Cambridge, Cambridge, United Kingdom*

Accepted 15 December 2009

Abstract. Presenilin-1 (PSEN1) is a primary component of the γ-secretase complex, and total levels of its holoprotein and endoproteolytic fragments are tightly regulated. We examined the effects of several types of endoplasmic reticulum (ER) stress on quantitative changes in the levels of *PSEN1* mRNA, holoprotein, and fragments. The ER stress-inducing chemical compounds tunicamycin, brefeldin-A, thapsigargin, and staurosporine were added to the culture media of various human cell lines. Tunicamycin treatment caused a doubling of PSEN1 holoprotein production in HEK293 cells and an increase in holoprotein production to approximately 180% in GOTO human neuroblastoma and KNS-42 human glioma cell lines, without changing the amounts of PSEN1 N- or C-terminal fragments. The elevated holoprotein level in HEK293 cells was accompanied by an increase in *PSEN1* mRNA expression. HEK293 cells that stably overexpressed PSEN1 holoprotein showed increased resistance to ER stress induced by tunicamycin, but they did not show resistance to ER stress caused by thapsigargin, a specific inhibitor of sarco ER calcium-ATPase (SERCA). In wild-type HEK293 cells under ER stress induced by tunicamycin, an increased amount of SERCA interacted with PSEN1 holoprotein. PSEN1 production varied among cell types and circumstances. The results suggested that the holoprotein forms a complex with the SERCA channel and participates in the regulation of intracellular calcium homeostasis. These findings provide support for the calcium hypothesis of Alzheimer's disease.

Keywords: Endoplasmic reticulum stress, presenilin, PS1, PSEN1, PS2, PSEN2, sarco ER calcium-ATPase, SERCA, tunicamycin

Supplementary data available online: http://dx.doi.org/10.3233/JAD-2010-1360

INTRODUCTION

Presenilin-1 (PSEN1) plays a pivotal role in the production of amyloid-β (Aβ) protein, which is central to the pathogenesis of Alzheimer's disease (AD). PSEN1 is a principal component of the γ-secretase

*Correspondence to: Nobuo Sanjo, Department of Neurology and Neurological Science, Graduate School of Medical and Dental Science, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo, 113-8519 Japan. Tel.: +81 3 5803 5234; Fax: +81 3 5803 0169; E-mail: n-sanjo.nuro@tmd.ac.jp.

ISSN 1387-2877/10/$27.50

complex that regulates intramembrane proteolysis of the amyloid-β protein precursor (AβPP) C-terminal fragment, which is the final step in Aβ processing [1–4]. Details of the pathway and mechanism of this complex activity have yet to be fully resolved, but its regulation appears to be related to inherent presenilin (PS) proteolytic activity and the action of certain modulators [3, 5–8].

PSEN1 also has γ-secretase-independent activities, such as neuroprotective and apoptotic effects [9–11], protein trafficking [12,13], modulation of cell-cell adhesion [14], and, recently, promotion of long-term potentiation (LTP) induced by presynaptic theta-burst stimulation, and glutamatergic neurotransmitter release [15]. It has also been proposed that presenilin holoprotein, but not their fragments, form passive endoplasmic reticulum (ER) calcium leak channels, and that loss of this function causes neurodegeneration that is independent of γ-secretase activity [16]. Two of the familial AD (FAD)-associated mutations (*PSEN1* M146V and *PSEN2* N141L) impair the formation of leak channels [16]. Other *PSEN1* mutations (L113P, G183V, M233L, V412I) and a single *PSEN2* mutation (Y231C) have been reported in families with frontotemporal dementia, in which amyloid pathology is lacking [17–22]. Moreover, another type of AD, characterized by *PSEN1* mutations, is accompanied by α-synuclein accumulation [23–25]. These observations suggest that loss or impairment of presenilin function plays an important role in neurodegeneration that may be independent of the Aβ cascade.

In our previous report, we demonstrated that PSEN1 is more abundant in astrocytes than in neurons in and around the ischemic focus [26]. This finding suggests that presenilins in cells other than neurons have pathological relevance to oxidative, ischemic, and/or ER stresses. Because this upregulation is restricted to astrocytes and is not observed in neurons, it seems likely that the roles and metabolism of PSEN1 differ among cell types. This is the case with the PSEN1-mediated unfolded protein response, which depends on the cell type under investigation [27,28]. To examine this phenomenon further, we challenged human embryonic kidney (HEK293), human neuronal, and human glial cell lines with various kinds of ER stress induced by chemical compounds. Our results indicated that the consistent change in response to carefully organized ER stress was a relative increase in the production of endogenous PSEN1 holoprotein by these cells. Further tests in HEK293 cells revealed that this was preceded by elevated transcription of *PSEN1* mRNA. Treatment of wild-type HEK293 cells with one of the chemical compounds, tunicamycin, led to overproduction of PSEN1 holoprotein and facilitated interaction of these proteins with sarco ER calcium-ATPase (SERCA), which has the highest affinity for Ca^{2+} removal from the cytosol in neuronal and non-neuronal cells in Ca^{2+} channels [29] and was recently reported to interact with PSEN1 [30]. SERCA and other Ca^{2+} pumps, along with Ca^{2+} release channels, are important for Ca^{2+} regulation in neuronal and non-neuronal cells. Moreover, these two proteins interacted to form a PSEN1-SERCA complex in cells stably overexpressing PSEN1 holoprotein, conferring increased resistance to the ER stress induced by tunicamycin. This suggests that PSEN1 holoprotein has a cytoprotective effect via the maintenance of calcium homeostasis under conditions of ER stress.

MATERIALS AND METHODS

Cell culture

The human neuroblastoma cell line GOTO [31] was cultured in Roswell Park Memorial Institute (RPMI) 1640 (Invitrogen, Carlsbad, CA, USA) medium supplemented with 20% fetal bovine serum (FBS; Cambrex Bio Science, MD, USA). The KNS-42 human glioma cell line [32] and HEK293 cell line were cultured in Dulbecco's modified Eagle's medium (Gibco, Gaithersburg, MD, USA) supplemented with 10% FBS. All cells were maintained in a humidified CO_2 incubator with 5% CO_2 at 37°C and subcultured at confluence in Hanks's balanced salt solution (HBSS; Gibco, Auckland, NZ) containing 0.02% ethylenediamine tetraacetate (EDTA) and 0.25% trypsin. To obtain HEK293 cells stably overexpressing wild-type PSEN1, a cDNA clone encoding human wild-type *PSEN1* (*PSEN1*wt) was generated by polymerase chain reaction from a human brain cDNA library. The cDNA was ligated into the expression vector pcDNA3.1 (Invitrogen), then transfected into HEK293 cells with the plasmids or with empty vector (as a negative control), using Lipofectamine 2000 (Invitrogen). Stably transfected cells were selected in 250 μg/ml of Geneticin.

Cell stimulation and immunoblotting

After 3 days of culture, various kinds of ER stress – inducing chemical compounds, namely tunicamycin (Sigma, St. Louis, MO, USA), brefeldin-A (Sigma), thapsigargin (Sigma), and staurosporine (Sigma), were

added directly to the culture medium at appropriate final concentrations such that ∼90% cell survival was expected (Supplementary Fig. 1; Supplement available online: http://www.j-alz.com/issues/20/vol20-1.html#supplementarydata01). The concentrations are given in Supplementary Table 1. The putative mechanisms of action of these chemical compounds are described in the Results section. The cells were washed twice with phosphate-buffered saline (PBS), and then harvested with harvest buffer [0.25 M sucrose, 5 mM HEPES (pH 7.2)], 1 mM EDTA, and protease inhibitors. Protein concentration was determined by Bio-Rad protein assay (Bio-Rad, Hercules, CA, USA).

Equal amounts of cell lysates were subjected to 4% to 20% Tris-glycine gradient gels (Invitrogen). The proteins were then transferred to a polyvinylidene difluoride (PVDF) membrane and the membrane was incubated with anti-PS1 amino-terminal polyclonal antibody [33] at a dilution of 1:2000 or loop antibody (Millipore, Billerica, MA, USA) at a dilution of 1:2000; anti-β-tubulin monoclonal antibody (BD Bioscience, San Jose, CA, USA) at a dilution of 1:500; anti-KDEL antibody (StressGen Biotechnologies, Victoria, BC, Canada) at a dilution of 1:1000; or anti-SERCA2b antibody (Affinity BioReagents, Golden, CO, USA) at a dilution of 1:2000. Bound primary antibodies were detected by horseradish peroxidase (HRP)-coupled goat anti-rabbit IgG secondary antibody (Pierce, Rockford, IL, USA) or with HRP-coupled goat anti-mouse IgG secondary antibody (Kirkegaard & Perry, Gaithersberg, MD, USA), and visualized using an Enhanced Chemiluminescence (ECL) Plus system (Amersham, Arlington Heights, IL, USA). After scanning of the immunoreactive bands (GT-8500, Epson, Nagano, Japan), the relative intensities of the bands were quantified by using image-analysis software (ImageJ; http://rsb.info.nih.gov/ij/).

Immunocytochemistry

For immunofluorescence microscopy, HEK293 cells were plated onto coverslips coated with mouse collagen type 4 (Becton Dickinson Labware, Bedford, MA, USA) in a 35-mm dish. After a brief rinse in PBS, the cells were fixed with 4% paraformaldehyde in PBS at room temperature for 20 min and then permeabilized with 0.03% Triton X-100 in PBS for 20 min at room temperature. Cells were blocked with 10% goat serum in PBS for 30 min and then incubated overnight with each primary antibody. The primary antibodies used were rabbit polyclonal antibody to PS1-amino terminal fragment (NTF) (Ab14) and SERCA2b monoclonal antibody (Affinity BioReagents). Fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG or tetra methyl rhodamine isothiocyanate (TRITC)-conjugated goat anti-rabbit IgG (Chemicon, Billerica, MA, USA) were used as secondary antibodies. Specimens were examined with a Carl Zeiss laser scanning confocal imaging system (LSM 510 META) equipped with an LSM Image Browser (Carl Zeiss, Oberkochen, Germany).

Co-immunoprecipitation

Immunoprecipitation was performed by using Dynabeads Protein G or Protein A (Dynal Biotech, Oslo, Norway) in accordance with the manufacturer's instructions. HEK293 cells were lysed with 1% CHAPSO (Calbiochem, Gibbstown, NJ) in buffer [20 mM HEPES (pH 7.2), 2 mM EDTA, and protease inhibitors], and the lysate was subjected to centrifugation at 21,000g for 10 min at 4°C. All immunoprecipitation steps were performed at 4°C. After preclearing of the solutions with protein G-Sepharose CL-4B (Sigma), the cell lysate from one 10-cm-diameter dish was incubated with antibodies that recognized PSEN1 or SERCA. Beads were washed six times with a wash buffer. Bound proteins were eluted by elution buffer and detected by Western blotting.

Assessment of cell death

Cell counting

For cell counting, cells were washed twice with HBSS, and then detached from the surface with HBSS containing 0.02% EDTA and 0.25% trypsin. Dead cells were excluded by 0.4% trypan blue, and viable cells were counted directly by cell counter. Triton X-100 was used as a positive control for dead cells.

LDH release assay

To measure the cytotoxicity induced by these chemical compounds, total amounts of lactate dehydrogenase (LDH) in the culture medium were analyzed by a LDH Cytotoxicity Detection Kit (Takara, Tokyo, Japan) in accordance with the manufacturer's instructions. Briefly, after 24 h of incubation of the cells with the chemical compound, the culture medium was collected and 3 samples of the medium were incubated with the reaction mixture from this kit at room temperature with shaking for 30 min. The reaction mixture was fixed with 1 N hydrochloric acid, and the absorbance measured at a wavelength of 490 nm was considered to represent the amount of LDH released.

TUNEL assay

Twenty-four hours after exposure to tunicamycin, apoptotic cells were detected by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) using an *In Situ* Cell Death Detection Kit, Fluorescein (Roche, Basel, Switzerland) in accordance with the manufacturer's instructions. After all the nuclei of the cells were stained with DAPI (4′, 6-diamino-2-phenylindole) (Vector Laboratories, Burlingame, CA, USA), TUNEL-positive cells were counted and the ratios of TUNEL-positive cells to DAPI-positive cells from three independent experiments were compared.

Calcium imaging

Cytosolic calcium concentration was measured with a Calcium KitII-Fluo 4 (Dojindo, Kumamoto, Japan) in accordance with the manufacturer's instructions. In brief, after 2 days of culture, cells were plated at 15,000 cells/well in a 96-well plate (BD Bioscience, San Jose, CA, USA), and Fluo 4-AM Loading Buffer (Dojindo) was added into each well. After 1 h of cell incubation at 37°C, the fluorescence intensity was measured with SkanIt RE for Varioskan Flash 2.4 (Thermo Fisher Scientific, Waltham, MA, USA) and analyzed with SkanIt Software version 2.4 (Thermo Fisher Scientific).

Analysis of presenilin transcription levels

Total RNA was extracted from cultured cells by using Isogen (Nippon Gene, Tokyo, Japan) in accordance with the manufacturer's instructions. First-strand cDNA was synthesized using random primer and Superscript III reverse transcriptase (Invitrogen Life Technologies, Carlsbad, CA, USA) after Turbo DNA-free (Ambion, Austin, TX, USA) treatment. The PCR reactions were performed in TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA) with primers for the *PSEN1* gene (Applied Biosystems; assay identification number Hs00997789_m1), and the human *GAPDH* gene was used as an endogenous control (Applied Biosystems, Warrington UK; part number 4352934E). Real-time PCR amplification was performed with an ABI PRISM 7700 sequence Detector (Applied Biosystems), and the relative amount of *PSEN1* mRNA was measured. The thermal cycler conditions were as follows: hold for 10 min at 95°C, followed by two-step PCR for 40 cycles of 95°C for 15 s, followed by 60°C for 1 min. All tests were performed in triplicate. Amplification data were analyzed with ABI PRISM 3100 Genetic Analyzer Data Collection Software Version 2.0 (Applied Biosystems).

Statistical analysis

All data are expressed as mean values ± SEM. Differences between groups were tested for statistical significance by using one-way Analysis of Variance (ANOVA) followed by the Bonferroni multiple comparison test. A P value < 0.05 was regarded as statistically significant.

RESULTS

Cytotoxicity of chemical compounds

Because the cytotoxicity of a compound depends on both the cell type and the mechanism by which the compound induces ER stress, it was necessary to determine which concentration of each compound was appropriate for observing both the molecular changes and the vulnerability of a cell line. Twenty-four hours after exposure of HEK293 cells to increasing concentrations of the different chemical compounds, cell mortality rates were quantified and compared with the amounts of LDH released into the culture medium (Supplementary Fig. 1). Similar experiments were performed on GOTO and KNS-42 cells (data not shown). The concentration of each compound that allowed 80% to 90% survival of each cell type was determined by three independent series of experiments. These appropriate concentrations, which differed according to cell type and compound, were used for subsequent experiments (Supplementary Table 1).

The mechanisms by which each compound induced ER stress differ. Tunicamycin blocks all N-glycosylation of proteins by inhibiting the enzyme GlcNAc phosphotransferase. Thapsigargin inhibits SERCA by tight binding and raises the cytosolic calcium concentration. Brefeldin-A administration leads to protein accumulation inside the ER by interfering with protein transport from the ER to the Golgi, and staurosporine inhibits protein kinases through prevention of the ATP binding sites of the kinases.

Quantitative changes in full-length PSEN1 under ER stress conditions

Tunicamycin treatment, but not treatment with the other chemical compounds (data not shown), at the appropriate concentrations for 24 h resulted in relative increases in PSEN1 holoprotein production (Fig. 1). At 24 h, production had more than doubled in HEK293

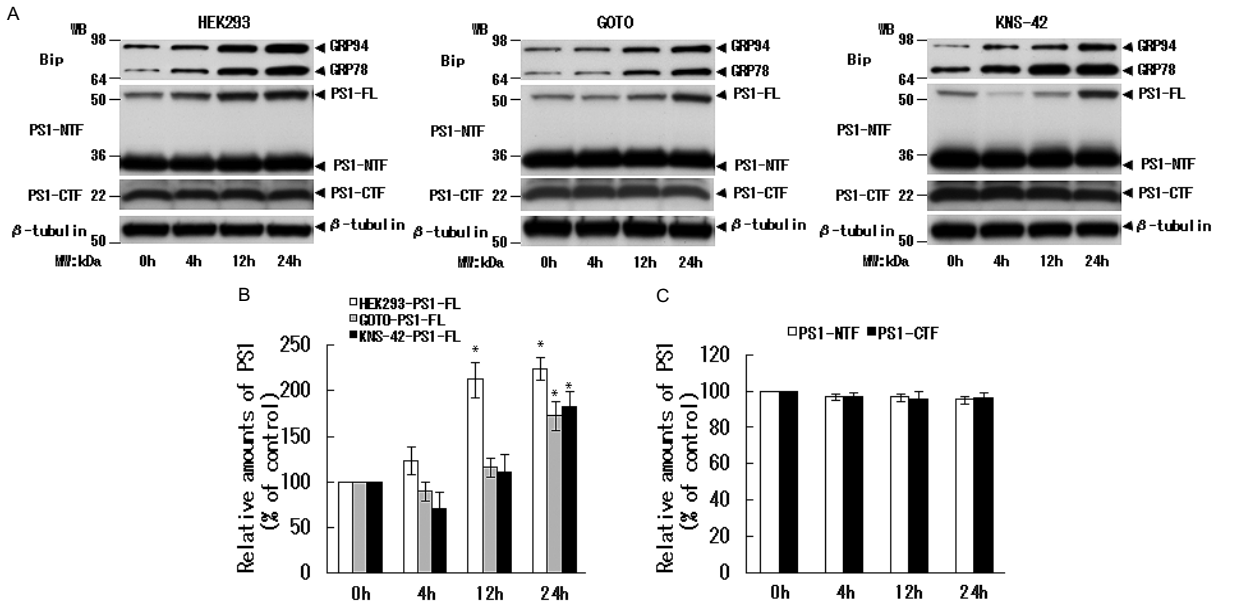

Fig. 1. Quantification of PSEN1 holoproteins and N-terminal fragments (NTFs) and C-terminal fragments (CTFs) after exposure to tunicamycin. Increase in the levels of PSEN1 holoprotein (PSEN1-FL) and GRP78/Bip after exposure to tunicamycin in HEK293 (A: left), GOTO (A: middle), and KNS-42 (A: right) cells. Proteins (total 20 μg) were loaded in each lane for Western blotting. Intensity of each band was quantified relative to that observed before the exposure, expressed as 1.0. The relative amounts of PSEN1 holoprotein in each cell line (B), and of CTFs and NTFs in HEK293 cells (C), are indicated as the mean values $\pm$ SEM. $n = 6$. $^*p < 0.05$.

cells ($P < 0.0001$) and increased to about 180% of the control values in both GOTO cells and ($P = 0.0001$) and KNS-42 cells ($P = 0.0031$) (Fig. 1A, B). ER stress-marker GRP78/Bip protein production significantly increased in all cell lines, relative to control. The three cell lines essentially reacted in the same manner; the only difference among them was in the rates of increase in PSEN1 holoprotein production. At 12 h, wild-type HEK293 cells had significantly higher holoprotein levels than in the other cell lines (HEK293 vs. GOTO: $P = 0.0001$; HEK293 vs. KNS-42: $P = 0.0017$; GOTO vs. KNS-42: $P = 0.802$), but at 24 h there were no significant differences in the levels of holoprotein between each of the cell lines (HEK293 vs. GOTO: $P = 0.066$; HEK293 vs. KNS-42: $P = 0.140$; GOTO vs. KNS-42: $P = 0.712$). The difference at 12 h may have been caused by differences in the origins of the cells: GOTO and KNS-42 cells originate in the central nervous system [31,32], whereas HEK293 cells are derived from the kidney. There were no significance differences from the control values in the amounts of PSEN1 NTFs or carboxyl terminal fragments (CTFs) in any of the cell lines (HEK293: Fig. 1C; GOTO and KNS-42: Supplementary Fig. 2). The quantitative increase in PSEN1 holoprotein production with quantitative stability in the amounts of both PSEN1 fragments indicated that the rate of endoproteolytic processing of PSEN1 holoprotein was not reduced under these conditions. The half-life of PSEN1 holoprotein is 1.5 h and that of their fragments is 24 h [34]. To conserve the amounts of both fragments over 24 h, the cells needed to maintain the endoproteolysis of PSEN1 holoprotein.

Cytoprotective effect of wild-type PSEN1 holoprotein

We hypothesized that overexpression of PSEN1 holoprotein could be cytoprotective against some ER stresses. To verify this hypothesis, we prepared a cell line stably overexpressing PSEN1 holoprotein, but not its fragments (Fig. 2A), and exposed the cells to tunicamycin. The cells showed enhanced resistance to ER stress induced by tunicamycin, and the cell death rate was reduced to less than half that in mock-transfected cells (Fig. 2B; $P = 0.0139$) on LDH release assay. A similar reduction in cell death with PSEN1 holoprotein overexpression was also confirmed by quantification of TUNEL-positive cells after exposure to tunicamycin: the ratio of TUNEL positivity to DAPI positivity was significantly less among PSEN1-overexpressing cells than among untransfected cells (Fig. 3A to F, G; $P = 0.0003$). Interestingly, this cytoprotective effect of PSEN1 overexpression in HEK293 cells was not detect-

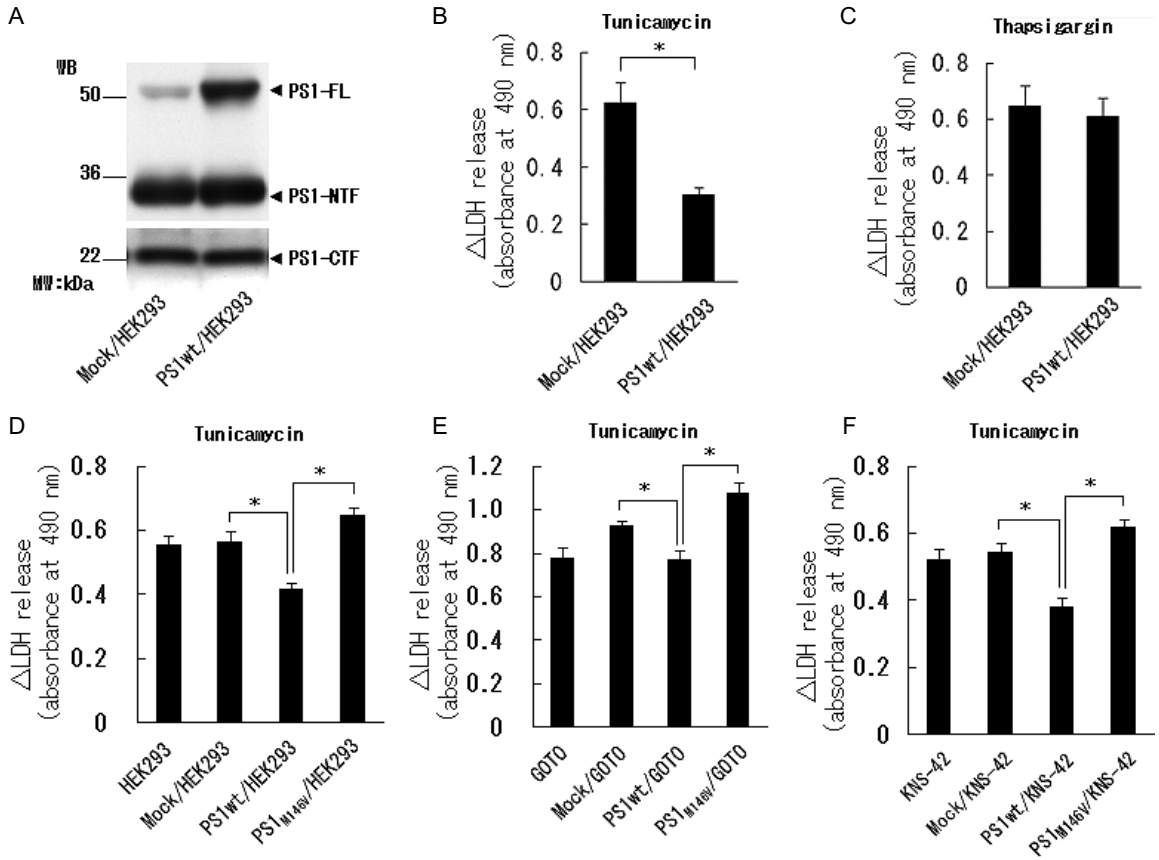

Fig. 2. PSEN1-overexpressing cells are resistant to ER stress. HEK293 cells stably overexpressing PSEN1 produced larger amounts of PSEN1 holoprotein than empty-vector stably transfected cells (A). Proteins (20 μg) were loaded in each lane for Western blotting. The total amounts of N- and C-terminal fragments were almost the same in both cell lines (A). The LDH release assay indicated that PSEN1-overexpressing cells had greater resistance to ER stress induced by tunicamycin (B), but not by thapsigargin (C), when compare to mock-transfected cells. Transient overexpression of wild-type PSEN1 or M146V FAD-mutation PSEN1 in HEK293 (D), GOTO (E), or KNS-42 (F) cells revealed that the FAD mutation negated the resistant effect of PSEN1. $n = 6$. $^*p < 0.05$.

ed following ER stress induced by thapsigargin, which is a SERCA inhibitor (Fig. 2C, 3H). SERCA pumps have the highest affinity for Ca^{2+} removal from the cytosol to ER in neuronal and non-neuronal cells in all types of Ca^{2+} pump [29]. The enhanced resistance to the ER stress induced by tunicamycin but not thapsigargin therefore strongly indicated that the cytoprotective effect in cells stably overexpressing PSEN1 holoprotein is associated with SERCA and intracellular Ca^{2+} homeostasis. As reported previously [30], the cytosolic Ca^{2+} concentration, as measured by Fluo4 fluorescence signals before and during stimulation with 1 μM thapsigargin, was lower in cells stably overexpressing PSEN1 than in mock-transfected cells (Supplementary Fig. 3).

In order to determine the cytoprotective effect of PSEN1 with an FAD-related mutation, we transiently overexpressed *PSEN1* M146V constructs in HEK293 cells and compared the viability of these cells with that of cells transiently overexpressing wild-type PSEN1 (Fig. 2D; Mock vs. PS1wt ($P = 0.0006$); PS1wt vs. $PS1_{M146V}$ ($P < 0.0001$)). Cells with the FAD mutation did not have the resistant effect seen in cells with PS1 overexpression. The cytoprotective effect of PSEN1 was also detected in neural and glial cells (Fig. 2E, GOTO cells, Mock vs. PS1wt ($P = 0.0099$); PS1wt vs. $PS1_{M146V}$ ($P < 0.0001$); Fig. 2F, KNS-42 cells, Mock vs. PS1wt ($P = 0.0006$); PS1wt vs. $PS1_{M146V}$ ($P < 0.0001$).

Quantitative changes in PSEN1 mRNA expression and protein production under ER stress conditions

To examine in more detail the quantitative changes in PSEN1 holoprotein production in untransfected

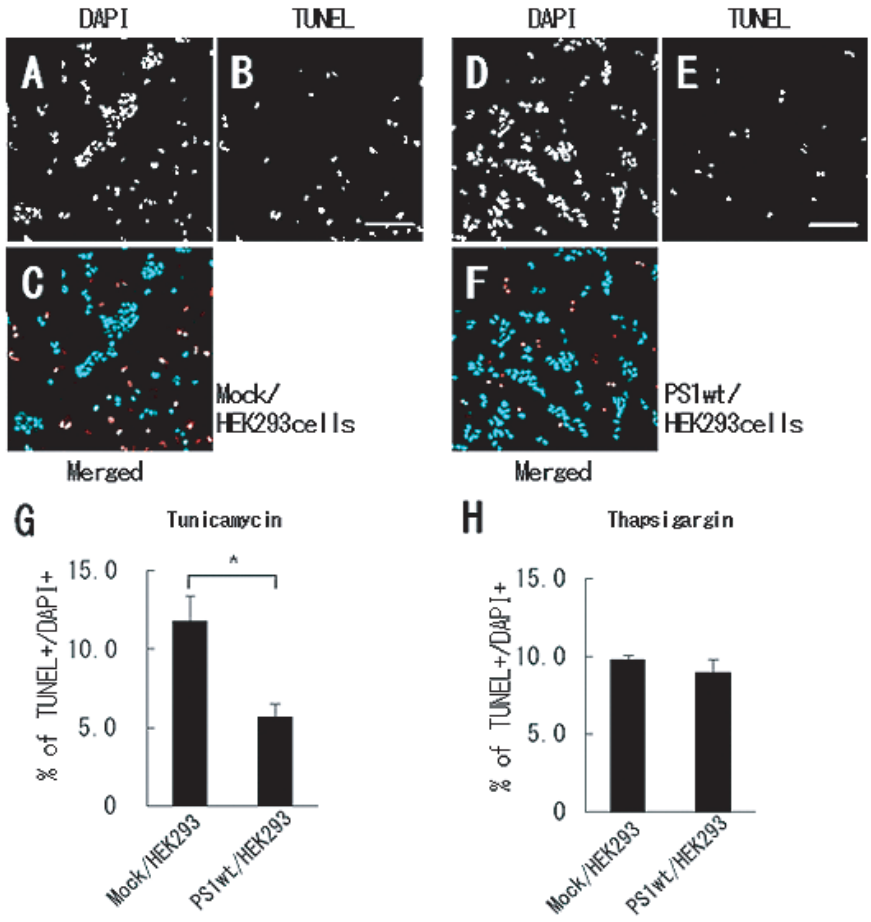

Fig. 3. PSEN1 overexpression has an anti-apoptotic effect. Apoptotic HEK293 mock-transfected and PSEN1wt-overexpressing cells were labeled by the TUNEL technique (B, E), and the abundance of these cells was compared with that of all cells, as labeled by DAPI (A, D). Nuclei of apoptotic cells appear yellow (C, F). There was less TUNEL positivity among HEK292 cells overexpressing PSEN1 than among empty-vector stably transfected cells under ER stress induced by tunicamycin (C, F, G), but not by thapsigargin (H). $n = 3$. Bars $= 100$ μm. $^{*}p < 0.05$.

HEK293 cells under ER stress induced by tunicamycin, PSEN1 holoprotein was quantified in cell lysates 6, 12, 18, 24, or 30 h after addition of the compound (Fig. 4A, B; 0 h vs. 12 h ($P = 0.0002$); 0 h vs. 18 h, 0 h vs. 18 h, 0 h vs. 30 h ($P < 0.0001$)). The gradual increase in PSEN1 holoprotein levels peaked at 18 h at approximately 250% of the baseline value and then gradually decreased. Because the increase in PSEN1 holoprotein level after exposure to tunicamycin was not accompanied by reciprocal changes in the amounts of PSEN1 fragments (Fig. 1), we hypothesized that increased production of PSEN1 protein may have accounted for the increase and/or decrease in the metabolic degradation of holoprotein. PSEN1 mRNA expression following tunicamycin exposure was examined by real-time PCR. There was a significant increase in message from 6 h; the peak value (about 125% of the baseline value) was recorded 12 h after addition of the compound. Expression then gradually returned to baseline levels (Fig. 4C; 0 h vs. 6 h ($P = 0.0296$), 0 h vs. 12 h ($P = 0.0042$), 0 h vs. 24 h ($P = 0.0328$)). A relative increase in mRNA expression following tunicamycin exposure was also detected in neural (Fig. 4D; 0 h vs. 12 h ($P = 0.0332$)) and glial (Fig. 4E; 0 h vs. 12 h ($P = 0.0401$), 0 h vs. 24 h ($P = 0.0443$)) cell lines. According to the data, ER stress induced by tunicamycin caused an increase in *PSEN1* mRNA transcription and then of PSEN1 holoprotein synthesis. If the increased amounts of PSEN1 holoprotein make more complexes with SERCA channels under tunicamycin exposure, then this greater number of complexes can be cytoprotective and reduce the disruption of intracellular Ca^{2+} homeostasis, as observed in HEK293 cells stably overexpressing PSEN1 holoprotein.

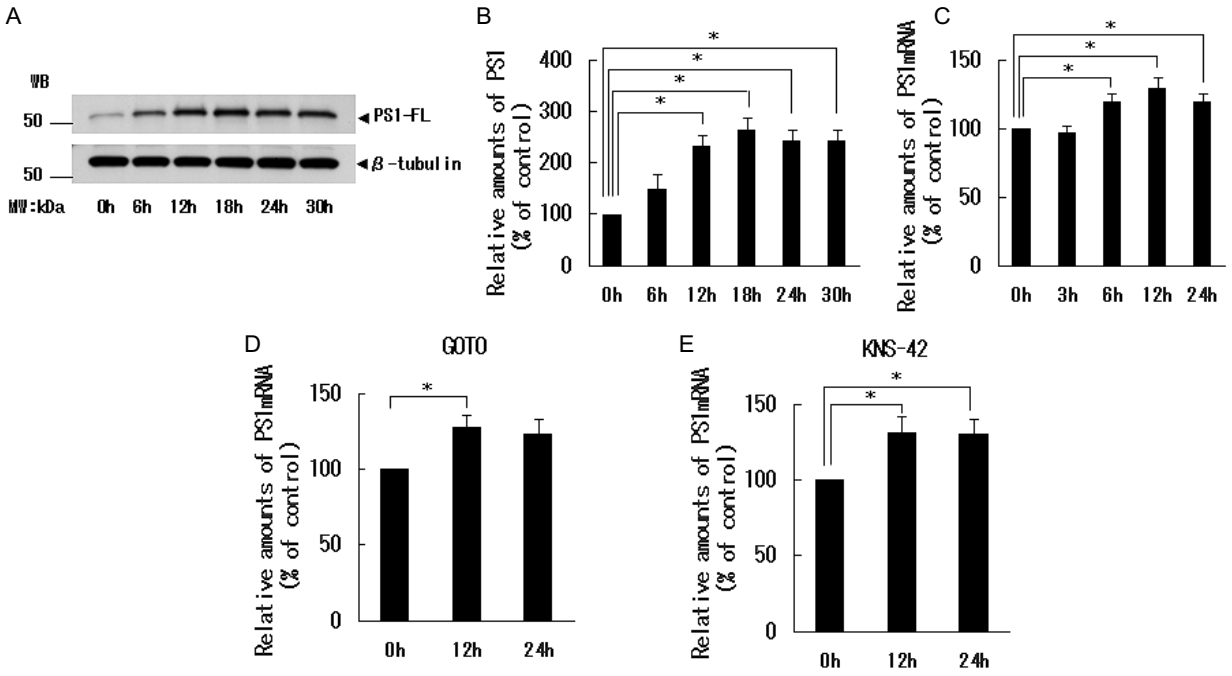

Fig. 4. Quantification of PSEN1 holoprotein and mRNA expression after exposure to tunicamycin. The maximum PSEN1 holoprotein level after the beginning of exposure to tunicamycin was detected at about 18 h in HEK293 cells (A, B), whereas the maximum increase in *PSEN1* mRNA was detected at about 12 h after the start of exposure in HEK293 (C), GOTO (D), and KNS-42 (E) cells. Proteins (20 μg) were loaded in each lane for Western blotting. $n = 6$ in A, B, $n = 3$ in C, D, E. $^*p < 0.05$.

Increased interaction between PSEN1 holoprotein and SERCA by exposure to tunicamycin

Finally, we analyzed the interacting partner of PSEN1 holoprotein under ER stress induced by tunicamycin to clarify the mechanism of the cytoprotective effect. It was recently reported that SERCA interacts with PSEN1 and that SERCA activity in the homeostasis of intracellular Ca^{2+} is regulated by PSEN1 [30]. Although the binding partner of SERCA was speculated in the same study to be PSEN1, it is unknown whether the association is primarily with the PS holoprotein or with its endoproteolytic fragments. We used co-immunoprecipitation to resolve this issue and to search for changes in PSEN1-SERCA binding following the tunicamycin-induced changes in PSEN1 holoprotein production.

In a manner similar to that in wild-type HEK293 cells (Figs 1, 4), a quantitative increase in PSEN1 holoprotein production was detected in mock-transfected HEK293 cells under tunicamycin exposure, whereas the total quantity of SERCA2b channels did not change in the same samples (Fig. 5A, left panels). However, tunicamycin failed to induce a quantitative change in the content of either PSEN1 holoprotein or SERCA2b channels in HEK293 cells stably overexpressing PSEN1 holoprotein (Fig. 5A right panels).

To examine this phenomenon further, we used co-immunoprecipitation to investigate both the potential partners interacting with SERCA and the changes induced by tunicamycin under ER stress. The co-immunoprecipitation data demonstrated clearly that PSEN1 holoproteins and CTFs interacted with SERCA2b channels in both untransfected HEK293 cells (Fig. 5B) and cells stably overexpressing PSEN1 holoprotein (Fig. 5C). The observed colocalization of PSEN1 and SERCA2b was confirmed by immunocytochemistry (Supplementary Fig. 4). This interaction was also detected in mock-transfected HEK293 cells (data not shown), GOTO cells (Supplementary Fig. 5A), and KNS-42 cells (Supplementary Fig. 5B). The percentage of colocalization of PSEN1 holoprotein with SERCA was difficult to determine from immunocytochemistry, but it was calculated to be 7.0% $\pm$ 2.7% (average $\pm$ standard deviation) on the basis of the co-immunoprecipitation data (i.e., the quantity of PSEN1-FL in the co-immunoprecipite compared to that in the input). Because tunicamycin exposure induced a near-doubling of both the amount of PSEN1 holoprotein and, in parallel, its binding with

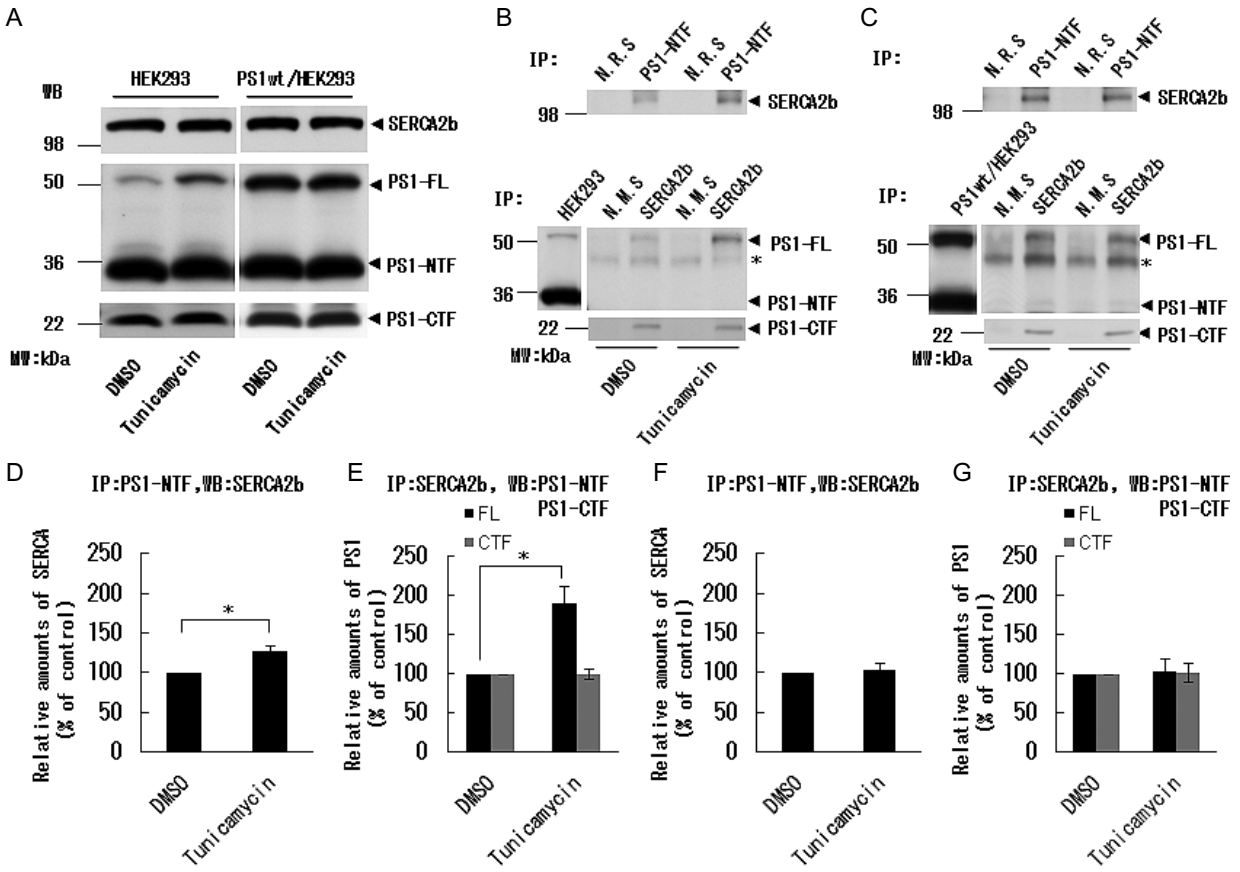

Fig. 5. Interaction of PSEN1 holoprotein with SERCA is increased under ER stress induced by tunicamycin. Total amount of SERCA channels was not changed under ER stress induced by tunicamycin in mock-transfected HEK293 cells or HEK293 cells stably overexpressing PSEN1 (A: upper panels). Proteins (5 μg) were loaded in each lane for Western blotting. The total amount of PSEN1 holoprotein (PSEN1-FL) was increased after exposure to tunicamycin in wild-type HEK293 cells, but was unchanged in HEK293 cells stably overexpressing PSEN1 (A: middle panels). Proteins (20 μg) were loaded in each lane for Western blotting. In a co-immunoprecipitation experiment, the interacting ratio between PSEN1 holoproteins and SERCA channels in untransfected HEK293 cells was increased under exposure to tunicamycin (B: upper and middle panels; D, E), but the interacting ratio between PSEN1 C-terminal fragments (CTFs) and SERCA channels was not changed (B: lower panel). In cells stably overexpressing PSEN1, neither the interacting ratio between PSEN1 holoproteins and SERCA channels, nor that between PSEN1 CTFs and SERCA channels, was changed under tunicamycin treatment. (C, F, G). In each lane, 5 μl (PSEN1) or 10 μl (SERCA) from 50 μl of immunoprecipitated sample buffer was loaded in B and C. $n = 3$, N.R.S.: normal rabbit serum, N.M.S.: normal mouse serum. *denotes non-specific band. $^*p < 0.05$.

SERCA, the stoichiometry between PSEN1 and SERCA remained stable under these experimental conditions. Very weak interaction was suspected between the PSEN1 NTFs and the SERCA2b channels, as determined by co-immunoprecipitation, but the bands were not clear enough for us to determine whether or not they represented background signals (Fig. 5B, C). In untransfected HEK293 cells, the amount of PSEN1 holoprotein interacting with SERCA2b doubled after exposure to tunicamycin (Fig. 5B, E; $^*P = 0.0251$), whereas the amount of interacting CTFs of PSEN1 remained stable (Fig. 5B, E). Conversely, the amount of SERCA2b channels interacting with PSEN1 holoproteins increased to about 120% (Fig. 5B, D; $^*P = 0.0392$). This result suggests that CTFs were not involved in the response to the ER stress induced by tunicamycin. In PSEN1-overexpressing cells the complex may already have been saturated because of the high levels of holoprotein already present. This would explain why treatment with tunicamycin had no appreciable effect on PSEN1-SERCA complex levels, even if an additional slight increase in PSEN1 holoprotein levels was induced by this treatment. To examine this further, cells stably overexpressing PSEN1 holoprotein were used for co-immunoprecipitation studies. The level of PSEN1-SERCA complex was indeed unchanged fol-

lowing treatment with tunicamycin (Fig. 5C upper and middle panels; Fig. 5F, G). These results suggest that the quantity of the complex was not regulated by the stress, but by the amount of PSEN1 holoprotein, and that the upper limit of the complex was probably restricted by the amount of SERCA channels.

DISCUSSION

Previous studies have demonstrated that the metabolism of PSEN1 and its fragments is dependent on cell type and condition [35–38]. Our study demonstrated that exposure to tunicamycin increased transcription of endogenous *PSEN1* mRNA and accumulation of PSEN1 holoprotein in HEK293 cells, neuroblastoma cell lines (GOTO), and glioma cell lines (KNS-42). However, in previous reports there have been no data showing an increase in either *PSEN1* mRNA or holoprotein levels under ER stress caused by chemical compounds, including tunicamycin. It is possible that the amount of PSEN1 holoprotein depends on the intensity and nature of the ER stress caused by chemical compounds, and that the total amount of PSEN1 holoprotein increases only when the cells are exposed to the maximum extent of their resistance. Initially, we determined the relationships between the concentrations of these chemical compounds and the viability of the cells. A level of 80% to 90% cell survival was used for these investigations. When HEK293, GOTO, and KNS-42 cells were exposed to tunicamycin at the appropriate concentrations, holoprotein levels were significantly increased. The rates of increase in PSEN1 holoprotein differed among the cell lines, perhaps due to cell of origin, but the final changes were similar in all instances. It is conceivable that the response to ER stress is required mainly in the central nervous system, but HEK293, which is of kidney origin, reacted in the same manner as GOTO and KNS-42, which are of central nervous system origin. The increase in PSEN1 holoprotein production was independent of the abundance of PSEN1 fragments, as indicated by the unchanged quantity of both NTFs and CTFs in all three cell lines.

PSEN1 holoprotein is degraded rapidly under normal circumstances [33,39] and the presence of other secretase components (nicastrin, Aph-1, and Pen-2) may facilitate their stabilization as holoprotein [3,40, 41]. This process of PSEN1 stabilization is also reported to be controlled by SEL-10, a member of the Skp1-Cdc53/CUL1-F-box protein (SCF) E2–E3 ubiquitin ligase family, which stimulates holoprotein ubiquitination [18]. Moreover, ubiquilin interacts with PSEN and facilitates increased PSEN synthesis without substantially changing PSEN half-life [42]. It is possible that these factors are involved in the PSEN1 holoprotein upregulation demonstrated here. The increase in *PSEN1* mRNA expression after exposure to tunicamycin, however, suggests that PSEN1 transcription is triggered, leading to an increase in PSEN1 protein levels. Thus the possible participation of stabilizing and facilitating factors does not explain the whole process of increase in PSEN1 holoprotein levels after increased transcription of *PSEN1* mRNA. An increase in *PSEN1* mRNA and holoprotein levels may instead represent some primary signal triggered in reaction to exposure to tunicamycin.

Several studies have indicated an interaction between PSEN1 and the calcium leak channel SERCA [16,30]. SERCA pumps have the highest affinity for Ca^{2+} removal from the cytosol and, together with plasma membrane Ca^{2+}-ATPases and transporters, determine the resting cytosolic Ca^{2+} concentration. Five isoforms have been found, and so far SERCA2b is the only isoform reported to be expressed in neurons [43]. Overexpression of PSEN1 results in accelerated Ca^{2+} clearance from the cytosol by increasing SERCA activity [30]. In our study, ER stress induced by tunicamycin caused an increase in PSEN1 holoprotein levels, and enhanced interaction between PSEN1 and SERCA was detected. Wild-type PSEN1 overexpression had a cytoprotective effect under ER stress induced by tunicamycin, but this cytoprotective effect was not detected under ER stress induced by thapsigargin, which is a specific inhibitor of SERCA. Taken together, these findings suggest that the increased PSEN1 holoprotein level prevented the disruption of intracellular Ca^{2+} homeostasis and subsequently stabilized cell viability by increasing the interaction between PSEN1 and SERCA, leading to upregulation of SERCA activity. Disruption of intracellular Ca^{2+}homeostasis induces calpain activation [44], and calpain activation induces the activation of caspase-12, which localizes on the outer membrane of the ER and is reported to be activated by ER stress induction. This process can be triggered by oxygen and glucose deprivation or by adding chemical compounds such as tunicamycin [45,46].

In addition to the calpain-caspase system, dysregulation of intracellular calcium signaling is implicated in the pathogenesis of AD. Accumulation of Aβ and hyperphosphorylation of tau may cause SERCA dysfunction, which maintains intracellular Ca^{2+} and thus elicits Ca^{2+} dysregulation in AD. Disruption of cal-

cium homeostasis in the initial phases of AD in humans and in mice with mutations in the AβPP or presenilin 1 genes has been reported to occur even before the development of overt symptoms [47–49]. Our experiment indicated a direct interaction between PSEN1 holoprotein and SERCA, thus partly supporting the calcium hypothesis of AD. PSEN1 holoprotein may act as a multifunctional anti-apoptotic factor under various stress condition. Indeed, a murine leukemic cell line was protected from p53-induced apoptosis by PSEN1 overexpression when PSEN1 holoprotein predominated [50]. In experimentally-induced transient ischemia in murine brains, *PSEN1* mRNA expression was induced in relatively resistant hippocampus cells, such as CA3 cells and cells in the dentate gyrus [51]. These findings are consistent with the hypothesis that upregulation of PSEN1 is a reaction that counteracts neuronal death. Moreover, PSEN1 can regulate neurotransmitter release via ryanodine receptor – mediated Ca^{2+} release from the ER, whereby PS deficiency attenuates LTP [15]. This experimental framework provides a unique opportunity to investigate the possible role of endogenous PSEN1 holoprotein. It will provide further insight into the metabolism of PSEN1 and its participation in Ca^{2+} homeostasis. It will also facilitate recognition of the relationship between PS holoprotein and intracellular calcium regulation and signaling in neurons and other types of cells, thus helping us to establish a more beneficial therapeutic approach to AD.

ACKNOWLEDGMENTS

This work is supported in part by grants (to TU and HM) from the Ministry of Health, Labor, and Welfare of Japan; the Canadian Institutes of Health Research; the Premier's Research Excellence Award (PREA) program; the Ontario Mental Health Foundation; and the Alzheimer's Society of Ontario. PStG-H is supported by the Wellcome Trust and the Howard Hughes Foundation.

Author's disclosures available online (http://www.j-alz.com/disclosures/view.php?id=244).

REFERENCES

[1] De Strooper B (2003) Aph-1, Pen-2, and Nicastrin with Presenilin generate an active gamma-secretase complex. *Neuron* **38**, 9-12.

[2] Edbauer D, Winkler E, Regula JT, Pesold B, Steiner H, Haass C (2003) Reconstitution of gamma-secretase activity. *Nat Cell Biol* **5**, 486-488.

[3] Takasugi N, Tomita T, Hayashi I, Tsuruoka M, Niimura M, Takahashi Y, Thinakaran G, Iwatsubo T (2003) The role of presenilin cofactors in the gamma-secretase complex. *Nature* **422**, 438-441.

[4] Kennedy JL, Farrer LA, Andreasen NC, Mayeux R, St George-Hyslop P (2003) The genetics of adult-onset neuropsychiatric disease: complexities and conundra? *Science* **302**, 822-826.

[5] Yu G, Nishimura M, Arawaka S, Levitan D, Zhang L, Tandon A, Song YQ, Rogaeva E, Chen F, Kawarai T, Supala A, Levesque L, Yu H, Yang DS, Holmes E, Milman P, Liang Y, Zhang DM, Xu DH, Sato C, Rogaev E, Smith M, Janus C, Zhang Y, Aebersold R, Farrer LS, Sorbi S, Bruni A, Fraser P, St George-Hyslop P (2000) Nicastrin modulates presenilin-mediated notch/glp-1 signal transduction and betaAPP processing. *Nature* **407**, 48-54.

[6] Gu Y, Chen F, Sanjo N, Kawarai T, Hasegawa H, Duthie M, Li W, Ruan X, Luthra A, Mount HT, Tandon A, Fraser PE, St George-Hyslop P (2002) APH-1 interacts with mature and immature forms of presenilins and nicastrin and may play a role in maturation of presenilin-nicastrin complexes. *J Biol Chem* **278**, 7374-7380.

[7] Araki W, Takahashi-Sasaki N, Chui DH, Saito S, Takeda K, Shirotani K, Takahashi K, Murayama KS, Kametani F, Shiraishi H, Komano H, Tabira T (2008) A family of membrane proteins associated with presenilin expression and gamma-secretase function. *FASEB J* **22**, 819-827.

[8] Chen F, Hasegawa H, Schmitt-Ulms G, Kawarai T, Bohm C, Katayama T, Gu Y, Sanjo N, Glista M, Rogaeva E, Wakutani Y, Pardossi-Piquard R, Ruan X, Tandon A, Checler F, Marambaud P, Hansen K, Westaway D, St George-Hyslop P, Fraser P (2006) TMP21 is a presenilin complex component that modulates gamma-secretase but not epsilon-secretase activity. *Nature* **440**, 1208-1212.

[9] Van Gassen G, Annaert W, Van Broeckhoven C (2000) Binding partners of Alzheimer's disease proteins: are they physiologically relevant? *Neurobiol Dis* **7**, 135-151.

[10] Giannakopoulos P, Bouras C, Kovari E, Shioi J, Tezapsidis N, Hof PR, Robakis NK (1997) Presenilin-1-immunoreactive neurons are preserved in late-onset Alzheimer's disease. *Am J Pathol* **150**, 429-436.

[11] Shen J, Bronson RT, Chen DF, Xia W, Selkoe DJ, Tonegawa S (1997) Skeletal and CNS defects in Presenilin-1-deficient mice. *Cell* **89**, 629-639.

[12] Nishimura M, Yu G, Levesque G, Zhang DM, Ruel L, Chen F, Milman P, Holmes E, Liang Y, Kawarai T, Jo E, Supala A, Rogaeva E, Xu DM, Janus C, Levesque L, Bi Q, Duthie M, Rozmahel R, Mattila K, Lannfelt L, Westaway D, Mount HT, Woodgett J, Fraser PE, St George-Hyslop P (1999) Presenilin mutations associated with Alzheimer disease cause defective intracellular trafficking of beta-catenin, a component of the presenilin protein complex. *Nat Med* **5**, 164-169.

[13] Efthimiopoulos S, Floor E, Georgakopoulos A, Shioi J, Cui W, Yasothornsrikul S, Hook VY, Wisniewski T, Buee L, Robakis NK (1998) Enrichment of presenilin 1 peptides in neuronal large dense-core and somatodendritic clathrin-coated vesicles. *J Neurochem* **71**, 2365-2372.

[14] Georgakopoulos A, Marambaud P, Efthimiopoulos S, Shioi J, Cui W, Li H-C, Schutte M, Gordon R, Holstein GR, Martinelli G, Mehta P, Friedrich VL, Jr., Robakis NK (1999) Presenilin-1 forms complexes with the cadherin/catenin cell-cell adhesion

system and is recruited to intercellular and synaptic contacts. *Mol Cell* **4**, 893-902.

[15] Zhang C, Wu B, Beglopoulos V, Wines-Samuelson M, Zhang D, Dragatsis I, Sudhof TC, Shen J (2009) Presenilins are essential for regulating neurotransmitter release. *Nature* **460**, 632-636.

[16] Tu H, Nelson O, Bezprozvanny A, Wang Z, Lee SF, Hao YH, Serneels L, De Strooper B, Yu G, Bezprozvanny I (2006) Presenilins form ER Ca2+ leak channels, a function disrupted by familial Alzheimer's disease-linked mutations. *Cell* **126**, 981-993.

[17] Bernardi L, Tomaino C, Anfossi M, Gallo M, Geracitano S, Costanzo A, Colao R, Puccio G, Frangipane F, Curcio SA, Mirabelli M, Smirne N, Iapaolo D, Maletta RG, Bruni AC (2008) Novel PSEN1 and PGRN mutations in early-onset familial frontotemporal dementia. *Neurobiol Aging* **30**, 1825-1833.

[18] Amtul Z, Lewis PA, Piper S, Crook R, Baker M, Findlay K, Singleton A, Hogg M, Younkin L, Younkin SG, Hardy J, Hutton M, Boeve BF, Tang-Wai D, Golde TE (2002) A presenilin 1 mutation associated with familial frontotemporal dementia inhibits gamma-secretase cleavage of APP and notch. *Neurobiol Dis* **9**, 269-273.

[19] Dermaut B, Kumar-Singh S, Engelborghs S, Theuns J, Rademakers R, Saerens J, Pickut BA, Peeters K, van den Broeck M, Vennekens K, Claes S, Cruts M, Cras P, Martin JJ, Van Broeckhoven C, De Deyn PP (2004) A novel presenilin 1 mutation associated with Pick's disease but not beta-amyloid plaques. *Ann Neurol* **55**, 617-626.

[20] Raux G, Gantier R, Martin C, Pothin Y, Brice A, Frebourg T, Campion D (2000) A novel presenilin 1 missense mutation (L153V) segregating with early-onset autosomal dominant Alzheimer's disease. *Hum Mutat* **16**, 95.

[21] Marcon G, Di Fede G, Giaccone G, Rossi G, Giovagnoli AR, Maccagnano E, Tagliavini F (2009) A novel Italian presenilin 2 gene mutation with prevalent behavioral phenotype. *J Alzheimers Dis* **16**, 509-511.

[22] Mendez MF, McMurtray A (2006) Frontotemporal dementia-like phenotypes associated with presenilin-1 mutations. *Am J Alzheimers Dis Other Demen* **21**, 281-286.

[23] Snider BJ, Norton J, Coats MA, Chakraverty S, Hou CE, Jervis R, Lendon CL, Goate AM, McKeel DW, Jr., Morris JC (2005) Novel presenilin 1 mutation (S170F) causing Alzheimer disease with Lewy bodies in the third decade of life. *Arch Neurol* **62**, 1821-1830.

[24] Ishikawa A, Piao YS, Miyashita A, Kuwano R, Onodera O, Ohtake H, Suzuki M, Nishizawa M, Takahashi H (2005) A mutant PSEN1 causes dementia with Lewy bodies and variant Alzheimer's disease. *Ann Neurol* **57**, 429-434.

[25] Houlden H, Crook R, Dolan RJ, McLaughlin J, Revesz T, Hardy J (2001) A novel presenilin mutation (M233V) causing very early onset Alzheimer's disease with Lewy bodies. *Neurosci Lett* **313**, 93-95.

[26] Miake H, Tsuchiya K, Nakamura A, Ikeda K, Levesque L, Fraser PE, St-George Hyslop PH, Mizusawa H, Uchihara T (1999) Glial expression of presenilin epitopes in human brain with cerebral infarction and in astrocytoma. *Acta Neuropathol* **98**, 337-340.

[27] Katayama T, Imaizumi K, Sato N, Miyoshi K, Kudo T, Hitomi J, Morihara T, Yoneda T, Gomi F, Mori Y, Nakano Y, Takeda J, Tsuda T, Itoyama Y, Murayama O, Takashima A, St George-Hyslop P, Takeda M, Tohyama M (1999) Presenilin-1 mutations downregulate the signalling pathway of the unfolded-protein response. *Nat Cell Biol* **1**, 479-485.

[28] Sato N, Urano F, Yoon Leem J, Kim SH, Li M, Donoviel D, Bernstein A, Lee AS, Ron D, Veselits ML, Sisodia SS, Thinakaran G (2000) Upregulation of BiP and CHOP by the unfolded-protein response is independent of presenilin expression. *Nat Cell Biol* **2**, 863-870.

[29] Berridge MJ, Lipp P, Bootman MD (2000) The versatility and universality of calcium signalling. *Nat Rev Mol Cell Biol* **1**, 11-21.

[30] Green KN, Demuro A, Akbari Y, Hitt BD, Smith IF, Parker I, LaFerla FM (2008) SERCA pump activity is physiologically regulated by presenilin and regulates amyloid beta production. *J Cell Biol* **181**, 1107-1116.

[31] Tsuneishi S, Sano K, Nakamura H (1993) Serum depletion increases the neurofilament protein mRNA levels in a neuroblastoma cell line, GOTO. *Brain Res Mol Brain Res* **17**, 119-128.

[32] Takeshita I, Sawa H, Nakamura T, Kuramitsu M, Kitamura K, Fukui M (1990) Contrary effect of lactic acid on expression of neuron-specific enolase and glial fibrillary acidic protein in human glioma cells. *Acta Neuropathol* (*Berl*) **79**, 506-512.

[33] Yu G, Chen F, Levesque G, Nishimura M, Zhang DM, Levesque L, Rogaeva E, Xu D, Liang Y, Duthie M, St George-Hyslop PH, Fraser PE (1998) The presenilin 1 protein is a component of a high molecular weight intracellular complex that contains beta-catenin. *J Biol Chem* **273**, 16470-16475.

[34] Ratovitski T, Slunt HH, Thinakaran G, Price DL, Sisodia SS, Borchelt DR (1997) Endoproteolytic processing and stabilization of wild-type and mutant presenilin. *J Biol Chem* **272**, 24536-24541.

[35] Counts SE, Lah JJ, Levey AI (2001) The regulation of presenilin-1 by nerve growth factor. *J Neurochem* **76**, 679-689.

[36] Park IH, Jung MW, Mori H, Mook-Jung I (2001) Zinc enhances synthesis of presenilin 1 in mouse primary cortical culture. *Biochem Biophys Res Comm* **285**, 680-688.

[37] Popescu BO, Cedazo-Minguez A, Popescu LM, Winblad B, Cowburn RF, Ankarcrona M (2001) Caspase cleavage of exon 9 deleted presenilin-1 is an early event in apoptosis induced by calcium ionophore A 23187 in SH-SY5Y neuroblastoma cells. *J Neurosci Res* **66**, 122-134.

[38] Pastorcic M, Das HK (2003) Ets transcription factors ER81 and Elk1 regulate the transcription of the human presenilin 1 gene promoter. *Brain Res Mol Brain Res* **113**, 57-66.

[39] Yu G, Chen F, Nishimura M, Steiner H, Tandon A, Kawarai T, Arawaka S, Supala A, Song YQ, Rogaeva E, Holmes E, Zhang DM, Milman P, Fraser PE, Haass C, George-Hyslop PS (2000) Mutation of conserved aspartates affects maturation of both aspartate mutant and endogenous presenilin 1 and presenilin 2 complexes. *J Biol Chem* **275**, 27348-27353.

[40] Luo WJ, Wang H, Li H, Kim BS, Shah S, Lee HJ, Thinakaran G, Kim TW, Yu G, Xu H (2003) PEN-2 and APH-1 coordinately regulate proteolytic processing of presenilin 1. *J Biol Chem* **278**, 7850-7854.

[41] Hu Y, Fortini ME (2003) Different cofactor activities in {gamma}-secretase assembly: evidence for a nicastrin-Aph-1 subcomplex. *J Cell Biol* **161**, 685-690.

[42] Mah AL, Perry G, Smith MA, Monteiro MJ (2000) Identification of ubiquilin, a novel presenilin interactor that increases presenilin protein accumulation. *J Cell Biol* **151**, 847-862.

[43] Baba-Aissa F, Raeymaekers L, Wuytack F, Dode L, Casteels R (1998) Distribution and isoform diversity of the organellar Ca2+ pumps in the brain. *Mol Chem Neuropathol* **33**, 199-208.

[44] Sergeev IN (2004) Calcium as a mediator of 1,25-dihydroxyvitamin D3-induced apoptosis. *J Steroid Biochem Mol Biol*

89–90, 419-425.

[45] Nakagawa T, Yuan J (2000) Cross-talk between two cysteine protease families. Activation of caspase-12 by calpain in apoptosis. *J Cell Biol* **150**, 887-894.

[46] Nakagawa T, Zhu H, Morishima N, Li E, Xu J, Yankner BA, Yuan J (2000) Caspase-12 mediates endoplasmic-reticulum-specific apoptosis and cytotoxicity by amyloid-beta. *Nature* **403**, 98-103.

[47] Etcheberrigaray R, Hirashima N, Nee L, Prince J, Govoni S, Racchi M, Tanzi RE, Alkon DL (1998) Calcium responses in fibroblasts from asymptomatic members of Alzheimer's disease families. *Neurobiol Dis* **5**, 37-45.

[48] Guo Q, Sebastian L, Sopher BL, Miller MW, Ware CB, Martin GM, Mattson MP (1999) Increased vulnerability of hippocampal neurons from presenilin-1 mutant knock-in mice to amyloid beta-peptide toxicity: central roles of superoxide production and caspase activation. *J Neurochem* **72**, 1019-1029.

[49] Leissring MA, Akbari Y, Fanger CM, Cahalan MD, Mattson MP, LaFerla FM (2000) Capacitative calcium entry deficits and elevated luminal calcium content in mutant presenilin-1 knockin mice. *J Cell Biol* **149**, 793-798.

[50] Amson R, Lassalle JM, Halley H, Prieur S, Lethrosne F, Roperch JP, Israeli D, Gendron MC, Duyckaerts C, Checler F, Dausset J, Cohen D, Oren M, Telerman A (2000) Behavioral alterations associated with apoptosis and down-regulation of presenilin 1 in the brains of p53-deficient mice. *Proc Natl Acad Sci U S A* **97**, 5346-5350.

[51] Tanimukai H, Imaizumi K, Kudo T, Katayama T, Tsuda M, Takagi T, Tohyama M, Takeda M (1998) Alzheimer-associated presenilin-1 gene is induced in gerbil hippocampus after transient ischemia. *Brain Res Mol Brain Res* **54**, 212-218.