Endoplasmic Reticulum Stress-induced Cysteine Protease Activation in Cortical Neurons

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Endoplasmic reticulum (ER) stress elicits protective responses of chaperone induction and translational suppression and, when unimpeded, leads to caspase-mediated apoptosis. Alzheimer’s disease-linked mutations in presenilin-1 (PS-1) reportedly impair ER stress-mediated protective responses and enhance vulnerability to degeneration. We used cleavage site-specific antibodies to characterize the cysteine protease activation responses of primary mouse cortical neurons to ER stress and evaluate the influence of a PS-1 knock-in mutation on these and other stress responses. Two different ER stressors lead to processing of the ER-resident protease procaspase-12, activation of calpain, caspase-3, and caspase-6, and degradation of ER and non-ER protein substrates. Immunocytochemical localization of activated caspase-3 and a cleaved substrate of caspase-6 confirms that caspase activation extends into the cytosol and nucleus. ER stress-induced proteolysis is unchanged in cortical neurons derived from the PS-1 P264L knock-in mouse. Furthermore, the PS-1 genotype does not influence stress-induced increases in chaperones Grp78/BiP and Grp94 or apoptotic neurodegeneration. A similar lack of effect of the PS-1 P264L mutation on the activation of caspasases and induction of chaperones is observed in fibroblasts. Finally, the PS-1 knock-in mutation does not alter activation of the protein kinase PKR-like ER kinase (PERK), a trigger for stress-induced translational suppression. These data demonstrate that ER stress in cortical neurons leads to activation of several cysteine proteases within diverse neuronal compartments and indicate that Alzheimer’s disease-linked PS-1 mutations do not invariably alter the proteolytic, chaperone induction, translational suppression, and apoptotic responses to ER stress.

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The endoplasmic reticulum (ER) is responsible for the synthesis, initial post-translational modification, and proper folding of proteins, as well as their sorting and export for delivery to appropriate cellular destinations. A variety of conditions, such as loss of the ER intraluminal oxidative environment or calcium content or the mutation or overexpression of relatively insoluble proteins, cause accumulation of misfolded proteins within the ER (1). Protein misfolding triggers three compensatory responses. One is the unfolded protein response (UPR), involving increased expression of molecular chaperones such as Grp94 and Grp78/BiP that promote proper protein folding (2), as well as SERCA2, an energy-dependent transporter for loading the ER lumen with calcium (3). A second response is the generalized suppression of translation mediated by the serine/threonine kinase PERK, which phosphorylates and inactivates the translation initiation factor eIF2α (4, 5). A third is ER-associated degradation, in which misfolded proteins are expelled from the ER and targeted for degradation by cytoplasmic proteasomes (6, 7). Although these three protective responses control transiently the accumulation of misfolded proteins within the ER, they can be overcome by sustained ER stress, which leads to apoptosis (8, 9). Molecular genetic and pharmacologic experiments have established that cells deficient in the UPR, ER-associated degradation or translational suppression responses are more vulnerable to ER stress-induced apoptosis (10–12), whereas increased expression of genes involved in the protective responses reduces apoptosis (13, 14).

Alzheimer’s disease (AD) is a slowly progressive cognitive and behavioral brain disorder characterized by neurodegeneration and abnormal accumulation of protein into neuritic plaques and neurofibrillary tangles. New insights into the pathogenesis of AD have been provided by the linkage of an inherited early onset form of AD to mutations in the presenilins (15, 16), two closely related genes (17) encoding polytopic integral membrane proteins that reside in the ER, intermediate compartment and Golgi (17–19). Several potential pathogenic mechanisms have been proposed for presenilin mutations, including impairments in ER stress signaling and increased sensitivity to stress-induced apoptosis. Evidence has been presented that mutant PS-1 enhances ER stress-induced apoptosis by reducing the UPR and PERK-mediated translational suppression not only in the context of overexpressing cell lines but also for primary mouse neurons bearing an AD-linked PS-1 knock-in mutation (13). Reduced responsiveness to ER stress may be particularly germane to AD, because the principal constituent of the insoluble neuritic plaques, the 42-residue amyloid Aβ protein, is synthesized partly in the ER and intermediate compartment (20, 21) and may progressively accumulate there (22, 23), where it can trigger ER stress responses and apoptosis (24). Nevertheless, the role of altered ER stress responsiveness as a neuropathogenic mechanism for presenilin...
mutations is uncertain. Another study failed to demonstrate an involvement of PS-1 or an AD-linked PS-1 mutant in protective responses to ER stress (25). Furthermore, signs of ER stress or aberrant apoptosis have not been reported so far for transgenic mouse lines expressing pathogenic presenilin mutations (25, 26). Finally, the influence of mutant PS-1 on ER stress-induced apoptotic signal transduction has not been investigated.

The signaling pathway that initiates ER stress-induced apoptosis has begun to be defined, and it involves activation of cytosine proteases distinct from those that trigger the widely studied mitochondrial and death receptor apoptotic pathways. ER stress-mediated apoptosis is dependent on an ER-resident cytosine protease, caspase-12 (24), for which activation may be regulated by processing of the pro-caspase-12 zymogen by the calcium-dependent cytosine protease calpain (27) and byzymogen clustering mediated by TRAF2 (28). There are conflicting reports on the role of mitochondrial release of cytochrome c in ER stress-induced apoptosis (29–31), and little is known about how caspase-12 activity leads to the execution of apoptosis. We describe here the further analysis of the cytosine protease activation responses to ER stress. The study has been carried out in primary cortical neurons and fibroblasts derived from mice, either wild type for PS-1 or homozygous for a pathogenic P264L knock-in mutation in PS-1 (32). After characterizing the activation of calpains and caspases in response to ER stress, we investigated the influences of the mutant PS-1 on proteolysis, apoptosis, and protective responses of UPR and PERK activation.

**EXPERIMENTAL PROCEDURES**

Antibodies and Other Materials—Dulbecco’s modified Eagle’s medium, Neurobasal, trypsin, and B27 supplement were from Life Technologies, Inc. Fetal calf serum was from Hyclone. Tunicamycin, thapsigargin, staurosporine, Hoechst 33342, and soybean trypsin inhibitor were from Sigma. Calpeptin was from Calbiochem. Ac-Asp-Glu-Val-Asp-aldehyde was from Enzyme Systems Products. Supplies for immunoblotting were from DuPont. Horseradish peroxidase-coupled secondary antibodies were from Santa Cruz Biotechnology. Biotinylated antibodies and avidin-biotin-peroxidase complex were from Vector. Anti-caspase-12 was a rat monoclonal antibody, generously provided by Drs. T. Nakagawa and J. Yuan (Boston). Anti-caspase-2 was a monoclonal antibody (clone 46) purchased from Transduction Laboratories. Anti-Grp94 and anti-Grp78/BiP were from Stresgen. Anti-cytochrome c was from Pharmingen and anti-actin (clone 4G5) from Roche Molecular Biochemicals. Anti-active caspase-3 was a rabbit anti-serum (Ab206) that reacts selectively with the p17 large subunit, but not with pro-caspase-3, and was prepared and characterized in this laboratory previously (32). Anti-calpain-cleaved a-spectrin (Ab38) reacts specifically with the ~150-kDa a-spectrin derivative formed by either calpain I or II but does not react with a-spectrin derivatives produced by caspases or other proteases, and it has been characterized extensively (32, 33). Anti-PERK was an affinity-purified rabbit anti-body generously provided by Dr. Takashi Kudo (Osaka, Japan). An antisera reactive specifically with the caspase-6-derived NH2-terminal fragment of lamin A (Ab255) was prepared as follows. The Hepa-1 cell line was transfected with a construct expressing human lamin A. The supernatant from a 10,000-g spin was counted as the crude post-mitochondrial supernatant. It was incubated 60 min at 37 °C and then stained with Hoechst 33342 at 37 °C for 15 min, rinsed, and examined under ultraviolet illumination. Images were captured using a Nikon Diaphot microscope and Spot CCD (charge-coupled device) camera. Quantitative analysis of neuronal death was conducted using neurons cultured for 14 days and trypsin blue staining as described previously (32). The number for each neuronal treatment was calculated as the mean of three randomly chosen fields and >300 cells were evaluated. The apoptotic index was calculated as the number of trypan blue-positive cells/total number of cells.

**Subcellular Fractionation**—Cultured neurons from two 100-mm dishes were washed twice in ice-cold 4% paraformaldehyde in phosphate-buffered saline, permeabilized with 0.05% Triton X-100, and stained with the biotin-avidin-peroxidase technique (33, 35). For detecting neuronal apoptosis, fixed cultures were permeabilized and were then stained with Hoechst 33342 at 37 °C for 15 min, rinsed, and examined under ultraviolet illumination. Images were captured using a Nikon Diaphot microscope and Spot CCD (charge-coupled device) camera. Quantitative analysis of neuronal death was conducted using neurons cultured for 14 days and trypsin blue staining as described previ-ously (32). The number for each neuronal treatment was calculated as the mean of three randomly chosen fields and >300 cells were evaluated. The apoptotic index was calculated as the number of trypan blue-positive cells/total number of cells.

**RT-PCR**—Levels of Grp78/BiP mRNA were determined by semi-quantitative RT-PCR analysis (25) of mRNA isolated from cultured mouse cortical neurons. Cultures were treated for 6 h with vehicle (0.1%...
Me₂SO), tunicamycin (0.5 μg/ml or 3 μg/ml), or thapsigargin (0.2 μM or 1 μM). Each 100-mm dish was washed twice in Dulbecco’s phosphate-buffered saline, and cells were scraped gently into the same buffer and collected by centrifugation. Total RNA was isolated using Triozol reagent (Life Technologies, Inc.). Aliquots of reverse-transcribed RNA were subjected to PCR using primer pairs specific for Grp78/BiP (5′-CTGGG-TACATTGGATCTGACTGGG-3′ and 5′-GCATCTGTGGCCCTCCAGC-CCATT-3′) and glyceraldehyde-3-phosphate dehydrogenase (5′-GATGACCATCAGAAAGTTGGAAG-3′ and 5′-GTAAGGAGAGATGCCTA- GTTG-3′). To establish a linear amplification range, equivalent aliquots were incubated in a PCR, and individual reactions were removed at 16, 18, 20, and 22 cycles. Based on the results, subsequent PCRs were incubated for 22 cycles. The PCR products were fractionated on agarose gels, stained, and quantified by fluorescent imaging (Typhoon 8600 Variable Mode Imager, Molecular Dynamics).

RESULTS

The blockade of N-linked glycosylation by tunicamycin leads to misfolding of newly synthesized proteins in the ER (2) and elicits the ER stress responses of chaperone induction (the UPR), transalational suppression, and caspase-mediated apoptosis in a variety of cultured cells, including neurons (8, 9). We examined the effects of tunicamycin on primary cortical neurons cultured from mice that were either wild type for PS-1 or homozygous for the AD-linked glycosylation by tunicamycin leads to misfolding of newly synthesized proteins in the ER (2) and elicits the ER stress responses of chaperone induction (the UPR), transalational suppression, and caspase-mediated apoptosis in a variety of cultured cells, including neurons (8, 9). We examined the effects of tunicamycin on primary cortical neurons cultured from mice that were either wild type for PS-1 or homozygous for the AD-linked point mutation PS-1P264L, which was introduced into the mouse genome by gene targeting (PS-1 knock-in (32)). The PS-1 P264L knock-in mutation is thought to be distinct from the “mitochondrial” and “death receptor” cascades triggered by caspase-9 and -8, respectively, and instead may be initiated by calpain (27), a family of calcium-dependent cysteine proteases (41). Subsequently, calpain reportedly cleaves and activates an ER-resident protease caspase-12 (24), triggering apoptosis through a poorly defined mechanism. We investigated this signaling pathway by subcellular fractionation and immunoblotting, and found that the ~60-kDa procaspase-12 was concentrated in the ER-enriched microsomal fraction (Fig. 2A). The fidelity of the fractions was confirmed by enrichment of cytochrome c in the mitochondrial fraction (Fig. 2A) and of calpain I in the cytosol fraction. To determine whether calpain can process procaspase-12, we incubated post-mitochondrial supernatants from mouse brain with calcium to activate the endogenous calpains I and II (36). Calpain activation diminished the level of procaspase-12 and produced a smaller ~35-kDa immunoreactive fragment (Fig. 2B), essentially identical in size to the calpain derivative of procaspase-12 reported previously (27). Calpain activation also caused degradation of α-spectrin, detected with a cleavage site-specific antibody reactive only with the calpain-derived NH₂-terminal ~150-kDa fragment. The loss of procaspase-12, appearance of the smaller derivative, and cleavage of α-spectrin were all blocked by calpeptin, a peptidyl aldehyde calpain in-

Presenilin Does Not Alter Stress-induced Proteolysis

FIG. 1. ER stress-induced apoptosis and caspase activation in primary mouse cortical neurons. Cortical neuron cultures were derived from mice either wild type for PS-1 or homozygous for a P264L knock-in mutation. Some cultures were treated for 24 h with tunicamycin, and then the neurons were either stained with Hoechst 33342 to visualize nuclear morphology (A) or immunostained with antibodies to active caspase-3 or -6, whereas few neurons under basal conditions exhibit chromatin condensation and fragmentation indicative of apoptosis, many neurons of both PS-1 genotypes underwent apoptosis during ER stress. A, immunoactivity for active caspase-3 was abundant in tunicamycin-treated PS-1 wild-type neurons, in which it filled the perikarya and extended into multiple neuritic processes. Immunoreactivity for caspase 6-cleaved lamin A also became prominent during tunicamycin treatment and was concentrated in neuronal nuclei.

 treatment but exhibited little neuronal labeling under control conditions (Fig. 1B). Active caspase-3 immunoreactivity extended throughout neuronal perikarya into multiple dendritic processes and their distal branches. Activation of caspase-6 was monitored using a cleavage site-specific antibody (Ab255) reactive with the NH₂-terminal fragment of lamin A, a nuclear matrix protein and preferential caspase-6 substrate that is cleaved during apoptosis (39, 40). Whereas little immunoreactivity for caspase-6-cleaved lamin A was observed in neurons under basal conditions, ER stress produced intense immunolabeling concentrated in neuronal nuclei. These data demonstrate that ER stress in cortical neurons causes the activation of at least two effector caspases and spreads from the ER to the cytosol and nucleus.

ER stress induces apoptosis through a signaling pathway thought to be distinct from the “mitochondrial” and “death receptor” cascades triggered by caspase-9 and -8, respectively, and instead may be initiated by calpain (27), a family of calcium-dependent cysteine proteases (41). Subsequently, calpain reportedly cleaves and activates an ER-resident protease caspase-12 (24), triggering apoptosis through a poorly defined mechanism. We investigated this signaling pathway by subcellular fractionation and immunoblotting, and found that the ~60-kDa procaspase-12 was concentrated in the ER-enriched microsomal fraction (Fig. 2A). The fidelity of the fractions was confirmed by enrichment of cytochrome c in the mitochondrial fraction (Fig. 2A) and of calpain I in the cytosol fraction. To determine whether calpain can process procaspase-12, we incubated post-mitochondrial supernatants from mouse brain with calcium to activate the endogenous calpains I and II (36). Calpain activation diminished the level of procaspase-12 and produced a smaller ~35-kDa immunoreactive fragment (Fig. 2B), essentially identical in size to the calpain derivative of procaspase-12 reported previously (27). Calpain activation also caused degradation of α-spectrin, detected with a cleavage site-specific antibody reactive only with the calpain-derived NH₂-terminal ~150-kDa fragment. The loss of procaspase-12, appearance of the smaller derivative, and cleavage of α-spectrin were all blocked by calpeptin, a peptidyl aldehyde calpain in-

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Concentrated in the mitochondrial/synaptosomal fraction (MITO). Calcium activation of calpain led to degradation of the calpain substrate processing of procaspase-12 and calpain-mediated cleavage of spectrin, detected by loss of the derivative (32, 33). In addition, ER stress caused activation of specific antibody reactive exclusively with this calpain/evidenced by the appearance of an immunoreactive fragment (arrow). These effects of calcium were reduced by the calpain inhibitor calpeptin (CALPEP) but not the caspase inhibitor Ac-Asp-Glu-Val-Asp-CHO (DEVD).

Immunoblotting was used to evaluate the processing of procaspase-12 and activation of calpain, caspase-3, and caspase-6 following ER stress to cortical neurons and to determine whether the PS-1P264L knock-in mutation alters apoptotic protease activity. In addition to tunicamycin, ER stress was induced by the sarcoplasmic-endoplasmic reticulum calcium ATPase inhibitor thapsigargin, which depletes intraluminal ER calcium and induces apoptosis (42). As shown in Fig. 3, tunicamycin and thapsigargin treatments for 24 h caused the processing of procaspase-12 and activation of calpain, caspase-3, and caspase-6. Although levels of the 60-kDa procaspase-12 were markedly reduced, smaller immunoreactive fragments could not be detected even with prolonged film exposures or varying durations of ER stress. This suggests that in cortical neurons, either processed caspase-12 is unstable or the proenzyme is processed to fragments undetectable by the antibody being used. Neuronal ER stress led to calpain activation and cytoskeletal protein degradation, manifested by appearance of an ~150-kDa NH₂-terminal fragment of the actin-binding protein α-spectrin and detected with a cleavage site-specific antibody, and to processing of procaspase-12 and formation of a smaller immunoreactive fragment (arrow). These effects of calcium were reduced by the calpain inhibitor calpeptin (CALPEP) but not the caspase inhibitor Ac-Asp-Glu-Val-Asp-CHO (DEVD).

The stress-induced activation of cysteine proteases from neurons bearing the PS-1P264L mutation was compared with that from neurons wild type for PS-1, and only minor differences were detectable (Fig. 3). The activation responses of calpain, caspase-3, and caspase-6 did not differ, and for the PS-1 mutant neurons only a very small enhancement in procaspase-12 processing was discernable at a submaximal dose of tunicamycin. In accordance with our previous report (32), there was no difference in staurosporine-induced cysteine protease activation between cortical neurons bearing the two PS-1 genotypes. Degenerated, apoptotic cortical neurons accumulated progressively during the 14-day culture period (34) and, consistent with the lack of significant effect of the PS-1 knock-in mutation on neuronal sensitivity to ER stress, there was only a minor, statistically insignificant difference between the two genotypes in the basal amount of apoptosis. Furthermore, the dose dependence for tunicamycin-induced apoptosis and its maximal extent were essentially unchanged (Table I). These data demonstrate that the PS-1 P264L knock-in mutation does not alter appreciably the sensitivity of cortical neurons to ER stress-induced proteolysis or apoptosis.

Pathogenic presenilin mutations reportedly impair the unfolded protein response to ER stress (13), although this effect has not been observed in all studies (25). To examine the influence of the PS-1P264L knock-in mutation on the UPR in cortical neurons, we measured by immunoblotting the levels of the inductive chaperones Grp94 and Grp78/BiP. Basal levels of Grp94 and Grp78/BiP were similar in neurons that are wild type for PS-1 and those expressing the P264L knock-in mutation. Treatment with tunicamycin for 24 h increased steady-state levels of Grp94 and Grp78/BiP, whereas thapsigargin had a more modest effect on levels of the chaperones, and no increase could be discerned with staurosporine treatment (Fig. 4A). The PS-1 knock-in mutation did not change either the basal levels of these ER chaperones or their increase evoked by tunicamycin or thapsigargin. When chaperone levels were normalized to those of actin for three independent experiments, tunicamycin increased Grp94 levels by 38 (wild type) and 35% (P264L knock-in) and elevated Grp78/BiP levels by 50 (wild type) and 54% (P264L knock-in). To investigate further the effect of mutant PS-1 on the UPR in cortical neurons, RT-PCR was used to evaluate basal and ER stress-induced levels of...
The results presented thus far demonstrate that for primary mouse cortical neurons, ER stress responses of cysteine protease activation, chaperone induction, and apoptosis are not altered significantly by an Alzheimer’s disease-linked PS-1 P264L knock-in mutation. To determine whether the lack of effect of the mutant PS-1 on ER stress responses is a peculiarity of cortical neurons, we examined embryonic fibroblasts derived from mice of the two PS-1 genotypes. Procaspase-12 and -3 were analyzed following treatment with tunicamycin, thapsigargin, or staurosporine. As shown in Fig. 5, the two ER stressors and staurosporine led to processing of procaspase-12 and -3 in fibroblasts. Activation of these proteases was virtually indistinguishable based on the PS-1 genotype. The basal levels of procaspase-12 and procaspase-3 also were not altered detectably by the PS-1 P264L knock-in mutation. Similarly, the ER stressors activated caspase 6-mediated lamin A degradation that did not differ based on PS-1 genotype.2 Next, the effect of PS-1 P264L on the UPR was evaluated by measuring the levels of Grp94 and Grp78/BiP. Basal levels of the two ER chaperones did not differ as a function of PS-1 genotype. Treatment with tunicamycin and, to a lesser extent, thapsigargin increased levels of the two ER chaperones, and there was no discernible reduction in chaperone levels from PS-1 mutant fibroblasts (Fig. 5). Quantitative analysis from four independent experiments confirmed that the tunicamycin-induced increases in Grp94 and Grp78/BiP were unaltered by the mutant PS-1 (38 and 35% increases, respectively, for PS-1 wild-type cells; 46 and 62% increases, respectively, for PS-1 mutant knock-in fibroblasts). Finally, the influence of PS-1 P264L on the translational suppression response to ER stress was assessed in fibroblasts. Stress-induced activation of the protein kinase PERK stimulates phosphorylation and inactivation of the translation initiation factor eIF2α and can be evaluated by the activation-induced autophosphorylation of PERK and its mobility shift upon SDS-PAGE (5, 25, 44). As shown in the immunoblot of Fig. 6, tunicamycin caused a time-dependent decrease in PERK mobility, indicative of activation-induced autophosphorylation. PERK activation was essentially complete by 7 h. Neither the rate nor the maximal amount of PERK activation was changed in PS-1 P264L mutant fibroblasts. Procaspase-12 processing was not initiated until after the peak of PERK autophosphorylation and did not differ in time course or magnitude between the two PS-1 genotypes.
Alzheimer’s disease-causing mutations in the presenilins reportedly increase the vulnerability of cultured cells to ER stress-induced apoptosis (13, 45), an endangering mechanism of potential relevance to the extensive aging-related neocortical neurodegeneration of AD. This effect is manifested by impaired cellular protective responses to ER stress, including induction of chaperones such as Grp94 and Grp78/BiP and translational suppression initiated by activation of the protein kinase PERK, as well as by increased stress-induced cell death. To investigate further the influence of mutant presenilin on ER stress responsiveness in cortical neurons, we first characterized the activation of cysteine proteases of the caspase and calpain families following ER stress to primary mouse cortical neurons and then compared stress-induced proteolysis between cells derived from PS-1 wild-type and homozygous PS-1 P264L knock-in mice. Our results provide evidence that in cortical neurons, ER stress leads to activation of proteases involved in both the initiation and execution of apoptosis, not only within the ER but also in other neuronal compartments, and leads to the degradation of ER, cytoskeletal, and nuclear protein substrates. The homozygous AD-linked PS-1 P264L mutation does not alter appreciably the ER stress-stimulated activation responses of calpain and caspase-3, -6, or -12 or influence significantly the Grp94 or Grp78/BiP induction, PERK activation, and apoptosis. The lack of effect of mutant PS-1 is observed with either of two ER stressors and in two different cell types. These findings are similar to the lack of effect of presenilin deletion or mutation on UPR and translational suppression observed for several cell lines in another study (25), extending them to primary cortical neurons and to the proteolytic signaling that mediates ER stress-induced apoptosis. Therefore, although certain presenilin mutants impair ER stress responses and enhance cell death, this effect does not occur for neurons expressing all AD-linked PS-1 mutations and so is unlikely to be a critical pathogenic mechanism by which presenilin mutations cause early onset AD.

Our study used the processing of procaspase-12 as a biochemical marker for activation of the ER apoptotic pathway. ER stress-induced apoptosis is dependent on this ER-resident protease (24, 27), activation of which likely involves proteolytic processing and oligomeric assembly in a manner similar to all other caspases (46). We confirmed that procaspase-12 is enriched in ER-containing microsomal fractions from the brain and is processed by the family of cytosolic calcium-dependent cysteine proteases, calpain (27). Furthermore, by using a cleavage site-specific antibody that reacts exclusively with a fragment of the cytoskeletal protein spectrin generated by calpain (32, 33), we provide evidence for activation of calpain and degradation of a cytoskeletal calpain substrate coincident with processing of procaspase-12 in response to cortical neuronal ER stress. These results support the concept that a rise in cytosolic free calcium concentration and activation of calpain trigger caspase-12 activation and ER apoptotic signaling, although they do not exclude the possibility that other proteases may be involved in activation of caspase-12. Neither calpain-mediated spectrin degradation nor procaspase-12 processing is altered appreciably in cortical neurons by the homozygous PS-1 P264L knock-in mutation, whether the stressor is tunicamycin, which impairs protein folding by preventing N-linked glycosylation, or thapsigargin, which interferes with protein folding by depleting ER calcium stores. This result is somewhat unexpected, given the evidence that mutant presenilins interfere with intracellular calcium homeostasis by enhancing stimulus-induced release of calcium from ER stores (45, 47), attenuating capacitative calcium entry (48), and elevating ER calcium stores (49). Any mutation-induced increase in calpain activation could have been readily detected, because the method has demonstrated already that necrotic stimuli evoke much stronger calpain activation than do apoptotic agents (32). The lack of effect of mutant PS-1 on calpain activation reported here may be attributable to differences between studies in presenilin mutation or levels of mutant PS-1 expression. Another possibility stems from the finding that stimulus-induced calpain activation depends not only on the magnitude of a calcium rise but also its source (50). Further study will be required to identify definitively the calcium pools and mechanisms contributing to ER stress-induced calpain activation. Nevertheless, the lack of effect of PS-1 P264L on activation of calpain and caspase-12 by either tunicamycin or thapsigargin indicates that an AD-linked mutant presenilin does not influence the initial triggering mechanism for ER stress-induced apoptotic signaling. This conclusion is substantiated by the lack of effect of the PS-1 knock-in mutation on procaspase-12 processing in a second cell type, the fibroblast, as well as on tunicamycin-induced neuronal apoptosis.

ER stress in cortical neurons not only initiates apoptotic signaling via processing of procaspase-12 within the ER but also activates cytosolic and nuclear caspases involved in the execution of apoptosis, all in a manner that is not altered by the pathogenic PS-1 P264L knock-in mutation. Immunocytochemistry with cleavage site-specific antibodies localize activated caspase-3 to the cytosol and caspase-6-cleaved lamin A to the nucleus of primary cortical neurons following ER stress (Fig. 1). The activation of caspase-3 and -6 is verified by immunoblot detection of the stress-induced loss of procaspase-3 and appearance of the p17 large subunit as well as the ~27-kDa NH2-terminal fragment of lamin A (Fig. 3). Caspase-3 and -6 are effector caspases characterized by their requirement for processing-induced activation by initiator caspases, abundance of cytosolic, membrane, cytoskeletal, and nuclear protein substrates, and prominent roles in morphological changes that characterize the execution phase of apoptosis (51, 52). There are several pathways by which ER stress could activate caspase-3 and -6. One possible route is through the well described release of mitochondrial cytochrome c, formation of the apoptosome, and activation of caspase-9, which in turn processes and activates downstream caspases such as -3 and -6. There are conflicting reports on activation of the mitochondrial pathway in response to ER stress (29–31), and should mitochondria be involved, the signals linking ER stress to cytochrome c release remain to be identified. An alternative possibility is the direct activation of caspase-3 and -6 by either caspase-12 or calpain. The former route has not been reported...
thus far, and the effect of calpain on caspase-3 is complex, leading either to processing of the prodomain, which facilitates activation of the caspase (53), or alternatively to inhibition of procaspace processing and caspase-3 activation (54). Currently, we are examining the pathway leading from ER stress to activation of executioner caspases. Whatever the mechanism, neither the loss of procaspace-3 nor the appearance of the activated p17 subunit and the caspase 6-cleaved lamin A is modified in cortical neurons or fibroblasts carrying the PS-1 P264L mutation. When coupled with the lack of effect of PS-1 P264L on the activation responses of calpain or caspase-12 or the dose-dependence for tunicamycin-induced apoptosis, these results indicate that several biochemical and morphological indices of stress-induced cortical neuronal apoptosis are not altered by this pathogenic presenilin mutation.

In addition to unaltered apoptotic protease signaling, cells bearing PS-1 P264L exhibit no appreciable impairment in their UPR or translational suppression response to ER stress. Protein levels for Grp94 and Grp78/BiP exhibit small but significant increases in cortical neurons treated with tunicamycin, effects that are not influenced by PS-1 P264L (Fig. 4). Additionally, Grp78/BiP mRNA levels were analyzed as a robust measure of the UPR, and for primary cortical neurons neither the basal mRNA levels nor their induction by tunicamycin or the dose of the UPR, and for primary cortical neurons neither the basal mRNA levels nor their induction by tunicamycin or the dose-dependence for tunicamycin-induced apoptosis, these results indicate that several biochemical and morphological indices of stress-induced cortical neuronal apoptosis are not altered by this pathogenic presenilin mutation.

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