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Original Article

Mitofusin 1 and 2 overexpression reduces AβO-mediated ER stress and apoptosis in N2a APPswe cells

Min Kyoung Kam[1,2,6,#] , Su-Min Jung[1,2,#] , Ga Eun Lee[1,2,#] , Sung Woo Lee[1,2], Hong Jun Lee[3,4], Young-Ho Park[5], Dong-Seok Lee[1,2,*]

[1] School of Life Sciences, BK21 FOUR KNU Creative BioResearch Group, Kyungpook National University, Daegu 41566, Republic of Korea
[2] School of Life Sciences & Biotechnology, College of Natural Sciences, Kyungpook National University, Daegu 41566, Republic of Korea
[3] Research of Medicine and Medical Research Institute, Chungbuk National University, Cheongju, Chungbuk, Republic of Korea
[4] Research Institute, huMetaCELL Inc., 220 Bugwang-ro, Bucheon-si, Gyeonggi-do, Republic of Korea
[5] Futuristic Animal Resource & Research Center (FARRC), Korea Research Institute of Bioscience and Biotechnology (KRIBB), Ochang-eup, Cheongju 28116, Republic of Korea
[6] Division of Brain Disease Research, Department for Chronic Disease Convergence Research, Korea National Institute of Health, 187 Osongsaengmyeong2-ro, Osong-eup, Cheongju-si, Chungcheongbuk-do, 363-951, Republic of Korea

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Abstract

Alzheimer's disease (AD) is the most common neurodegenerative disorder, and amyloid beta oligomers (AβO), which are pathological markers of AD, are known to be highly toxic. AβO increase mitochondrial dysfunction, which is accompanied by a decrease in mitochondrial fusion. Although mitofusin (Mfn) 1 and Mfn2 are mitochondrial fusion proteins, Mfn2 is known to regulate endoplasmic reticulum (ER) function, as it is located in the ER. Several studies have shown that AβO exacerbates ER stress, however, the exact mechanism requires further elucidation. In this study, we used mouse neuroblastoma cells stably overexpressing the amyloid precursor protein (APP) with the Swedish mutation (N2a APPswe cells) to investigate the role of Mfn in ER stress. Our results revealed that amyloid beta (Aβ) caused cellular toxicity in N2a APPswe cells, upregulated ER stress-related proteins, and promoted ER expansion. The AβO-mediated ER stress was reduced when Mfn1 and Mfn2 were overexpressed. Moreover, Mfn1 and Mfn2 overexpressed resulted in reduced apoptosis of N2a APPswe cells. In conclusion, our results indicate that both Mfn1 and Mfn2 reduce ER stress and apoptosis. Our data provide a foundation for future studies on the roles of Mfn1 and Mfn2 in the molecular mechanisms underlying AβO-mediated ER stress and the pathogenesis of AD.

Keywords: Alzheimer's disease, APP Swedish mutation, Endoplasmic reticulum stress, Apoptosis, Mitofusin.

1. Introduction

Alzheimer's disease (AD) is the most common neurodegenerative disorder and is characterized by cognitive impairment and memory loss. One of the hallmarks of AD is the presence of amyloid beta oligomers (AβO), which are composed of amyloid beta (Aβ) peptides [1]. Aβ is produced from amyloid precursor protein (APP) upon cleavage by β- and γ-secretases. The APP Swedish mutation (K595N/M596L) is a double mutation that causes familial AD [2]. This mutation increases Aβ production due to abnormal β-secretase cleavage. Recent studies have suggested that Aβ production impairs mitochondrial dynamics. Additionally, several studies have determined that Aβ induces endoplasmic reticulum (ER) stress [3, 4].

The ER is a vital organelle that participates in protein synthesis, folding, modification, and secretion. Post-translational protein modifications are essential for cellular homeostasis and survival. Uncontrolled homeostasis induces the accumulation of misfolded or unfolded proteins, which is referred to as ER stress. ER stress upregulates the unfolded protein response (UPR) to restore ER function by activating transcriptional factors or chaperone proteins [5]. The UPR involves three ER transmembrane receptors, namely, PKR-like ER kinase (PERK), inositol requiring enzyme 1α/β (IRE1), and activating transcription factor 6α/β (ATF6) [6]. However, prolonged ER stress upregulates C/EBP homologous protein (CHOP) and leads to apoptosis [7]. A growing body of evidence suggests that Aβ increases ER stress-mediated neuronal cell death [4]. However, the specific mechanisms underlying Aβ-induced

* Corresponding author.
E-mail address: lee1@knu.ac.kr (D.-S. Lee).
# These authors contributed equally
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ER stress and apoptosis require further investigation.

Mitochondria are highly dynamic organelles that continuously fuse and divide to meet cellular energy requirements. Therefore, maintaining mitochondrial quality through fusion, fission, and mitophagy is important. Mitofusin (Mfn) 1 and 2 are two GTPases involved in outer mitochondrial membrane fusion [8]. Only Mfn2 is located in both mitochondria and the ER. It tethers the two organelles to regulate Ca2+ homeostasis [9]. The regulation of Ca2+ flux is critical for ER stress-induced apoptosis. In our previous studies, we showed that AβO-mediated oxidative stress increases mitochondrial dysfunction and reduces Mfn1 and Mfn2 expression levels [10, 11]. Furthermore, we previously reported that AβO treatment increases ER stress in mouse hippocampal neuronal cells [3, 12]. As the impact of Aβ toxicity on several types of cellular stress and diseases has already been elucidated, we focused on the role of Mfn in the AβO-mediated ER stress mechanism. Thus, in this study, we investigated whether the upregulation of Mfn1 or Mfn2 alters the expression of ER stress-related proteins or stress-mediated apoptosis. Using an in vitro model of AD, namely N2a cells overexpressing Swedish mutant APP (N2a APPswe cells), we investigated the effect of ER stress caused by the accumulation of Aβ. Moreover, we overexpressed Mfn1 and Mfn2 and confirmed the role of Mfn in APP Swedish mutation induced ER stress. Our results showed that restoring Mfn1 and Mfn2 levels reduced AβO-mediated ER stress and apoptosis. Further study of the role of Mfn in ER stress may reveal novel molecular mechanisms underlying AβO-mediated stress associated with AD.

2. Materials and methods

2.1. Reagents

Tunicamycin (Tm) and tauroursodeoxycholic acid (TUDCA) were purchased from Merck (Rahway, NJ, USA).

2.2. Cell culture

Mouse neuroblastoma N2a cells were purchased from the American Type Culture Collection (Manassas, VA, USA) and maintained in modified minimum medium (Welgene, Daegu, Korea) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific, Waktham, MA, USA) and 1% penicillin/streptomycin (Welgene) at 37°C under 5% CO2 in a humidified incubator (Panasonic Corporation, Osaka, Japan). N2a cells stably expressing the APP Swedish mutation (K670N/M671L) were cultured as described in our previous studies [10, 11].

2.3. Lentivirus construction and transduction

Mouse Mfn1 and Mfn2 (Addgene, Watertown, MA, USA) were amplified via polymerase chain reaction (PCR) using the LA Taq™ polymerase kit (TaKaRa, Kyoto, Japan). The amplified genes were cloned into the pCR8/GW/TOPO vector (Thermo Fisher Scientific) to generate expression clones using LR recombination between the entry vector and pLenti 6.3/V5-DEST vector (Thermo Fisher Scientific). The sequences of the constructed vectors were confirmed via DNA sequencing. Plasmid pLenti 6.3-Mfn1 or pLenti 6.3 Mfn2 vector was transfected into HEK293FT cells with the psPAX2 packaging vector and pMD.2G enveloping vector to construct the lentivirus [13]. The lentiviral vector was transduced into N2a APPswe cells using Effectene™ (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

2.4. Western blot analysis

Whole protein lysates were prepared using an ice-cold PRO-PREP protein extraction solution (iNtRON Biotechnology, Seongnam, Korea) according to the manufacturer's instructions. Proteins were quantified using the Bradford assay (Bio-Rad, Hercules, CA, USA), and 10–30 μg of protein lysate was subjected to 8–15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The proteins were then transferred onto nitrocellulose membranes (BD Biosciences, Franklin Lakes, NJ, USA). After blocking with 5% skimmed milk (BD Biosciences), the membranes were incubated overnight at 4°C with primary antibodies against β-actin, Mfn1, Mfn2 (Santa Cruz Biotechnology, Dallas, TX, USA), IRE1α, BiP, eIF2α, p-eIF2α, ATF4, CHOP, cleaved caspase-3, PARP (Cell Signaling Technology, Danvers, MA, USA), 6E10 (BioLegend, San Diego, CA, USA), and p-IRE1α (Abcam, Cambridge, UK) at 1:1,000–1:5,000 dilutions. The membranes were washed five times with 10 mM Tris-HCl (pH 7.5) containing 150 mM NaCl and 0.1% Tween-20 (TBST) and then incubated with horseradish peroxidase conjugated goat anti-rabbit or anti-mouse IgG (1:5,000 dilution; Thermo Fisher Scientific) for 2 h at room temperature. After removing excess secondary antibodies, the membranes were washed six times with TBST, and specific binding was detected using the Clarity™ Western ECL substrate (Bio-Rad) according to the manufacturer's instructions.

2.5. Analysis of cell toxicity

Cell toxicity was measured by monitoring lactate dehydrogenase (LDH) release using the CytoTox 96® Non-Radioactive Cytotoxicity Assay Kit (Promega Corporation, Madison, WI, USA), according to the manufacturer's instructions. The cell culture media were collected, and LDH levels were measured at 490 nm using a Synergy™ H1 microplate reader (Agilent, Santa Clara, CA, USA).

2.6. Confocal microscopy

Cells were seeded on 0.1% poly-D-lysine-coated 24 mm round coverslips (Marienfeld, Lauda-Königshofen, Germany) and incubated for 24 h. Mfn1- and Mfn2-transduced cells were washed with phosphate buffered saline (PBS), fixed with 4% paraformaldehyde in PBS for 1 h, again washed with PBS, and then stained with ER-Tracker™ Red and Hoechst (Thermo Fisher Scientific). After washing with PBS, the coverslips were mounted on slides using the VECTASHIELD® mounting medium (Vector Laboratories, Newark, CA, USA). Images were obtained using an LSM-800 confocal microscope (Carl Zeiss, Oberkochen, Germany).

2.7. Annexin V and propidium iodide staining

An annexin V-fluorescein isothiocyanate (FITC)/ propidium iodide (PI) apoptosis detection kit (BD Biosciences) was used to detect apoptosis using flow cytometry. Staining was conducted according to the manufacturer's instructions. After culturing for 12 h, cells were harvested and washed with PBS. Annexin V (5 μL) and PI (5 μL) were added to the cell suspensions, and cells were incubated for 15 min at room temperature in the dark. The cells were analyzed using flow cytometry (FACSverse; BD

Biosciences).

2.8. Statistical analysis

Data are presented as the mean ± standard deviation of values from at least three independent experiments (n ≥ 3). Experimental differences were tested for statistical significance via a one-way analysis of variance using the GraphPad Prism 8 software (GraphPad, San Diego, CA, USA). A p-value < 0.05 was considered statistically significant and is indicated on the graphs with an asterisk. P-values < 0.01 and < 0.001 are indicated by two and three asterisks, respectively.

3. Results

3.1. Aβ overexpression resulted in toxicity in N2a APPswe cells

The APP Swedish mutation (K595N/M596L) causes increased Aβ levels via abnormal β-secretase cleavage [2]. To investigate the increase in Aβ levels, we performed western blot analysis using anti- Aβ (6E10) and anti-APP antibodies (Figure 1a) to assess whether Aβ levels are increased in N2a cells stably expressing APPswe. Compared with those in normal N2a cells, several Aβ peptides showed increased levels in N2a APPswe cells. Aβ1-42 is highly toxic and leads to the upregulation of oxidative stress and apoptosis [14]. Recent studies have demonstrated that the toxicity of AβO results in increased apoptosis [15]. We previously reported that APPswe increases the levels of reactive oxygen species and apoptosis-related proteins (Bax, AIF, Bcl-2, and Cyt C) [10]. In this study, we investigated the intracellular toxicity and levels of apoptosis-related proteins (cleaved caspase-3 and cleaved PARP) at 12 and 24 h (Figure 1b and 1c). LDH assay results indicated that intracellular toxicity was increased in a time dependent manner by APPswe mutation. In addition, the levels of the cleaved caspase-3 and cleaved PARP increased in a time dependent manner. To measure the rate of apoptosis, we analyzed annexin V/PI staining using flow cytometry (Figure 1d). Strikingly, APPswe mutations promoted early and late apoptosis. These results suggested that elevated levels of AβO are highly toxic and result in increased neuronal apoptotic cell death.

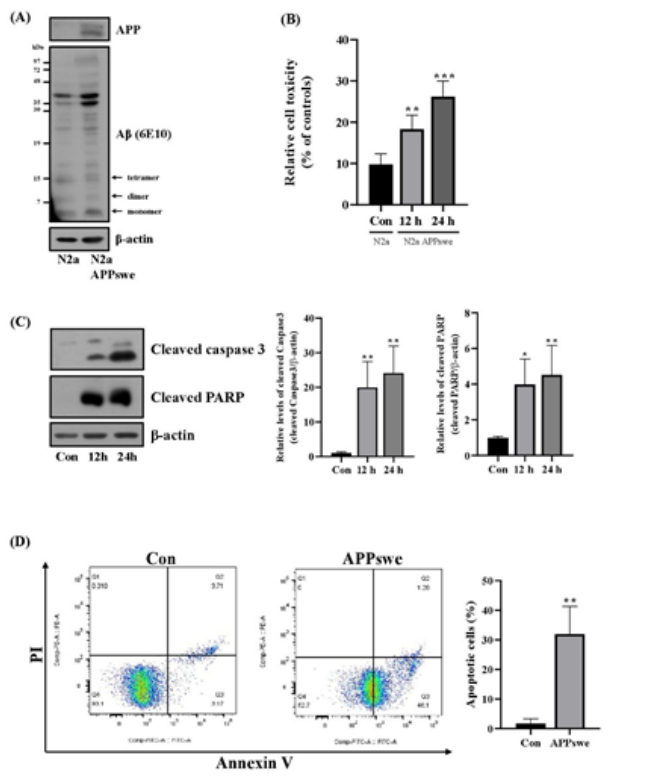

Fig. 1. Aβ results in cellular toxicity in N2a APPswe cells. (a) The level of Aβ was assessed by western blotting in N2a and N2a APPswe cells. (b) Cell toxicity was evaluated by assessing the LDH levels over a time period. (c) The levels of cleaved caspase-3 and cleaved PARP were assessed by western blotting. The graphs depict the quantification of cleaved caspase-3/β-actin and cleaved PARP/β-actin. (d) Flow cytometry results with Annexin V-FITC/PI staining. The ratio of apoptosis was early apoptosis percentage plus late apoptosis percentage. The data are presented as means ± the SD (n ≥ 3). *p < 0.05, **p < 0.01, and ***p < 0.001.

3.2. Aβ upregulated ER stress-related proteins and promoted ER expansion

A growing body of evidence suggests that AβO are associated with an increase in neuronal death; however, the specific mechanism has not yet been elucidated. We previously demonstrated that AβO upregulate oxidative stress in hippocampal neurons [16]. AβO also increases ER stress in HT-22 cells [3, 12]. In this study, we investigated whether APPswe mutation increases ER stress in N2a cells. The levels of ER stress-related proteins (eIF2α, ATF4, IRE1α, and CHOP) increased after 12 h (Figure 2a). However, BiP expression levels decreased in a time-dependent manner. To investigate whether APPswe mutation increases ER stress, we treated N2a APPswe cells with the

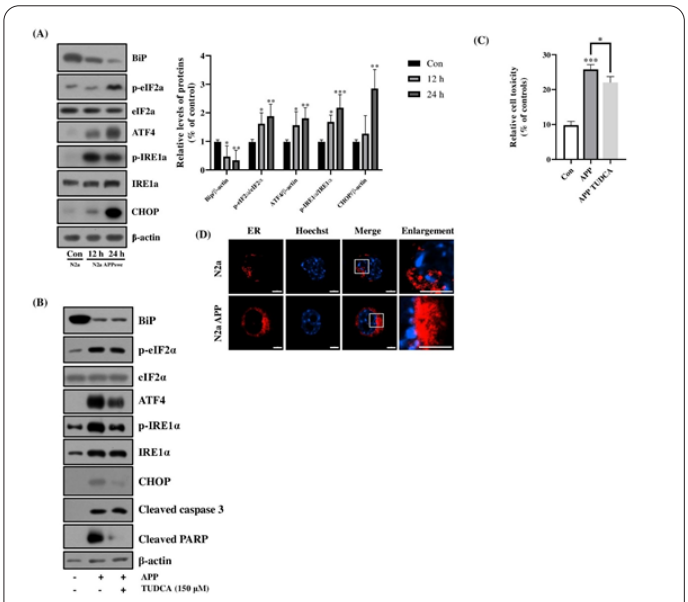

Fig. 2. Aβ upregulates the levels of ER stress-related proteins and ER expansion. (a) The levels of ER stress-related proteins (BiP, p-eIF2α, eIF2α, p-IRE1α, IRE1α, ATF4, and CHOP) were evaluated by western blot analysis in N2a cells cultured for 12–24 h and in APPswe cells cultured for 12 h and 24 h. The graphs show quantification of BiP/β-actin, p-IRE1α/IRE1α, p-eIF2α/eIF2α, ATF4/β-actin, and CHOP/β-actin. (b) N2a APPswe cells were treated with TUDCA (150 μM), and the levels of proteins related to ER stress were determined by western blotting. The graphs display the quantification of BiP/β-actin, p-IRE1α/IRE1α, p-eIF2α/eIF2α, ATF4/β-actin, and CHOP/β-actin. (c) LDH assay-based cell toxicity in N2a, APPswe, and TUDCA-treated APPswe cells. (d) ER morphologies were observed under a confocal microscope using ER-Tracker™-Red fluorescent dye and Hoechst stain; scale bar = 5 μm. The data are presented as means ± the SD (n ≥ 3). *p < 0.05, **p < 0.01, and ***p < 0.001.

ER stress inhibitor TUDCA (Figure 2b). Based on the expression levels of UPR-related proteins, as assessed using western blotting, we found that TUDCA treatment reduced the AβO-mediated ER stress. Furthermore, TUDCA also reduced the cellular toxicity induced by AβO-mediated ER stress (Figure 2c). To examine the ER morphology associated with AβO-triggered ER stress, we performed confocal microscopy staining using ER-Tracker™ Red (Figure 2d). Results indicated that the shape of the ER was expanded by APPswe mutation. Thus, APPswe mutation increase ER stress and ER stress-mediated cellular toxicity.

3.3. Upregulation of Mfn1 and Mfn2 reduced Aβ-mediated ER stress and apoptosis in N2a APPswe cells

The toxicity of AβO is known to impair mitochondrial function. Mitochondrial dysfunction can occur through decrease in mitochondrial fusion or an increase in mitochondrial fission [17]. We previously demonstrated that AβO impair mitochondrial dynamics in neuronal cells, including N2a cells. In addition, Mfn2 is located in the ER, and it tethers the mitochondria and ER, thereby facilitating Ca2+ homeostasis [18]. Therefore, in this study, we hypothesized that Mfn2 regulates ER stress despite the presence of mitochondrial fusion proteins. We confirmed that Mfn1 and Mfn2 levels decreased in a time-dependent manner in N2a APPswe cells (Figure 3a). As the transcriptional levels of Mfn1 and Mfn2 were reduced in N2a APPswe cells, we overexpressed these proteins through lentiviral vector transduction (Figure 3b). We then determined the changes in ER stress-related protein levels and apoptosis at 12 h using western blotting (Figure 3c and 3d). Western blot analysis indicated that both Mfn1 and Mfn2 had an effect on UPR signaling. The reduction in BiP levels by AβO was reversed by the overexpression of both Mfn1 and Mfn2. Moreover, p-IRE1α and CHOP levels were reduced upon Mfn1 and Mfn2 overexpression; however, the levels of other proteins remained unchanged. The levels of apoptosis related proteins were reduced in N2a APPswe cells upon Mfn1 and Mfn2 overexpression. Moreover, Mfn1 and Mfn2 overexpression reduced the intracellular toxicity of APPswe cells (Figure 3e). Accordingly, we evaluated the apoptosis rate after Mfn1 and Mfn2 overexpression (Figure 3f). After 12 h of culture, the rate of apoptosis was reduced in cells overexpressing Mfn1 and Mfn2. We also observed changes in ER morphology following Mfn1 and Mfn2 overexpression (Figure 3g). Confocal microscopy revealed a reduction in ER expansion resulting in ER morphology similar to that observed in the control N2a cells. Consistent with these observations, overexpression of Mfn1 and Mfn2 reduced ER stress levels and the apoptosis triggered by APPswe mutation.

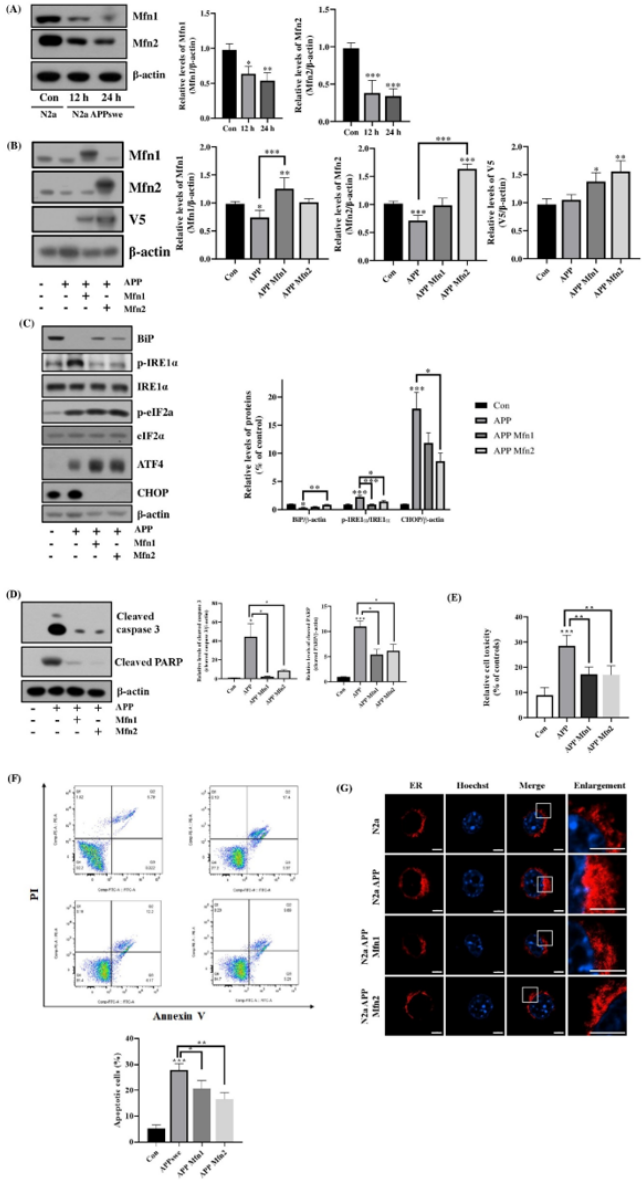

Fig. 2. Upregulation of Mfn1 and Mfn2 reduced Aβ-mediated ER stress and apoptotic cell death in N2a APPswe cells. (a) Western blot analysis of the levels of Mfn1 and Mfn2 in N2a and N2a APPswe cells. (b) Western blot analysis of the levels of Mfn1, Mfn2, and V5 in N2a, APPswe, and Mfn1/2-overexpressing APPswe cells. (c) Western blot analysis of ER-stress related proteins (BiP, p-eIF2α, eIF2α, p-IRE1α, IRE1α, ATF4, and CHOP) in cells cultured for 12 h. The graphs depict the quantification of BiP/β-actin, p-IRE1α/IRE1α, p-eIF2α/eIF2α, ATF4/β-actin, and CHOP/β-actin. (d) The levels of cleaved caspase-3 and cleaved PARP were evaluated by western blotting. The graphs present the quantification of cleaved caspase-3/β-actin and cleaved PARP/β-actin. (e) The cell toxicity levels were measured using LDH assay. (f) Flow cytometry analysis of cell apoptosis using Annexin V-FITC/PI staining. The ratio of apoptosis was early apoptosis percentage plus late apoptosis percentage. (g) ER morphologies of N2a, APPswe, and Mfn1/2-overexpressing APPswe cells by confocal microscopy; scale bar = 5 μm. The data are presented as means ± the SD (n ≥ 3). *p < 0.05, **p < 0.01, and ***p < 0.001.

4. Discussion

In this study, we obtained evidence that an increase in Mfn levels causes a reduction in the ER stress triggered by AβO in cells expressing APP swedish mutation. The failure of proteostasis is associated with several neurodegenerative disorders, including Alzheimer's disease (AD), Parkinson's disease, and Huntington's disease [19]. ER stress can be induced by Aβ [4]. GRP78, also known as BiP, is known to regulate UPR signaling. In many cases, ER stress upregulates BiP in response to the accumulation of misfolded proteins. The BiP protein activates PERK and IRE1 through autophosphorylation. The activation of PERK then leads to the phosphorylation of eIF2α. IRE1 activation induces XBP1 mRNA splicing [6]. However, our results indicated that BiP protein levels decreased in a time-dependent manner (Figure 2a). Moreover, BiP protein levels increased gradually until 12 h but decreased after 12 h (data not shown). N2a APPswe cells, which continuously express Aβ, may have a state of chronic stress. Some

previous studies found that chronic ER stress downregulates BiP but increases the levels of other UPR related proteins [20, 21]. In addition, BiP silencing increases apoptosis by upregulating UPR signaling [22]. These findings are consistent with our observation that AβO can increase CHOP levels and induce apoptosis, which were concomitant with the decrease in BiP levels.

Several lines of evidence suggest that AβO also induces mitochondrial fragmentation, which is related to neuronal cell death in AD models [23]. Loss of Mfn2 results in an increase in oxidative stress and the degeneration of dopaminergic neurons [24]. In this study, the accumulation of AβO in N2a APPswe cells resulted in reduced levels of both Mfn1 and Mfn2 (Figure 3a). Although Mfn1 and Mfn2 are mitochondrial proteins, also mediate tethering between mitochondria and the ER [9]. Therefore, to confirm whether Mfn1 and Mfn2 affect ER stress in a state of AβO-accumulation, we overexpressed both Mfn1 and Mfn2. The overexpressing of Mfn1 and Mfn2, to reverse their reduced expression levels in response to AβO accumulation, resulted in reduction in the levels of p-IRE1α and CHOP (Figure 3c). In addition, Mfn1 and Mfn2 overexpression reduced ER stress induced apoptosis (Figures 3d and 3f). We initially hypothesized that only Mfn2, and not Mfn1, affects the ER stress response, as some studies have demonstrated that the loss of Mfn2 upregulates ER stress [25]. However, our results indicated that both Mfn1 and Mfn2 reduced ER stress and apoptosis. CHOP is regulated by the PERK-eIF2α-ATF4 pathway. In addition to PERK, ATF6 and IRE1 are also known to regulate the expression of CHOP along with XBP1 [7, 26, 27]. Thus, AβO-mediated apoptosis is likely induced by CHOP. Mfn2 has been reported to regulate mitochondrial fusion as well as ER morphology [25]. Therefore, we observed ER morphology after Mfn1 and Mfn2 overexpression, the results indicated that ER expansion was reduced to the level observed in the control cells (Figure. 3g). Mfn1 and Mfn2 have different roles in mitochondrial regulation [8, 28]. However, we found that both these Mfn proteins could reduce ER stress. A previous study demonstrated that Mfn1 and Mfn2 are ubiquitinated via the Parkin/Pink1-mediated pathway [29]. Notably, the ubiquitination of Mfn2 is known to regulate ER-mitochondrial tethering [30]. Moreover, Mfn1 regulates the ER-mitochondria contact sites through fusion activity [31]. Collectively, these results suggest that Mfn1 and Mfn2 form heterodimers [32], and thus, Mfn1 can affect the ER through the regulation of Mfn2 or mitochondrial function. The mitochondrial network is highly associated with mitochondrial dynamics. Our previous study showed that morphological changes in the ER, including expansion are related to mitochondrial dynamics [33]. In addition, ER stress induces both ER and mitochondrial expansion, accompanied by an increase in the number of ER-mitochondria contact sites in yeast [34]. Taken together, these finding demonstrate that ER stress increases ER expansion and ER-mitochondria contact, which in turn affects the mitochondrial network. However, further studies are required to elucidate the connection between ER stress and mitochondrial morphology and dynamics. Additionally, the underlying relationship between Mfn1 and AβO-mediated ER stress should be investigated. We confirmed that both Mfn1 and Mfn2 reduce the ER stress and apoptosis triggered by AβO. Mfn proteins are known to affect mitochondrial dynamics. In addition, ER-associated degradation and the ER tubules are also directly associated with mitochondrial dynamics [35]. Taken together, our finding indicate that the Aβ-mediated decrease in Mfn levels exacerbates the imbalance in mitochondrial dynamics via ER stress.

5. Conclusion

In conclusion, our findings indicate that the overexpression of Mfn1 and Mfn2 ameliorates AβO-mediated ER stress and apoptosis. Based on the results of our study, the characterization of Mfn may help reveal the relationship between the ER and mitochondria. The results of this study provide a basis for the identification of novel molecular mechanisms targeting AβO-mediated stress and the pathogenesis of AD.

Highlights

Aβ increased ER stress and promoted ER expansion in N2a APPswe cells.
Mfn1 and Mfn2 overespression in N2a APPswe cells reduced AβO-mediated ER stress.
Mfn1 and Mfn2 reduced N2a APPswe cell apoptosis.

Conflict of interests

The authors declare that they have no conflicts of interest.

Consent for publications

All authors have read and approved the final manuscript for publication.

Ethics approval and consent to participate

No human participants or aminals were involned in the present research.

Availability of data and material

Data generated or analyzed during this study are provided in full within the published article.

Authors' contributions

Min Kyoung Kam, Su-Min Jung and Ga Eun Lee contributed equally to this work. Min Kyoung Kam, Su-Min Jung and Ga Eun Lee conceived the study, performed cell-based experiments, western blotting analysis, and confocal imaging, collected and interpreted the data, and compiled the manuscript. Sung Woo Lee designed the study and performed cell-based experiments. Hong Jun Lee and Young-Ho Park designed the study and secured financial support. Dong-Seok Lee conceived and designed the study, analyzed and interpreted the data, wrote the manuscript, secured financial support, and approved the manuscript.

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