

# Activation of PERK Signaling Attenuates A $\beta$ -Mediated ER Stress

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## Abstract

Alzheimer's disease (AD) is characterized by the deposition of aggregated beta-amyloid (A $\beta$ ), which triggers a cellular stress response called the unfolded protein response (UPR). The UPR signaling pathway is a cellular defense system for dealing with the accumulation of misfolded proteins but switches to apoptosis when endoplasmic reticulum (ER) stress is prolonged. ER stress is involved in neurodegenerative diseases including AD, but the molecular mechanisms of ER stress-mediated A $\beta$  neurotoxicity still remain unknown. Here, we show that treatment of A $\beta$  triggers the UPR in the SK-N-SH human neuroblastoma cells. A $\beta$  mediated UPR pathway accompanies the activation of protective pathways such as Grp78/Bip and PERK-eIF2 $\alpha$  pathway, as well as the apoptotic pathways of the UPR such as CHOP and caspase-4. Knockdown of PERK enhances A $\beta$  neurotoxicity through reducing the activation of eIF2 $\alpha$  and Grp8/Bip in neurons. Salubrinal, an activator of the eIF2 $\alpha$  pathway, significantly increased the Grp78/Bip ER chaperone resulted in attenuating caspase-4 dependent apoptosis in A $\beta$  treated neurons. These results indicate that PERK-eIF2 $\alpha$  pathway is a potential target for therapeutic applications in neurodegenerative diseases including AD.

**Citation:** Lee DY, Lee K-S, Lee HJ, Kim DH, Noh YH, et al. (2010) Activation of PERK Signaling Attenuates A $\beta$ -Mediated ER Stress. PLoS ONE 5(5): e10489. doi:10.1371/journal.pone.0010489

**Editor:** Mel B. Feany, Brigham and Women's Hospital, Harvard Medical School, United States of America

**Received:** January 25, 2010; **Accepted:** April 5, 2010; **Published:** May 5, 2010

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**Funding:** This work was supported by a grant from the BioGreen 21 Program (No. 20100301061075), Rural Development Administration, Republic of Korea. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

**Competing Interests:** The authors have declared that no competing interests exist.

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## Introduction

Alzheimer's disease (AD), the most common form of dementia, is a chronic neurodegenerative disease causing progressive impairment of memory and other cognitive functions. Neuritic plaques, neurofibrillary tangles, and neuronal loss represent the main pathological characters in AD brains. Amyloid  $\beta$ -protein (A $\beta$ ), the central component of senile plaques, is produced from sequential proteolytic cleavages of the type 1 transmembrane  $\beta$ -amyloid precursor protein (APP) by  $\beta$ - and  $\gamma$ -secretase [1,2]. Aggregated A $\beta$  has been shown to interfere with several cellular processes and results the endoplasmic reticulum (ER) stress. ER stress triggers a cellular stress response called the unfolded protein response (UPR) intended to protect the cell against the toxic aggregated proteins [3].

The UPR is initiated by the binding of the ER chaperone GRP78/BiP to the misfolded proteins. Under normal conditions, GRP78/Bip sequester three key signal transducers at the ER membrane by forming the inactive complex; double-stranded RNA-activated protein kinase-like ER kinase (PERK), transcription factor ATF-6, and endoribonuclease IRE-1 [4,5,6]. Although the activation mechanisms of these ER-stress sensors are not fully

understood, dissociation from GRP78/Bip seems to be required for the activation of three key signal transducers. One probable hypothesis is that the accumulating unfolded-protein preferentially binds GRP/Bip, which dissociates from PERK, ATF-6, and IRE-1. GRP78/Bip dissociation leads to autophosphorylation of PERK and IRE-1, and mobilization of ATF-6 to the Golgi for activation [7]. The activation of the UPR results in an overall decrease in translation, increased protein degradation and increased levels of ER chaperones, including GRP78/Bip [8], which consequently increases the protein folding capacity of the ER. Eventually, the cell might return to normal ER homeostasis or, under prolonged ER stress, continue towards apoptosis. As neurons are highly susceptible to the toxic effects of aggregated A $\beta$  of AD, ER-stress-mediated cell death might have an important role in the pathogenesis of this disease [5]. Recently, several reports showed that the activation of UPR in neurons of AD brain [3] and oligomeric A $\beta$  aggregates of A $\beta$ 1-42 peptide induce mild ER stress in neuronal cells [9]. Recent studies have demonstrated that activation of the UPR is a one of representative marker in both brain aging and age-related diseases of the brain. For example, the activation of the PERK pathway has been reported in the aged rodent models [10,11,12]. Similarly, studies have demonstrated in

neurons of Alzheimer's disease [3] and in models of Parkinson's disease [13] that there is evidence for activation of the PERK pathway. Interestingly, phosphor-PERK stained neurons were overlapped with tau positive neurons [3,14]. These data suggest that the PERK pathway is participated in the pathogenesis of aged related neurodegenerative diseases.

Activated PERK phosphorylates eukaryotic translation initiation factor 2 subunit  $\alpha$  (eIF2 $\alpha$ ). After stress-induced phosphorylation of eIF2 $\alpha$ , global protein translation of normal cellular mRNAs is repressed [15]. In parallel, translational initiation of transcription factor ATF4 is selectively stimulated. ATF4 induces the expression of downstream target genes such as GADD34, CHOP/GADD153 and others, which participate in the control of cellular redox status and cell death [12]. Importantly, the protein phosphatase-1 (PP1) complex is inhibited by small molecule drug Salubrinal (Sal), which selectively blocks dephosphorylation of phospho-eIF2 $\alpha$  [16]. Maintaining levels of p-eIF2 $\alpha$  by Sal enhances cell survival in various cell lines against apoptosis induced by the ER stressors [8,16].

However, the role of the UPR pathway, e.g. PERK signaling pathway, has not been elucidated in ER stress mediated A $\beta$  neurotoxicity. Here, we demonstrate that the selective activation of PERK pathway is an early event of A $\beta$  induced ER stress. PERK-eIF2 $\alpha$  pathway promotes the induction of ER chaperones and confers resistant to aggregated protein toxicity in neuronal cells.

## Results

### A $\beta$ activates UPR in SK-N-SH cells

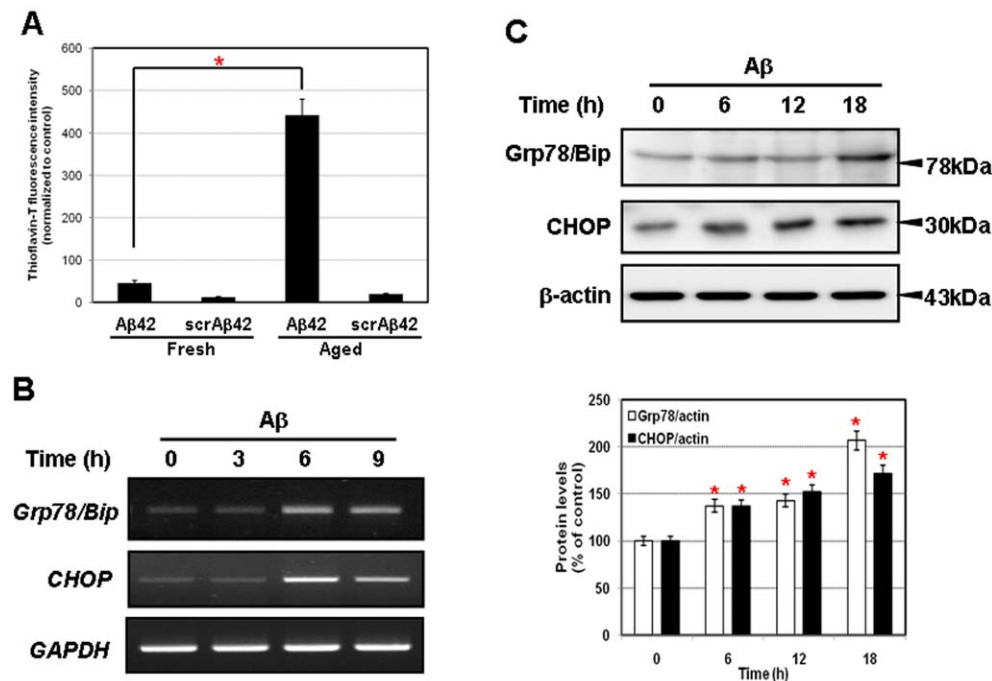
Characterization of the aggregation status of A $\beta$ 42 is one of the critical issues in understanding the role of A $\beta$  in the Alzheimer's

disease. When acting on neuronal cells, whether it is the fibrillar or the non-fibrillar peptides shows different effect in neurotoxicity. Reports from in vitro toxicity studies have suggested that aggregated A $\beta$  is more toxic agent than soluble A $\beta$  in cultured neurons [1,17,18]. In this study, we analyzed the effect of aggregation status of A $\beta$ 42 on UPR in neuronal cell. For that purpose, we prepared fresh and aged A $\beta$  peptide solutions and their aggregation status were characterized by thioflavin-T (ThT) fluorescence. As shown in Fig. 1A, fluorescence intensity of aged A $\beta$ 42, prepared after incubation of the peptide solution for 7 days at 4°C, was significantly higher in comparing with fresh A $\beta$ 42. In contrast, the scrambled A $\beta$ 42 (scrA $\beta$ 42) did not lead to any significant increase in the Th-T fluorescence levels (Fig. 1A).

To test whether UPR is activated in SK-N-SH human cholinergic neuroblastoma cells by aged A $\beta$ 42, we analyzed the levels of two known ER stress markers, Grp78/BiP and CHOP [19], using semi-quantitative RT-PCR and Western blot analysis. As shown in Fig. 1, Grp78/BiP and CHOP mRNA expression significantly up-regulated by aged 10  $\mu$ M A $\beta$ 42 treatment from 6 h (Fig. 1B), consistent changes of protein levels were observed (Fig. 1C). On the contrary, the expression of Grp78/BiP and CHOP did not change by treatment of fresh A $\beta$ 42 peptide and the scrA $\beta$ 42 peptide. (Figure S1A). These results demonstrate that the aggregation status and the sequence of amino acids of A $\beta$  peptide are critical for the activation of UPR in neuronal cells.

### A $\beta$ preferentially induces PERK-eIF2 $\alpha$ pathway

To determine the activation status of three major ER stress sensors, PERK, IRE1 $\alpha$ , and ATF6 $\alpha$ , we performed the Western blot analysis with the antibodies against the phosphor-PERK,



**Figure 1. A $\beta$ 1-42 induces ER stress in SK-N-SH cells.** **A**, Relative aggregation state of A $\beta$  peptides were measured by thioflavin-T fluorometric assay in a cell-free system. Increased thioflavin-T fluorescence in aged A $\beta$ 42 peptide revealed greater  $\beta$ -sheet content in comparison with the fresh A $\beta$ 42 peptide but not in both fresh and aged of scrambled A $\beta$ 42 peptides (scrA $\beta$ 42). Thioflavin-T fluorescence was monitored at 450 nm excitation and 482 nm emission. The expression levels of mRNA (**B**) and protein (**C**) of human Grp78/Bip, CHOP were increased in SK-N-SH cells treated with aged A $\beta$  time dependent manner. GAPDH and  $\beta$ -actin were used as the loading controls. Data were presented as means  $\pm$  SD from at least three independent experiments. \* $P$ <0.001. doi:10.1371/journal.pone.0010489.g001

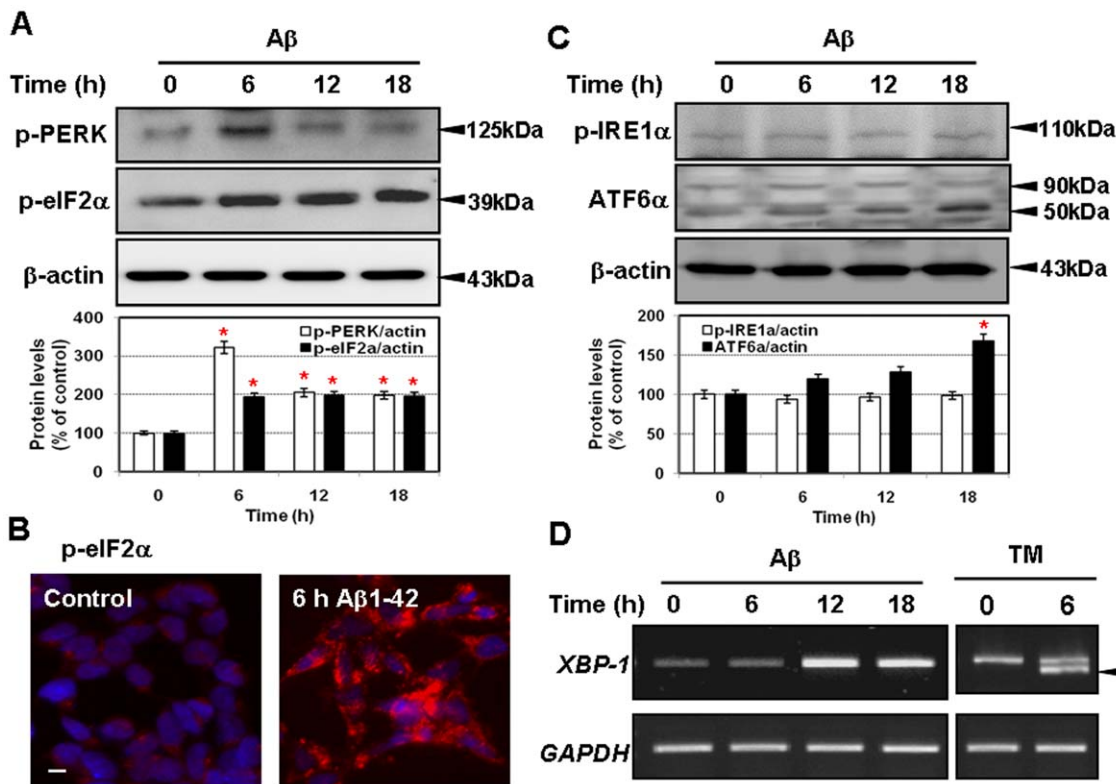
phosphor-IRE1 $\alpha$  and ATF-6 in A $\beta$ -treated neuronal cells. Interestingly, the levels of p-PERK and p-eIF2 $\alpha$  in neurons were significantly increased after 6 h by A $\beta$  treatment (Fig. 2A). In contrast, the level of p-PERK and p-eIF2 $\alpha$  did not change by fresh A $\beta$ 42 peptide and the scrA $\beta$ 42 peptide. (Figure S1B).

The activation of p-eIF2 $\alpha$  was also detected in the immunostaining analysis. Staining intensity of cytoplasmic p-eIF2 $\alpha$  level was significantly increased by A $\beta$  treatment (Fig. 2B). These data showed that PERK-eIF2 $\alpha$  pathway was definitely induced by A $\beta$  in neuronal cells. In contrast, the activities of the other two UPR sensors, IRE1 $\alpha$  and ATF6, did not change in A $\beta$  treated neuronal cells within 12 h (Fig. 2C). After 18 h, cleavage form of ATF6 $\alpha$  was slightly increased by A $\beta$  treatment but its induction was not as prominent as that of PERK-eIF2 $\alpha$  (Fig. 2C). These results indicate that A $\beta$  preferentially induces PERK-eIF2 $\alpha$  pathway. The expression level of XBP-1 mRNA was increased by A $\beta$  treatment but, unconventional splicing of XBP-1 mRNA, which is mediated by the endonuclease activity of IRE1 $\alpha$ , was not detected in A $\beta$  treated neuronal cell (Fig. 2D). These results indicate that the induction of ER stress by A $\beta$  is not mediated via the IRE1-XBP1 pathway at least by 18 h after A $\beta$  treatment. In summary, the activation of UPR in A $\beta$  treated neuronal cells was detected as phosphorylation of PERK, p-eIF2 $\alpha$ , and cleavage of ATF6. However, short-term treatment of A $\beta$  (within 6 h) selectively augmented activation of the PERK pathway in neurons.

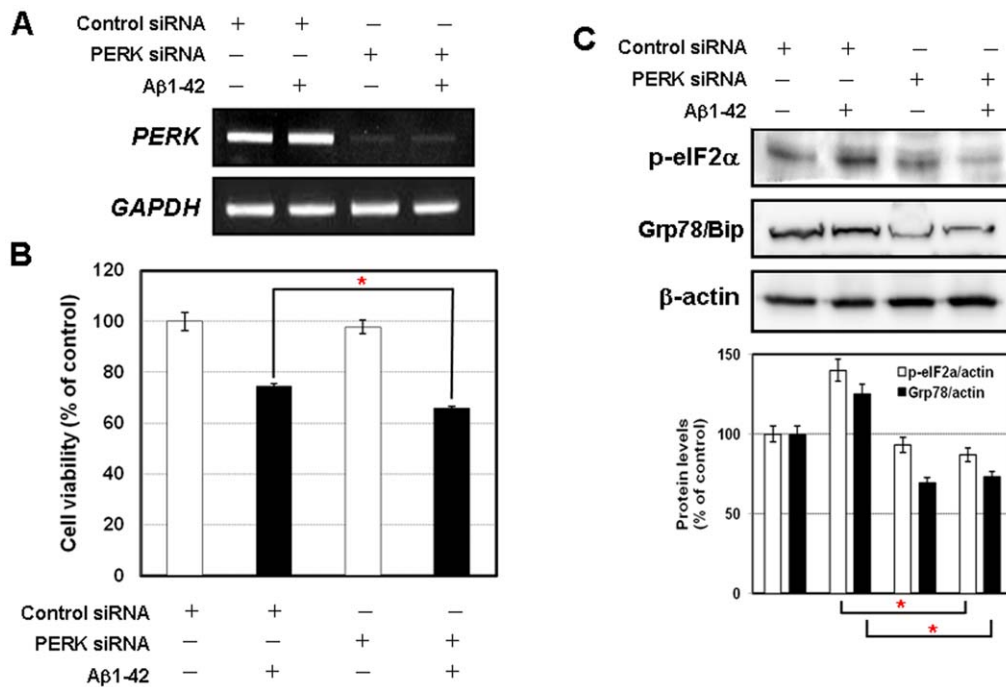
### Effects of PERK knockdown on A $\beta$ -induced neuronal cell death

To elucidate the role of PERK-eIF2 $\alpha$  pathway in ER stress-mediated neuronal cell death by A $\beta$  treatment, we knocked down expression of PERK by using siRNA against PERK. Transfection of PERK siRNA, but not control siRNA significantly reduced endogenous PERK mRNA levels (Fig. 3A). We then have assessed the role of PERK on ER stress mediated A $\beta$  neurotoxicity. When treated with A $\beta$  in SK-N-SH cells, silencing of PERK showed slightly enhanced cell death in comparison with those transfected with the control siRNA (Fig. 3B). These results indicate that PERK may play a role in cell survival mechanism rather than apoptosis on ER stress mediated A $\beta$  neurotoxicity.

Next, we further tested whether knockdown of PERK abolishes eIF2 $\alpha$  phosphorylation induced by A $\beta$ . As expected, A $\beta$ -induced eIF2 $\alpha$  phosphorylation was significantly reduced in PERK knockdown cells (Fig. 3C). Grp78/Bip, ER-resident chaperone protein, is crucial for the modulation of UPR pathway under ER stress condition and functions as a cytoprotective protein in stressed cells [5]. Also, we tested the effect of siRNA silencing of PERK on the levels of Grp78/Bip. PERK knockdown significantly repressed the levels of Grp78/Bip induced by A $\beta$  (Fig. 3C). These data indicate that PERK participates in the activation of p-eIF2 $\alpha$  and Grp78/Bip in A $\beta$ -mediated ER stress response in neuronal cells.



**Figure 2. A $\beta$ 1-42 induces activation of PERK and eIF2 $\alpha$  in SK-N-SH cells.** **A**, Western blotting with anti-p-PERK (top) and anti-p-eIF2 $\alpha$  (middle) in SK-N-SH cells treated with A $\beta$  show the activation of PERK and eIF2 $\alpha$  from 6 h. **B**, Immunostaining of p-eIF2 $\alpha$  after 6 h A $\beta$  treatment show the increased staining in cytoplasm. **C**, Western blotting with anti-p-IRE1 $\alpha$  and anti-ATF-6 in SK-N-SH cells treated with A $\beta$ . **D**, Unconventional splicing of the *XBP1* mRNA was not detected after A $\beta$  treatment whereas Tunicamycin (TM; 2  $\mu$ g/ml) treatment used as the positive control generated the spliced form of the *XBP1* mRNA. GAPDH and  $\beta$ -actin were used as the loading controls. Scale bar: 10  $\mu$ m. \* $P$ <0.05. doi:10.1371/journal.pone.0010489.g002



**Figure 3. PERK siRNA reduces the neuronal cell viability by A $\beta$  treatment.** **A**, PERK siRNA transfection was confirmed by the PERK RT-PCR analysis. **B**, The cell viability in PERK siRNA transfection with A $\beta$  treatment was reduced compared with A $\beta$  treatment alone. Data were presented as means  $\pm$  SD from at least three independent experiments. **C**, PERK siRNA transfection with A $\beta$  treatment suppressed the activated eIF2 $\alpha$  and Grp78/Bip by A $\beta$  treatment alone. \* $P$ <0.05, control siRNA + A $\beta$  versus PERK siRNA + A $\beta$ . doi:10.1371/journal.pone.0010489.g003

### Effects of Salubrinal, a selective activator of eIF2 $\alpha$ , on A $\beta$ -induced neuronal cell death

Salubrinal (Sal), a small molecule that protects cells from ER stress induced apoptosis by selectively activating an eIF2 $\alpha$  branch of the UPR pathway [16]. When cells are challenged with ER stress, phosphorylated eIF2 $\alpha$  is increased which mediates both a transient decrease in global translation and the translational up-regulation of selected stress-induced mRNAs. Phospho-eIF2 $\alpha$  (p-eIF2 $\alpha$ ) is dephosphorylated by protein phosphatase-1 (PP1) complex. Importantly, the PP1 complex is inhibited by Sal, which selectively blocks dephosphorylation of p-eIF2 $\alpha$  but not other PP1 substrates [16].

To investigate whether Sal has the ability to prevent neuronal apoptosis induced by A $\beta$ , we treated various concentration of Sal for 2 h before A $\beta$  treatment and assessed cell viability using alamarBlue assay. While cell viability was decreased by treatment of A $\beta$ , pre-treatment with Sal significantly attenuated A $\beta$ -induced neuronal cell death from 25  $\mu$ M. Pre-treatment with 100  $\mu$ M Sal reduced A $\beta$ -induced neuronal cell death by 36.3 $\pm$ 2.8% (Fig. 4A and Figure S2). In addition, A $\beta$ -mediated cell death was significantly reduced by pre-treatment with 100  $\mu$ M Sal compared to A $\beta$  treatment alone from 24 h (Fig. 4B).

In humans, caspase-4, which was identified as the homologous gene to mouse caspase-12, has been shown to be specifically activated in ER stress-induced apoptosis and A $\beta$ -induced neuronal cell death [20]. To determine whether A $\beta$ -induced neuronal cell death required activation of apoptotic proteases, we measured the activities of caspase-4 and caspase-3. A $\beta$  treatment increased caspase-4 activity by 1.4-fold, whereas pre-treatment with Sal reduced the caspase-4 activity to half of those in cells treated with A $\beta$  only (Fig. 4C). This protective effect on neuronal cell death was also detected in the activity of caspase-3

(Fig. 4D) and the apoptotic morphological changes of nuclei (Figure S3).

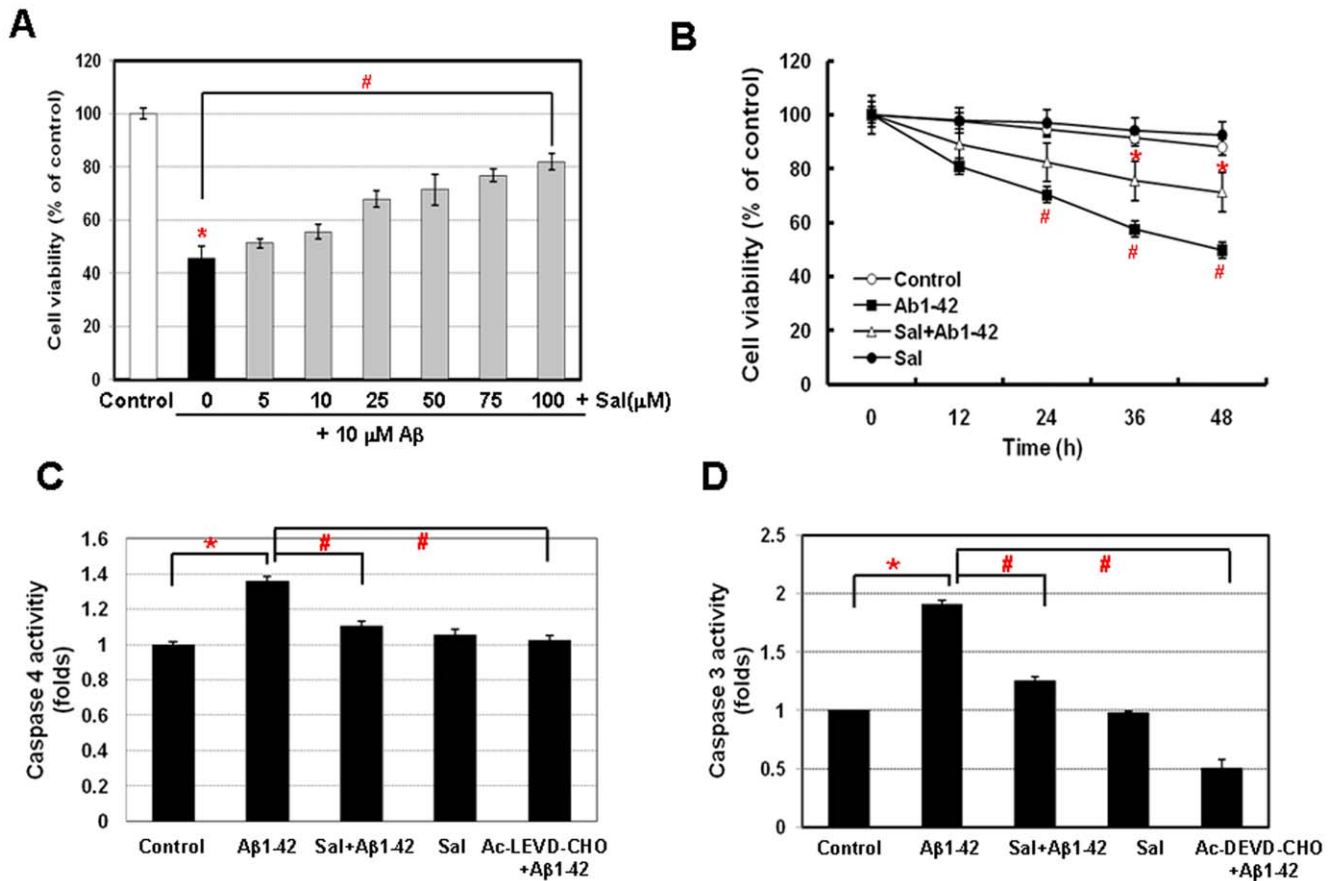
### Effects of Salubrinal on UPR modulator Grp78/Bip

Since PERK-eIF2 $\alpha$  pathway may play a crucial role in cell survival rather than apoptosis in A $\beta$ -induced neuronal cell death, we examined whether Sal affects on the induction of p-eIF2 $\alpha$  and Grp78/Bip in SK-N-SH cells exposed to A $\beta$  and/or Sal. As shown in Fig. 5, in the presence of Sal, A $\beta$  increased eIF2 $\alpha$  phosphorylation from 3 h, whereas A $\beta$  only increased eIF2 $\alpha$  phosphorylation after 6 h. In the absence of Sal, the level of Grp78/Bip expression was not changed by 6 h after A $\beta$  treatment. In contrast, pre-treatment of Sal caused the highest induction of Grp78/Bip compared to A $\beta$  alone. Taken together, these findings suggest that Sal, the selective activator of eIF2 $\alpha$ , enhances Grp78/Bip expression in neuronal cells. Up-regulation of Grp78/Bip dependent PERK-eIF2 $\alpha$  pathway seems to be a neuroprotective role against A $\beta$ -induced neurotoxicity.

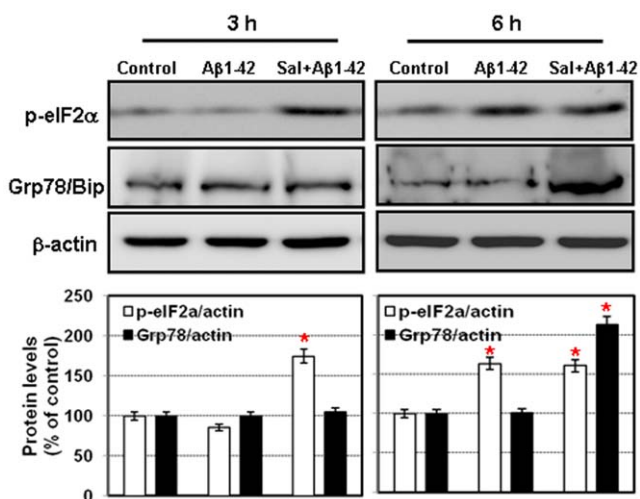
### Discussion

The results of this study are the first to show the protective effect of PERK pathway in A $\beta$  mediated neuronal cell death. We show that treatment of A $\beta$ 1-42 triggers the UPR in the SK-N-SH human neuroblastoma cells. This event accompanies the activation of protective pathways of the UPR such as Grp78/Bip and PERK-eIF2 $\alpha$  pathway, as well as the apoptotic pathways of the UPR such as CHOP and caspase-4. Previous studies reported that the induction of Grp78/Bip and phosphorylated PERK appeared in the brain of AD patients which is regarded as an early phenomenon in the pathology of AD [14]. ER stress specific caspase-4 is involved in neurotoxicity induced by A $\beta$ 25-35 and A $\beta$ 1-42 [20].





**Figure 4. PERK activator Salubrin attenuates A $\beta$ 1-42-induced neuronal apoptosis through the regulation of Grp78/Bip and caspase-4.** **A**, Salubrin protects neuronal cells against A $\beta$ 1-42-induced cell death. Dose-dependent protection by Salubrin of SK-N-SH cells treated with A $\beta$  and various concentrations of Salubrin as indicated. **B**, Co-treatment of Salubrin and A $\beta$  increased the neuronal cell viability compared with A $\beta$  treatment. Caspase-4 (**C**) and -3 activity (**D**) induced by A $\beta$  was suppressed by the co-treatment of Salubrin and A $\beta$ . Data were presented as means  $\pm$  SD from at least three independent experiments. \* $P$ <0.05, control versus vehicle alone; # $P$ <0.05, control versus A $\beta$  alone. doi:10.1371/journal.pone.0010489.g004



**Figure 5. Western blot analyses with anti-p-eIF2 $\alpha$  and anti-Grp78/Bip in SK-N-SH cells show that the co-treatment of Salubrin and A $\beta$  activated eIF2 $\alpha$  at 3 h and Grp78/Bip at 6 h.**  $\beta$ -actin was used as the loading controls. \* $P$ <0.05. doi:10.1371/journal.pone.0010489.g005

Mutations in the Presenilin genes PS-1 and PS-2 are the most common causes of early onset familial AD. It has been shown that these proteins are located predominantly within the ER [21]. The ER has been identified as the site where the highly toxic amyloidogenic A $\beta$  peptide 1–42 is generated [22], and in brains manifesting sporadic AD, the ER-resident protein disulfide-isomerase (PDI) activity has been shown to be suppressed by S-nitrosylation [23]. Furthermore, PS-1 mutations linked to AD impair UPR signaling by inhibiting activation of PERK, IRE1 and ATF6 [24,25]. Conversely, other studies have reported that phosphorylation of PERK and eIF2 $\alpha$  has been found in neurons of AD, suggesting activation of UPR [14,26].

During the last years, several evidences suggested that early intraneuronal accumulation of A $\beta$  peptides is one of the key events leading to neuronal dysfunction in AD patients [27]. Synthetic A $\beta$ 1-40 and A $\beta$ 1-42 are amyloidogenic and neurotoxic peptides that have been widely used to mimic *in vitro* the degenerative process that occurs in the brain of AD patients [28]. Previous reports have been shown that extracellularly applied A $\beta$  can be taken up by cultured neuronal cell lines [27,29,30] and colocalized in the endosomes/lysosomes or mitochondria [31,32]. In addition, extracellularly treated A $\beta$  aggregates induce mild ER stress in neuronal cells [9]. We thought that several mechanisms are involved in the A $\beta$ -induced ER stress. Recently, Oh et al. [33] suggested that extracellular A $\beta$  peptides enter the cell and inhibit

the proteasome activity. Proteasome is the important machinery for ER-associated degradation (ERAD), which carries out eliminating the misfolded protein [34]. Another possible mechanism is the effect on calcium channel currents. A $\beta$  affects calcium homeostasis by blocking Calcium influx at the plasma membrane or by perturbing Calcium storage in the ER [35,36]. Therefore, it is possible that the proteasome dysfunction and the disturbance of calcium homeostasis result in the activation of UPR. The PERK-eIF2 $\alpha$  pathway is the immediately early response among three UPR pathways, which leads to global translational attenuation [37]. These results suggest that the activation of PERK-eIF2 $\alpha$  pathway induced by the internalized A $\beta$  in the cytoplasm. Here we showed that the induction of eIF2 $\alpha$  phosphorylation and Grp78/Bip by the treatment of eIF2 $\alpha$  activator, Salubrinal, attenuated A $\beta$ -induced neuronal cell death. These results suggests that PERK-eIF2 $\alpha$  pathway is necessary for cell survival mechanism rather than cell death in this event, in agreement with results of other reports [16,38]. Up-regulation of the ER chaperone protein Grp78/Bip has been recently noted in investigating the action mechanism of novel small molecules for diseases related to ER stress [8,39]. Under conditions associated with ER stress, misfolded proteins accumulate in the ER lumen, a pathologic process resulting in the activation of the UPR pathway to combat the harmful effects of ER stress through the activation of ER chaperones such as Grp78/Bip. The Grp78/Bip was discovered as cellular proteins induced by glucose starvation [40]. Residing primarily in the ER, Grp78/Bip plays critical roles in the cellular stress of various diseases. In addition to facilitating proper protein folding, preventing intermediates from aggregating, and targeting misfolded protein for proteasome degradation, Grp78/Bip also binds Ca<sup>2+</sup> and serves as an ER stress signaling regulator [40,41]. Grp78/Bip is induced by ER stress for protecting against tissue or organ damage under pathologic conditions such as neurotoxic stress, myocardial infarction, and arteriosclerosis [41,42]. Indeed, overexpression of ER chaperones such as Grp78/Bip, calnexin, and Grp170/Orp150 suppressed the production A $\beta$ , a major component of extracellular senile plaques in AD [43].

Up-regulation of Grp78/Bip dependent PERK-eIF2 $\alpha$  pathway is supposed to function as a neuroprotective role against A $\beta$  neurotoxicity. In supporting this hypothesis, Sal, an activator of eIF2 $\alpha$  of the UPR pathway, enhances Grp78/Bip expression for maintaining the normal ER homeostasis and cell viability. However, it is possible that Sal would impact differently on the other cellular signaling pathway. Additional further studies will be required in various cell system and animal models to understand fully the precise mechanism of Sal.

In summary, our results show that ER stress could be an important mechanism of early pathogenesis in A $\beta$ 1-42 induced neurotoxicity. In particular, the initial activation of the UPR mediated by PERK-eIF2 $\alpha$  pathway might play a neuroprotective role to restore cellular homeostasis against A $\beta$ -induced ER stress, thereby increase cell survival. On the basis of these data, we propose that the PERK-eIF2 $\alpha$  pathway be part of the potential target for therapeutic applications in several neurodegenerative diseases related to pathological ER stress including AD.

## Materials and Methods

### Cell culture

SK-N-SH human neuroblastoma cells were obtained from American Type Culture Collection and cultured at 37°C in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% heat-inactivated FBS in a humidified 95% air, 5% CO<sub>2</sub>

incubator. Cell culture reagents were purchased from Gibco BRL. Salubrinal (Sal) and Tunicamycin (TM) were purchased from Tocris and Assay designs, respectively.

### A $\beta$ 1-42 preparation

The synthetic peptide A $\beta$ 1-42 (A $\beta$ 42) and scrambled A $\beta$ 1-42 (scrA $\beta$ 42) were purchased from Biosource and rPeptide, respectively. The peptides were dissolved in D.W to 500  $\mu$ M or in a diluted ammonia solution for facilitating peptide solubilization. A $\beta$ 1-42 or scrambled-A $\beta$ 42 aliquots were then store at -20°C until being used (fresh samples; A $\beta$ 42 or scrA $\beta$ 42 peptide), or were incubated for 1 week at 4°C before use (aged samples; A $\beta$ 42 or scrA $\beta$ 42 peptide).

### Aggregation state analysis of A $\beta$ peptide

The fibril formation of A $\beta$  peptides was measured by a thioflavin-T fluorometric assay as previously described with some modifications [44,45,46]. Fresh or aged A $\beta$  peptide-containing samples were added to 3  $\mu$ M thioflavin-T solution in a 50 mM glycine-NaOH buffer (pH 8.5). Fluorescence was measured at 450 nm excitation and 482 nm emission using a fluorescence spectrometer (Perkin-Elmer LS50). Each sample was determined in triplicates.

### Cell viability (amarBlue assay)

For assessing apoptosis, amarBlue assay was performed as described previously [47]. SK-N-SH cells were plated on 96-well plates (Nunc) at a density of 15,000 cells/well, in 100  $\mu$ l of 10% FBS/MEM and incubated for 24 h. 2 h before 10  $\mu$ M A $\beta$  treatment, the media was replaced with 1% FBS/MEM. At the end of the treatment, 10  $\mu$ l of amarBlue agent (Serotec) was added. The cells were incubated for 3 h and then absorbance of the cells was measured at a wavelength of 570 nm using a microtiter plate spectrophotometer (FLUOstar Optima). The background absorbance was measured at 600 nm and subtracted. The cell viability was defined as [(test sample count)-(blank count)/(untreated control count)-(blank count)] $\times$ 100.

### Hoechst 33258 staining

SK-N-SH cells were fixed with 4% paraformaldehyde for 20 min and then stained with 8  $\mu$ g/ml of Hoechst dye 33258 (Sigma-Aldrich) for 5 min. They were washed twice with phosphate-buffered saline and observed using Axiovert 200 M equipped with ApoTom (Carl Zeiss). Dead cells and apoptotic bodies were characterized by condensed or fragmented nuclei.

### Caspase substrate cleavage assays

Caspase-3 and -4 activities were measured using colorimetric assay kits (BioVision Lab) as described previously [48]. Briefly, cells were collected and washed with ice-cold PBS and then resuspended in chilled lysis buffer for 20 min on ice. The supernatant was collected by centrifugation at 10,000 g for 5 min and assayed for protein content. For caspase activity measurements, cell extracts (20  $\mu$ g protein) were incubated with 0.5 mM Ac-DEVD- $\rho$ NA (caspase-3) or 0.5 mM Ac-LEVD- $\rho$ NA (caspase-4) in a final volume of 100  $\mu$ l at 37°C for 1 h. The release of the chromogenic compound  $\rho$ NA from the parent substrates was measured by absorbance at 405 nm using a microtiter plate spectrophotometer (FLUOstar Optima). Ac-DEVD-CHO (caspase-3) or Ac-LEVD-CHO (caspase-4) was used as a caspase-specific inhibitor (Sigma-Aldrich). Enzymatic activity is expressed as arbitrary units of relative value.

## RT-PCR analysis

Total RNA was isolated from the cells by using Trizol Reagent (Invitrogen) according to the manufacturer's instructions. cDNA was synthesized by using Superscript II Reverse Transcription system (Invitrogen). For RT-PCR, *AccuPower* PCR premix (Bioneer) was mixed with each primer. CHOP primers 5'-TTCTCTGGCTTGGCTGACTG-3' (forward), 5'-CTGCGT-ATGTGGGATTGAGG-3' (reverse); Grp78/Bip primers 5'-GCTCGACTCGAATTCCAAAG-3' (forward), 5'-TTTGTC-AGGGGTCTTTCACC-3' (reverse); XBP-1 primers 5'-TAAGACAGCGCTTGGGGATC-3' (forward), 5'-CTGGGGAAAGGG-CATTTGAAG-3' (reverse); PERK primers 5'-ATCCCCCATG-GAACGACCTG-3' (forward), 5'-ACCCGCCAGGGACAAAA-ATG-3' (reverse); GAPDH primers 5'-GGGGCTCTCCAGAA-CATCAT-3' (forward), 5'-AAGTGGTCGTTGAGGGCAAT-3' (reverse). Amplification conditions were as follows: single cycle of 94°C for 5 min followed by 30 cycles of 94°C for 30 s, 58°C for 30 s and 72°C for 30 s, and the final single cycle of 72°C extension for 7 min.

## Western blot analysis

Total proteins from SK-N-SH cells were isolated using the PROPREP protein extraction buffer (iNtRon biotechnology). Protein preparation and SDS-PAGE/immunoblotting were performed as previously described [48]. The cell homogenate was centrifuged at 1,000 g at 4°C for 10 min to discard unbroken or coarse cell debris and the resulting supernatant (RIPA lysate) was used for immunoblotting. Protein concentrations of RIPA lysates were determined by a modified Bradford method using BSA as a standard. Sample buffer (5%  $\beta$ -mercaptoethanol, 15% glycerol, 3% SDS, 0.1 M Tris, pH 6.8) was added to the aliquots (50  $\mu$ g of protein) of the lysates, boiled for 3 min, and then resolved by 8~12% SDS-polyacrylamide gel electrophoresis (PAGE) under reducing conditions. The resolved proteins were transferred onto nitrocellulose membranes (Amersham Pharmacia Biotech, Littel Chalfont, UK) using a semidry trans-blot system (Schleicher & Schuell, Dassol, Germany). The blots were blocked for 2 h at room temperature with tris-buffered saline (TBS) (10 mM Tris, pH 7.5, 100 mM NaCl) containing 5% nonfat dry milk. The blots were washed three times with TBS, and then incubated at room temperature overnight with Anti-KDEL (1:1000, Assay designs), anti-GADD153/CHOP (1:1000, Santa Cruz Biotechnology), anti-phospho-PERK (1:800, Santa Cruz Biotechnology), anti-phospho-eIF2 $\alpha$  (1:1000, Cell Signaling), anti-phospho-Ire1 $\alpha$  from (1:800, Abcam), anti-ATF6 $\alpha$  (1:1000, Santa Cruz Biotechnology), or  $\beta$ -actin (1:2000, Abcam) were used for primary antibodies in TBST (10 mM Tris, pH 7.5, 100 mM NaCl, 0.05% Tween 20) containing 1% nonfat dry milk. The next day, the blots were washed three times with TBST, and then incubated for 1 h at room temperature with horseradish peroxidase (HRP)-conjugated secondary antibodies (1:2000 dilution) (Santa Cruz Biotechnology) in TBST containing 1% nonfat dry milk. After washing three times with TBST, the protein was visualized using the ECL detection system (Amersham Pharmacia Biotech).

## Immunocytochemistry

Immunostaining was performed as described previously [49]. Cells grown on glass cover slides, were washed with PBS and fixed in 10% formalin solution containing 4% formaldehyde for 20 min at RT, then incubated with Phospho-eIF2 $\alpha$  (1:1000, Cell Signaling) primary antibody, revealed with anti-rabbit IgG Alexa 594 secondary antibody (1:200, Molecular Probe). After reaction with secondary antibodies, the cells were stained with 100 nM

DAPI (4',6-diamidino-2-phenylindole)(Molecular Probes) for 5 min, and mounted. Fluorescence-labeled cells were visualized using Axiovert 200 M equipped with ApoTom (Carl Zeiss).

## Small interference RNA (siRNA) for PERK

SK-N-SH cells were seeded onto 6-well plates and allowed to reach 50% confluence on the day of transfection. The small interfering RNA (siRNA) constructs used were obtained as the siGENOME SMARTpool reagents (Dharmacon), the siGENOME SMARTpool PERK (M-004883-03-0020). The non-targeting siRNA control, SiConTRol non-targeting siRNA pool (D-001206-13-20) was also obtained from Dharmacon. Cells were transfected with 100 nM siRNA diluted in Opti-Eagle's Minimal Essential Medium (MEM) using Lipofectamine reagent (Invitrogen) according to the manufacturer's transfection protocol.

## Statistical analysis

All data are expressed as the means  $\pm$  SD. To determine the significance of differences between the means of two groups, an unpaired two-tailed Student's *t*-test was applied to study the relationship between the different variables. To determine the significance of differences among the means of several groups, one-way analysis of variance (ANOVA) followed by Scheffé's post-hoc tests were applied. Statistical significance was determined via ANOVA followed by Scheffé's post-hoc tests. A *p*-value of <0.05 was considered to be significant.

## Supporting Information

**Figure S1** Aged A $\beta$ 1-42 peptide induces ER stress and activation of PERK-eIF2 $\alpha$  in SK-N-SH cells. Cells were treated with A $\beta$ 42 peptide or scrambled A $\beta$ 42 peptide (scrA $\beta$ 42) in fresh or aged condition. A, The expression levels of protein of human Grp78/Bip (top) and CHOP (middle) were increased in SK-N-SH cells treated with aged A $\beta$ 42 but not fresh A $\beta$ 42 at 18 h. B, Western blotting with anti-p-PERK (top) and anti-p-eIF2 $\alpha$  (middle) in SK-N-SH cells treated with aged A $\beta$ 42 show the activation of PERK and eIF2 $\alpha$  from 6 h but not fresh A $\beta$ 42. The scrambled A $\beta$ 42 peptides (scrA $\beta$ 42) did not lead to any significant increase in both fresh and aged condition, demonstrating that the specific sequence of amino acids of A $\beta$  peptide is needed for the induction of ER stress and the activation of PERK-eIF2 $\alpha$ .  $\beta$ -actin was used as the loading control (bottom).  
Found at: doi:10.1371/journal.pone.0010489.s001 (2.23 MB TIF)

**Figure S2** Effects of Salubrinal on cell viability in SK-N-SH cells. Cells were treated with various concentrations of Salubrinal as indicated. Tunicamycin (TM, 2  $\mu$ g/ml) was used as the positive control. Cell viability was measured by alamarBlue assay from 48 h after each treatment. \**P*<0.01, versus control (vehicle alone).  
Found at: doi:10.1371/journal.pone.0010489.s002 (1.18 MB TIF)

**Figure S3** Salubrinal inhibits A $\beta$ 1-42-induced neuronal apoptosis. Cells were stained with Hoechst 33258 staining. Dead cells were identified by morphological changes, such as nuclei fragmentation (arrowhead), compared with normal cell nuclei. Scale bar: 10  $\mu$ m.  
Found at: doi:10.1371/journal.pone.0010489.s003 (2.50 MB TIF)

## Author Contributions

Conceived and designed the experiments: DYK KSL SSK. Performed the experiments: DYK. Analyzed the data: DYK KSL HJL KY WBL SSK. Contributed reagents/materials/analysis tools: HJL DHK YHN HYJ SHL JYL YCY YJ DKK. Wrote the paper: DYK KSL SSK.

## References

- Hardy J, Selkoe DJ (2002) The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics. *Science* 297: 353–356.
- Sisodia SS, Annaert W, Kim SH, De Strooper B (2001) Gamma-secretase: never more enigmatic. *Trends Neurosci* 24: S2–6.
- Hoozemans JJ, van Haastert ES, Nijholt DA, Rozemuller AJ, Eikelenboom P, et al. (2009) The unfolded protein response is activated in pretangle neurons in Alzheimer's disease hippocampus. *Am J Pathol* 174: 1241–1251.
- Bertolotti A, Zhang Y, Hendershot LM, Harding HP, Ron D (2000) Dynamic interaction of BiP and ER stress transducers in the unfolded-protein response. *Nat Cell Biol* 2: 326–332.
- Lindholm D, Wootz H, Korhonen L (2006) ER stress and neurodegenerative diseases. *Cell Death Differ* 13: 385–392.
- Zhou J, Liu CY, Back SH, Clark RL, Peisach D, et al. (2006) The crystal structure of human IRE1 luminal domain reveals a conserved dimerization interface required for activation of the unfolded protein response. *Proc Natl Acad Sci U S A* 103: 14343–14348.
- Todd DJ, Lee AH, Glimcher LH (2008) The endoplasmic reticulum stress response in immunity and autoimmunity. *Nat Rev Immunol* 8: 663–674.
- Kim I, Xu W, Reed JC (2008) Cell death and endoplasmic reticulum stress: disease relevance and therapeutic opportunities. *Nat Rev Drug Discov* 7: 1013–1030.
- Chafekar SM, Hoozemans JJ, Zwart R, Baas F, Scheper W (2007) Abeta 1-42 induces mild endoplasmic reticulum stress in an aggregation state-dependent manner. *Antioxid Redox Signal* 9: 2245–2254.
- Hussain SG, Ramaiah KV (2007) Reduced eIF2alpha phosphorylation and increased proapoptotic proteins in aging. *Biochem Biophys Res Commun* 355: 365–370.
- Naidoo N, Ferber M, Master M, Zhu Y, Pack AI (2008) Aging impairs the unfolded protein response to sleep deprivation and leads to proapoptotic signaling. *J Neurosci* 28: 6539–6548.
- Paz Gavilan M, Vela J, Castano A, Ramos B, del Rio JC, et al. (2006) Cellular environment facilitates protein accumulation in aged rat hippocampus. *Neurobiol Aging* 27: 973–982.
- Ryu EJ, Harding HP, Angelastro JM, Vitolo OV, Ron D, et al. (2002) Endoplasmic reticulum stress and the unfolded protein response in cellular models of Parkinson's disease. *J Neurosci* 22: 10690–10698.
- Hoozemans JJ, Veerhuis R, Van Haastert ES, Rozemuller JM, Baas F, et al. (2005) The unfolded protein response is activated in Alzheimer's disease. *Acta Neuropathol* 110: 165–172.
- Yoneda T, Urano F, Ron D (2002) Transmission of proteotoxicity across cellular compartments. *Genes Dev* 16: 1307–1313.
- Boyce M, Bryant KF, Jousse C, Long K, Harding HP, et al. (2005) A selective inhibitor of eIF2alpha dephosphorylation protects cells from ER stress. *Science* 307: 935–939.
- Allaman I, Gavillet M, Belanger M, Laroche T, Viert D, et al. Amyloid-beta aggregates cause alterations of astrocytic metabolic phenotype: impact on neuronal viability. *J Neurosci* 30: 3326–3338.
- Dahlgren KN, Manelli AM, Stine WB, Jr., Baker LK, Krafft GA, et al. (2002) Oligomeric and fibrillar species of amyloid-beta peptides differentially affect neuronal viability. *J Biol Chem* 277: 32046–32053.
- Oyadomari S, Mori M (2004) Roles of CHOP/GADD153 in endoplasmic reticulum stress. *Cell Death Differ* 11: 381–389.
- Hitomi J, Katayama T, Eguchi Y, Kudo T, Taniguchi M, et al. (2004) Involvement of caspase-4 in endoplasmic reticulum stress-induced apoptosis and Abeta-induced cell death. *J Cell Biol* 165: 347–356.
- Walter J, Capell A, Grunberg J, Pesold B, Schindzielorz A, et al. (1996) The Alzheimer's disease-associated presenilins are differentially phosphorylated proteins located predominantly within the endoplasmic reticulum. *Mol Med* 2: 673–691.
- Hartmann T, Bieger SC, Bruhl B, Tienari PJ, Ida N, et al. (1997) Distinct sites of intracellular production for Alzheimer's disease A beta40/42 amyloid peptides. *Nat Med* 3: 1016–1020.
- Uehara T, Nakamura T, Yao D, Shi ZQ, Gu Z, et al. (2006) S-nitrosylated protein-disulphide isomerase links protein misfolding to neurodegeneration. *Nature* 441: 513–517.
- Katayama T, Imaizumi K, Honda A, Yoneda T, Kudo T, et al. (2001) Disturbed activation of endoplasmic reticulum stress transducers by familial Alzheimer's disease-linked presenilin-1 mutations. *J Biol Chem* 276: 43446–43454.
- Katayama T, Imaizumi K, Sato N, Miyoshi K, Kudo T, et al. (1999) Presenilin-1 mutations downregulate the signalling pathway of the unfolded-protein response. *Nat Cell Biol* 1: 479–485.
- Unterberger U, Hoftberger R, Gelpi E, Flicker H, Budka H, et al. (2006) Endoplasmic reticulum stress features are prominent in Alzheimer disease but not in prion diseases in vivo. *J Neuropathol Exp Neurol* 65: 348–357.
- Knauer MF, Soreghan B, Burdick D, Kosmoski J, Glabe CG (1992) Intracellular accumulation and resistance to degradation of the Alzheimer amyloid A4/beta protein. *Proc Natl Acad Sci U S A* 89: 7437–7441.
- Pereira C, Santos MS, Oliveira C (1999) Involvement of oxidative stress on the impairment of energy metabolism induced by A beta peptides on PC12 cells: protection by antioxidants. *Neurobiol Dis* 6: 209–219.
- Bi X, Gall CM, Zhou J, Lynch G (2002) Uptake and pathogenic effects of amyloid beta peptide 1-42 are enhanced by integrin antagonists and blocked by NMDA receptor antagonists. *Neuroscience* 112: 827–840.
- Yazawa H, Yu ZX, Takeda, Le Y, Gong W, et al. (2001) Beta amyloid peptide (Abeta42) is internalized via the G-protein-coupled receptor FPRL1 and forms fibrillar aggregates in macrophages. *FASEB J* 15: 2454–2462.
- Hansson Petersen CA, Alikhani N, Behbahani H, Wiehager B, Pavlov PF, et al. (2008) The amyloid beta-peptide is imported into mitochondria via the TOM import machinery and localized to mitochondrial cristae. *Proc Natl Acad Sci U S A* 105: 13145–13150.
- Hu X, Crick SL, Bu G, Frieden C, Pappu RV, et al. (2009) Amyloid seeds formed by cellular uptake, concentration, and aggregation of the amyloid-beta peptide. *Proc Natl Acad Sci U S A* 106: 20324–20329.
- Oh S, Hong HS, Hwang E, Sim HJ, Lee W, et al. (2005) Amyloid peptide attenuates the proteasome activity in neuronal cells. *Mech Ageing Dev* 126: 1292–1299.
- Schubert U, Anton LC, Gibbs J, Norbury CC, Yewdell JW, et al. (2000) Rapid degradation of a large fraction of newly synthesized proteins by proteasomes. *Nature* 404: 770–774.
- Pierrot N, Ghisdal P, Caumont AS, Octave JN (2004) Intraneuronal amyloid-beta1-42 production triggered by sustained increase of cytosolic calcium concentration induces neuronal death. *J Neurochem* 88: 1140–1150.
- Sun XD, Mo ZL, Taylor BM, Epps DE (2003) A slowly formed transient conformer of Abeta(1-40) is toxic to inward channels of dissociated hippocampal and cortical neurons of rats. *Neurobiol Dis* 14: 567–578.
- Scheuner D, Song B, McEwen E, Liu C, Laybutt R, et al. (2001) Translational control is required for the unfolded protein response and in vivo glucose homeostasis. *Mol Cell* 7: 1165–1176.
- Sokka AL, Putkonen N, Mudo G, Pryazhnikov E, Reijonen S, et al. (2007) Endoplasmic reticulum stress inhibition protects against excitotoxic neuronal injury in the rat brain. *J Neurosci* 27: 901–908.
- Kim AJ, Shi Y, Austin RC, Werstuck GH (2005) Valproate protects cells from ER stress-induced lipid accumulation and apoptosis by inhibiting glycogen synthase kinase-3. *J Cell Sci* 118: 89–99.
- Lee AS (2001) The glucose-regulated proteins: stress induction and clinical applications. *Trends Biochem Sci* 26: 504–510.
- Lee AS (2007) GRP78 induction in cancer: therapeutic and prognostic implications. *Cancer Res* 67: 3496–3499.
- Ni M, Lee AS (2007) ER chaperones in mammalian development and human diseases. *FEBS Lett* 581: 3641–3651.
- Hoshino T, Nakaya T, Araki W, Suzuki K, Suzuki T, et al. (2007) Endoplasmic reticulum chaperones inhibit the production of amyloid-beta peptides. *Biochem J* 402: 581–589.
- Hashioka S, Monji A, Ueda T, Kanba S, Nakanishi H (2005) Amyloid-beta fibril formation is not necessarily required for microglial activation by the peptides. *Neurochem Int* 47: 369–376.
- LeVine H, 3rd (1993) Thioflavine T interaction with synthetic Alzheimer's disease beta-amyloid peptides: detection of amyloid aggregation in solution. *Protein Sci* 2: 404–410.
- Resende R, Ferreiro E, Pereira C, Resende de Oliveira C (2008) Neurotoxic effect of oligomeric and fibrillar species of amyloid-beta peptide 1-42: involvement of endoplasmic reticulum calcium release in oligomer-induced cell death. *Neuroscience* 155: 725–737.
- Lee do Y, Lee KS, Lee HJ, Jung HY, Lee JY, et al. (2008) Alcohol enhances Abeta42-induced neuronal cell death through mitochondrial dysfunction. *FEBS Lett* 582: 4185–4190.
- Lee do Y, Lee KS, Lee HJ, Noh YH, Kim do H, et al. (2008) Kynurenic acid attenuates MPP(+)-induced dopaminergic neuronal cell death via a Bax-mediated mitochondrial pathway. *Eur J Cell Biol* 87: 389–397.
- Lee KS, Kwon OY, Lee JH, Kwon K, Min KJ, et al. (2008) Drosophila short neuropeptide F signalling regulates growth by ERK-mediated insulin signalling. *Nat Cell Biol* 10: 468–475.