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RESEARCH PAPER

Inhibition of Endoplasmic Reticulum Stress-induced Apoptosis by Silkworm Storage Protein 1

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Abstract The endoplasmic reticulum (ER) plays essential roles indispensable for cellular activity and survival, including functions such as protein synthesis, secretory and membrane protein folding, and Ca^{2+} release in cells. The ER is sensitive to stresses that can lead to the aggregation and accumulation of misfolded proteins, which eventually triggers cellular dysfunction; severe or prolonged ER stress eventually induces apoptosis. ER stress-induced apoptosis causes several devastating diseases such as atherosclerosis, neurodegenerative diseases, and diabetes. In addition, the production of biopharmaceuticals such as monoclonal antibodies requires the maintenance of normal ER functions to achieve and maintain the production of high-quality products in good quantities. Therefore, it is necessary to develop methods to efficiently relieve ER stress and protect cells from ER stress-induced apoptosis. The silkworm storage protein 1 (SP1) has anti-apoptotic activities that inhibit the intrinsic mitochondrial apoptotic pathway. However, the role of SP1 in controlling ER stress and ER stress-induced apoptosis has not been investigated. In this paper, we demonstrate that SP1 can inhibit apoptosis induced by a well-known ER stress inducer, thapsigargin, by alleviating the decrease in cell viability and mitochondrial membrane potential. Interestingly, SP1 significantly blocked increases in CHOP and GRP78 expression as well as ER Ca^{2+} leakage into the cytosol following ER stress induction. This indicates that SP1 protects cells from ER stress-induced apoptosis by functioning as an upstream inhibitor of apoptosis. Therefore, studying SP1 function can offer new insights into protecting cells against ER stress-induced apoptosis for future applications in the biopharmaceutical and medicine industries.

Keyword: endoplasmic reticulum (ER), ER stress, apoptosis, silkworm, storage protein 1

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1. Introduction

The endoplasmic reticulum (ER) is a membranous network within cells that plays a key role in several cellular functions, including protein synthesis, secretory and membrane protein folding, lipid biogenesis, and Ca^{2+} sequestration [1,2]. Efficient ER function is essential for most cellular activities and cell survival. The ER lumen contains numerous proteins such as chaperones, folding enzymes, oxidizing enzymes, and glycosylation enzymes, which are involved in the folding and posttranslational modification of newly synthesized proteins [3]. Many of these proteins' functions are Ca^{2+}-dependent, and hence the ER is highly sensitive to stresses that disturb Ca^{2+} levels such as Ca^{2+} depletion and hypoxia, or N-terminal glycosylation [1]. Such stresses lead to the aggregation and/or accumulation of misfolded or unfolded proteins, triggering the dislocation of a 78-kDa glucose-regulated protein (GRP78, also known as BiP), an ER chaperonin, from the ER membrane where it is bound to three ER transmembrane receptors. Once dislocated, GRP78 enters the ER lumen and induces the unfolded protein response (UPR) [4]. Therefore, GRP78 expression is widely considered as a marker for ER stress [5,6]. The UPR is a pro-survival response to alleviate the accumulation of unfolded proteins and restore regular ER function [7]. However, signaling through ER transmembrane receptors can also trigger pro-apoptotic signals. During severe and prolonged ER stress, this signaling initiates the activation

of downstream molecules such as the C/EBP-homologous protein (CHOP) [8]. CHOP ends the anti-apoptotic effect of Bcl-2 by interrupting the expression of Bcl-2 [9]. Overexpression of CHOP has been shown to induce cell death associated with the activation and mitochondrial translocation of Bax [10]. In addition, an extended period of elevated matrix levels of Ca^{2+} beyond a critical threshold is the strongest factor for triggering pro-apoptotic mitochondrial membrane permeabilization that results in the release of cytochrome c from the mitochondria [11]. The release of cytochrome c from the mitochondria is a key event in the intrinsic mitochondrial apoptotic pathway and is hypothesized to be mediated by mitochondrial permeability transition during ER stress-induced apoptosis [12,13]. Thus, cell death induced by prolonged or severe ER stress mainly occurs via the intrinsic mitochondrial apoptotic pathway. In the biopharmaceutical industry, growth-inhibiting conditions such as nutrient deprivation, shear stresses, hypoxia, and elevated pCO_2 can cause ER stress in cells that can promote apoptosis [14]. Reduction in cell viability due to ER stress-mediated apoptosis limits recombinant protein production. Thus, maintaining ER homeostasis is critical for cellular function and cell survival.

Recently, we have reported that silkworm storage protein 1 (SP1) exhibits anti-apoptotic effects [15]. A stable cell line expressing SP1 and *E. coli*-produced recombinant SP1 showed resistance to apoptosis induced by staurosporine (STS), an inducer of intrinsic mitochondria-mediated apoptosis [16,17]. However, the inhibitory effect of SP1 on ER stress-induced apoptosis has not yet been investigated. Since apoptotic signals from the ER are upstream of apoptotic signals from the mitochondria, we hypothesized that SP1 could inhibit ER stress and thereby control the downstream effects of mitochondrial damage. In this study, we investigated the inhibitory effect of silkworm SP1 on ER stress induced by thapsigargin (Tg). We found that SP1 inhibits apoptosis by maintaining Ca^{2+} homeostasis and suppressing GRP78 and CHOP expression, all of which are initial steps in the induction of ER stress-mediated pro-apoptotic signals.

2. Materials and Methods

2.1. Cell culture and treatment

Recombinant HeLa cells expressing SP1 (HeLa/SP1) and control cells lacking SP1 expression (HeLa/CTRL) were constructed based on the methods described in our previous report [15]. Only difference is that pcDNA3.1 plasmid (Thermo Fisher Scientific, USA) was used instead of pEGFP-N1 plasmid for SP1 expression. As not all the cell lines have the same level of anti-apoptotic effect probably due to the different SP1 expression level, the cell line that has the most anti-apoptotic resistance was chosen as the final cell line. The cells were maintained in Dulbecco's Modified Eagle's medium (DMEM) (Biowest, France) containing 10% fetal bovine serum (FBS) (Young In Frontier, Korea) and 1% penicillin/streptomycin (Life Technologies, USA) in a humidified tissue culture incubator maintained at 37°C and 5% CO_2. Thapsigargin (Tg) (Sigma, USA) was dissolved in dimethyl sulfoxide (DMSO) (Duchefa, Netherlands) and used at different concentrations for each experiment.

2.2. Analysis of apoptosis by trypan blue staining, flow cytometry, and fluorescence microscopy

Cells were seeded onto 24-well plates at a density of 3×10^4 cells/cm^2 and treated with 10 μM of Tg. Dead and live cells were counted by the trypan blue dye exclusion method using a hemocytometer. For flow cytometry, cells were stained with 10 μg/mL propidium iodide (PI) (Sigma, USA), and fluorescence levels were analyzed with a flow cytometer (Beckman Coulter, Inc.). To examine nuclear condensation and fragmentation, which are the major features of apoptosis, cells were visualized by fluorescence microscopy after staining with 2 μg/mL of Hoechst 33342 fluorescent dye.

2.3. Measurement of mitochondrial membrane potential

To check mitochondrial function, cells were treated with 3 μM of Tg for 8 h and stained with 100 μL/well of the prepared Mito-ID® MP Dye Loading Solution (Mito-ID® Membrane Potential Cytotoxicity Kit, Enzo, USA) for 30 min at 37°C. Fluorescence was measured with excitation at 490 nm and emission at 580 nm using Varioskan™ Flash Multimode Reader (Thermo Scientific, USA).

2.4. Quantitative real-time PCR (qPCR) analysis

Total RNA from cells was isolated with Trizol reagent (Favorgen, Taiwan), and the first-strand of cDNA was synthesized with the ReverTra Ace qPCR RT master mix (TOYOBO, Japan). Primers in this study were synthesized by Cosmogenetech (Korea) and the sequences were: 5′-ACT GTT ACA ATC AAG GTC TAT GAA GG-3′ (forward primer) and 5′-CAA AGG TGA CTT CAA TCT GTG G-3′ (reverse primer) for GRP78, 5′-AAG GAA AGT GGC ACA GCT-3′ (forward primer) and 5′-CTG GTC AGG CGC TCG ATT-3′ (reverse primer) for CHOP, and 5′-GGG AGC CAA AAG GGT CAT-3′ (forward primer) and 5′-GAG GGG CCA TCC ACA GTC-3′ (reverse primer) for GAPDH. All qPCRs were performed on the Step One Plus real-time PCR system (Applied

Biosystem, USA) with SYBR Green PCR kit (Qiagen, Germany), and the relative mRNA levels were calculated using the $2^{-\Delta\Delta Ct}$ method.

2.5. Western blotting

Cells were lysed using RIPA buffer (Elpis biotech, Korea) with protease inhibitors (Thermo Scientific, USA). Following extraction, 20 μg proteins of each sample were separated by polyacrylamide SDS gel and transferred onto a PVDF membrane (Bio rad, USA). The membrane was then blocked for 2 h in Tris-buffered saline with 0.1% tween 20 (TBS-T) containing 5% skim milk and incubated with monoclonal mouse anti-CHOP antibody (Cell Signaling Technology, USA), monoclonal rabbit anti-BiP (GRP78) antibody (Cell Signaling Technology, USA) and monoclonal rabbit anti-GAPDH antibody (Cell Signaling Technology, USA) at 4°C overnight. The membranes were washed using TBS-T and incubated with anti-mouse IgG, HRP-linked antibody (Abcam, USA) or anti-rabbit IgG, HRP-linked antibody (Cell Signaling Technology, USA) for 1 h at room temperature. Finally, the protein bands were visualized using ECL reagent (GE healthcare, UK).

2.6. Measurement of intracellular free Ca^{2+} levels

Since Tg disrupts Ca^{2+} homeostasis in cells, the free Ca^{2+} concentrations in the cytosol of Tg-treated cells were examined. HeLa cells treated with 10 μM of Tg for 1 h were stained with 5 μM of the Ca^{2+}-sensitive dye Fluo-3, AM (Invitrogen, USA) for 30 min at 37°C. After washing, the cells were incubated with Ca^{2+}-free Dulbecco's PBS (Biowest, France) for another 30 min at room temperature for de-esterification. Fluorescence was measured by excitation at 506 nm and emission at 526 nm using Varioskan™ Flash Multimode Reader.

3. Results and Discussion

3.1. Inhibition of ER stress-induced apoptosis by SP1

Prolonged exposure of cells to ER stress is known to result in apoptosis. To investigate if SP1 can inhibit apoptosis caused by ER stress, a cell line stably expressing SP1 (HeLa/SP1) was constructed and used for experiments. Cell viabilities were measured using trypan blue exclusion assay before and after thapsigargin (Tg) treatment. Tg activates the UPR by disrupting Ca^{2+} homeostasis in the ER. As shown in Fig. 1A, treatment with 10 μM of Tg drastically reduced viability in HeLa/CTRL to 84.3% at 24 h post-treatment, which further decreased to 45.6% at 48 h post-treatment. On the contrary, cell viability after Tg treatment in HeLa/SP1 decreased only to 93.4 and 80.8% at 24 and 48 h post-treatment, respectively. This result indicates that SP1 is capable of inhibiting ER stress-induced apoptosis. Further assessment of the effect of SP1

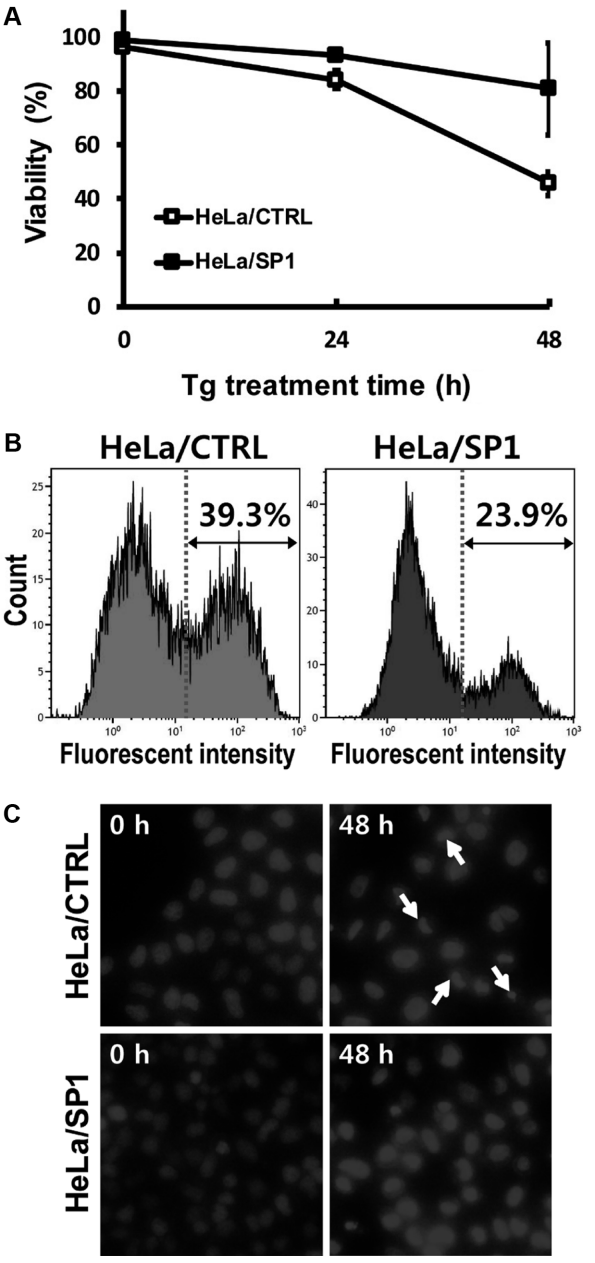

Fig. 1. Effect of SP1 on ER stress-induced apoptosis. HeLa cell lines not expressing SP1 (HeLa/CTRL) and those stably expressing SP1 (HeLa/SP1) were treated with or without 10 μM Tg for various periods of time. (A) Cell viability was measured at 0, 24, and 48 h after Tg treatment (all values are mean values ± SD, n = 3) (B) HeLa/CTRL (Left) and HeLa/SP1 (Right) cells were stained with propidium iodide (PI) at 0 and 80 h after Tg treatment. Cells with higher fluorescence intensity than that indicated by the dotted line were counted as dead cells. (C) Cells were treated with Tg for 48 h and analyzed for anti-apoptotic effect by Hoechst 33342 staining for the observation of nuclear fragmentation. Arrows indicate fragmented apoptotic nuclei. (C) Cells were stained with PI at 0 (left) and 15 h (right) after the STS treatment. The cells with fragmented nuclei were counted as apoptotic cells.

on inhibiting ER stress-induced apoptosis was carried out using flow cytometry (Fig. 1B). When cells were treated with Tg for 80 h, stained with PI, and analyzed *via* flow cytometry, 39.3% of HeLa/CTRL cells were found to be dead while only 23.9% of HeLa/SP1 cells were dead. This indicates that HeLa/SP1 showed an enhanced resistance to ER stress-induced apoptosis. As nuclear fragmentation is an important feature of the apoptotic process, we checked for signs of nuclear fragmentation in Tg-treated HeLa/CTRL and HeLa/SP1 cell lines *via* fluorescence microscopy. After 48 h of Tg treatment, many HeLa/CTRL cells were observed with small fragmented nuclei, whereas only a few HeLa/SP1 cells showed fragmented nuclei (Fig. 1C). Overall, these results clearly indicate that SP1 can inhibit ER stress-induced apoptosis.

3.2. Effect of SP1 on Tg-induced collapse of mitochondrial membrane potential

Mitochondrial membrane potential collapse is an apoptotic event that occurs upstream of nuclear fragmentation [18]. Once we had established that SP1 inhibited nuclear fragmentation, we further investigated the effects of SP1 on mitochondrial membrane potential collapse, which is an event upstream of nuclear fragmentation during ER stress-induced apoptosis. The mitochondrial membrane potentials of HeLa/CTRL and HeLa/SP1 cells treated with Tg for 0 or 8 h and stained with the Mito-ID® dye were analyzed using a fluorometer (Fig. 2). In the absence of SP1, mitochondrial membrane potential decreased to 48.0% after 8 h of Tg treatment as compared to that in untreated cells. In contrast, mitochondrial membrane potential levels were significantly higher at 65.2% in Tg-treated cells expressing SP1, than in cell lines not expressing SP1. These results indicate that SP1 is capable of inhibiting the collapse of mitochondrial membrane potential during ER stress-induced apoptosis.

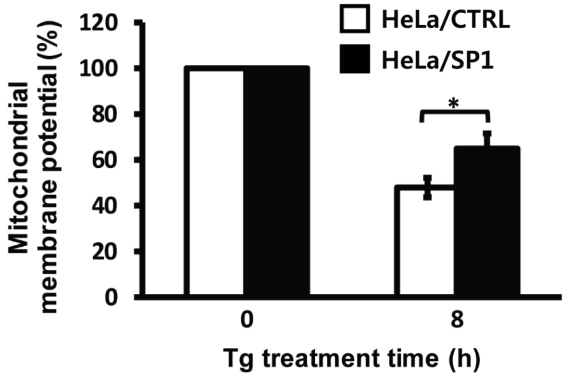

Fig. 2. Effect of SP1 on collapse of mitochondrial membrane potential induced by ER stress. Cells were cultured and treated with Tg for 0 and 8 h, following which, mitochondrial membrane potentials were measured by a fluorometer (all values are mean values ± SD, n = 3, $*p < 0.05$). Note that cells expressing SP1 showed higher mitochondrial membrane potential than cells without SP1 expression.

3.3. Regulation of CHOP and GRP78 expression levels by SP1 after ER stress induction

Induction of ER stress leads to increased expression levels and release of GRP78, which in turn triggers pro-apoptotic signals, especially when cells experience severe and/or prolonged ER stress. This is mediated by increases in levels of CHOP expression, which eventually leads to intrinsic mitochondria-dependent apoptosis. Since SP1 seems to inhibit ER stress-induced apoptosis by protecting cells against mitochondrial membrane potential collapse, it is plausible to hypothesize that SP1 enhances cellular resistance to ER stress by suppressing the expression of CHOP and/or GRP78. Therefore, we analyzed the expression of these genes by qPCR before and after Tg-treatment in HeLa/CTRL and HeLa/SP1 cell lines. Basal levels of CHOP in both cell lines were almost the same in the absence of Tg-induced ER stress (Fig. 3A). Tg treatment of HeLa/CTRL cells caused a 47.3-fold increase in CHOP expression levels. However, the increase in CHOP expression levels was significantly suppressed by the presence of SP1, and HeLa/SP1 only showed a 21.8-fold increase in CHOP expression levels after Tg treatment. The CHOP protein expression levels were also analyzed by Western blot (Fig. 3B). CHOP protein expression was drastically increased upon Tg treatment in the absence of SP1 expression. However, the level increase was slightly lower in HeLa/SP1 than that in HeLa/CTRL. All of these results demonstrate that SP1 can inhibit ER stress-induced apoptosis by inhibiting upstream event of CHOP expression and this subsequently inhibits CHOP expression as a result.

GRP78 expression can be induced by a variety of physiological and environmental stress conditions that disturb ER function and homeostasis [19,20]. In addition, the transcriptional activation of the gene encoding for GRP78 in culture system is used as an indicator for ER stress and the initiation of the UPR [21]. Thus, the effect of SP1 on the expression of GRP78, an ER stress marker and upstream effector of CHOP expression, post ER stress induction was also analyzed. As expected, there were no significant differences in GRP78 mRNA and protein expression levels in the absence of ER stress in either HeLa/CTRL or HeLa/SP1 cell lines (Figs. 3C and 3D). Post Tg treatment, GRP78 mRNA levels drastically increased (8.4-fold) in HeLa/CTRL cells, whereas HeLa/SP1 cells exhibited only a 3.3-fold increase in GRP78 expression. The increase in GRP78 protein level was also suppressed in HeLa/SP1 as compared to that in HeLa/CTRL. This finding supports the hypothesis that SP1 inhibits increases in CHOP expression by reducing GRP78 expression levels after ER stress induction, which

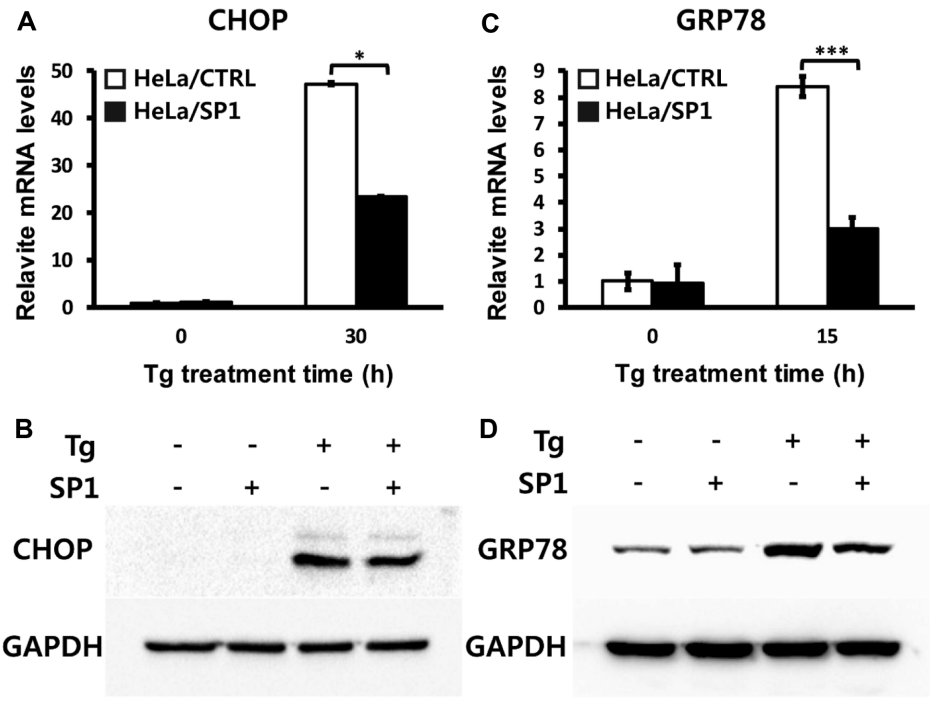

Fig. 3. Cellular mRNA and protein levels of genes affected by ER stress, as analyzed by qPCR (A, C) or Western blot (B, D) in HeLa/CTRL and HeLa/SP1 cells after Tg treatment. (A, C) CHOP (Left) and GRP78 (Right) mRNA levels were measured and normalized by actin mRNA levels in each cell line at the indicated time intervals (all values are mean ± SD, n = 3, $*p < 0.05$). Although both CHOP and GRP78 levels drastically increased during ER stress, SP1 expression significantly alleviated the increased expression of these genes. (B, D) CHOP (Left) and GRP78 (Right) protein levels were analyzed in each cell line. GAPDH was used as internal control.

subsequently inhibits apoptosis. From our results, it is clear that SP1 protects cells from ER stress-induced apoptosis by regulating an upstream event, namely, GRP78 expression.

3.4. Effect of SP1 on intracellular Ca^{2+} levels

Since the ER is involved in Ca^{2+} homeostasis, exposing cells to ER stresses leads to altered Ca^{2+} homeostasis. Tg treatment induces the release of ER-stored Ca^{2+} into the cytosol, affecting the Ca^{2+} balance in cells to trigger protein aggregation [22]. Since SP1 expression in cells inhibits upstream cellular responses to ER stress, including increases in GRP78 expression, we wanted to check if SP1 also modulates ER Ca^{2+} levels and blocks the leakage of Ca^{2+} from the ER into the cytosol. As the leakage of Ca^{2+} from the ER is an early event in ER stress-induced apoptotic pathway, cytosolic Ca^{2+} level was assessed at the early time point as compared to other events shown above. Cells treated with Tg for 1 h were loaded with Fluo-3, AM to assess cytosolic Ca^{2+} levels. As shown in Fig. 4, there was a significant increase in cytosolic Ca^{2+} levels in HeLa/CTRL cells, indicating an efflux of Ca^{2+} from the ER. On the contrary, cytosolic Ca^{2+} levels did not show any increase in HeLa/SP1 cells, even after exposure to Tg. This result demonstrates that SP1 maintains ER Ca^{2+} levels and helps to maintain Ca^{2+} homeostasis in cells, which subsequently protects cells from progressing to ER stress-induced apoptosis.

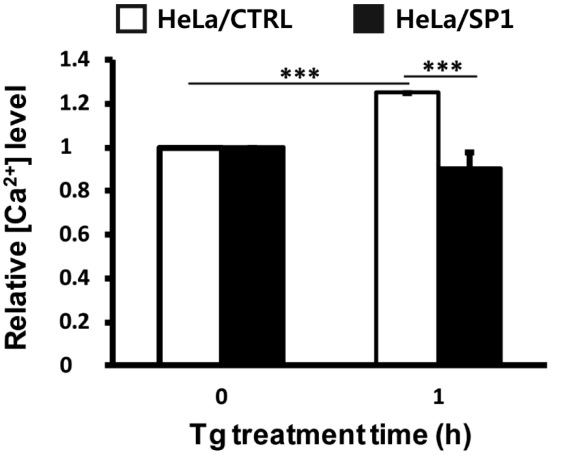

Fig. 4. Cytosolic calcium (Ca^{2+}) levels before and after Tg treatment. Cells treated with Tg for 0 and 1 h were analyzed for cytosolic Ca^{2+} levels with Fluo-3, AM dye. Increased Ca^{2+} levels in the cytosol indicate decreased ER Ca^{2+} levels. While the cytosolic Ca^{2+} level increased significantly after ER stress induction in HeLa/CTRL cells, there was no increase in cytosolic Ca^{2+} levels in HeLa/SP1 cells (all values are mean ± SD, n = 4, $***p < 0.001$).

To rule out the possibility that SP1 only inhibits ER stress-induced apoptosis caused by Tg treatment, cells

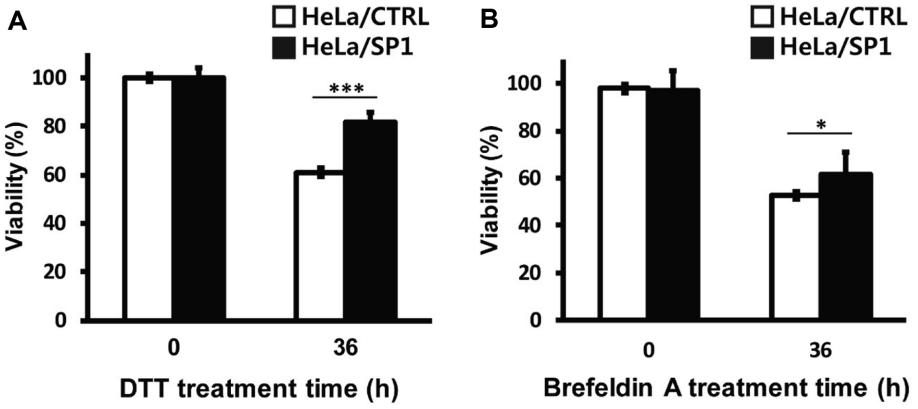

Fig. 5. Effect of SP1 on apoptosis induced by other types of ER stress inducers. HeLa cell lines not expressing SP1 (HeLa/CTRL) and those stably expressing SP1 (HeLa/SP1) were treated with (A) 2 mM DTT or (B) 2 μM Brefeldin A. Cell viability was measured at 0 and 36 h after treatment using trypan blue exclusion assay (all values are mean values ± SD, n = 3, $*p < 0.05$, $***p < 0.001$).

were treated with other types of ER stress inducers to investigated the anti-apoptotic effect of SP1. Cell viabilities were measured using trypan blue exclusion assay before and after 2 mM of DTT or 2 μM of Brefeldin A treatment. DTT blocks disulfide-bond formation, and this leads to ER stress. Brefeldin A induces the accumulation of unfolded proteins in the ER by inhibiting protein transport from ER to Golgi. As shown in Fig. 5A, treatment with DTT drastically reduced cell viability in HeLa/CTRL to 60.9% at 36 h post-treatment. On the contrary, cell viability after treatment in HeLa/SP1 decreased only to 81.4% at 36 h post-treatment. The similar anti-apoptotic effect of SP1 was observed when Brefeldin A was used as ER stress inducer (Fig. 5B). These results indicate that the effect of SP1 is not limited to one kind of ER stress inducer and can be recognized as a general inhibitor of ER stress-induced apoptosis.

GRP78 dislocation and expression are both crucial events in the cells suffering ER stress. In the absence of stress, GRP78 binds to IRE1α, PERK, and ATF6. Upon Tg treatment, the dislocation of GRP78 and the accumulation of unfolded proteins in ER activate UPR. The GRP78 dislocation is required to act as a molecular chaperone to those accumulated proteins, and ATF6 activation after GRP78 dislocation increases GRP78 protein expression level to upregulate protein folding capacity. Thus, both GRP78 dislocation and expression are closely related events in ER stress-mediated cellular events. From our results, SP1 inhibited Ca^{2+} leakage from ER after Tg treatment and suppressed GRP78 mRNA and protein expression levels. We assume that the inhibition of Ca^{2+} leakage by SP1 subsequently suppressed GRP78 dislocation from ER membrane. As Ca^{2+} leakage after Tg treatment is also known to increase the GRP78 expression [23], the inhibition of Ca^{2+} leakage may also reduce the amount of dislocated GRP78 protein levels, which in turn indirectly inhibits CHOP expression. Further studies should be followed to explore the exact mechanism of SP1's effect on the Ca^{2+} leakage and GRP78 dislocation.

Perturbations such as protein misfolding can disrupt normal ER function to generate ER stress. Cells have the ability to cope with such stresses for short periods of time, but severe or prolonged ER stress eventually promotes CHOP-mediated apoptosis usually *via* the mitochondrial apoptotic pathway. ER stress and ER stress-induced apoptosis can cause several devastating conditions such as atherosclerosis, neurodegenerative diseases, and diabetes [24,25]. In the biopharmaceutical industry, the production of high quality and quantity of therapeutic proteins such as monoclonal antibodies, EPO (erythropoietin), and fusion proteins in bioreactors requires that normal ER functions are maintained during cell culture [26]. In this context, it is crucial to develop methods that can efficiently relieve ER stress and protect cells from ER stress-induced effects. Silkworm SP1 has shown interesting biological activities that block mitochondria-dependent intrinsic apoptotic pathways in mammalian cells. The anti-oxidative effects of SP1 were also demonstrated and it was found that SP1 protected cells from oxidative stress and enhanced the production of superoxide dismutase 1 (SOD1) [16,17]. In this study, we report that SP1 can inhibit cells from undergoing ER stress-induced apoptosis. We used Tg to induce ER stress by specifically inhibiting ER Ca^{2+}-ATPase, which activates GRP78 transcription through the depletion of Ca^{2+} in the ER [19]. In protecting cells from ER stress, SP1 acts as an upstream inhibitor of apoptosis, as is indicated by its ability to inhibit ER Ca^{2+} depletion and GRP78 expression after Tg treatment. SP1 can be highly beneficial in maintaining normal ER function, including protein folding when cells are under stress, since

SP1 not only inhibits ER stress-induced apoptosis but also relieves ER stress itself. Further functional studies to discover the detailed molecular mechanism by which SP1 acts to inhibit ER stress-induced apoptosis will enhance the practical applications of this protein.

4. Conclusion

ER stress is caused by a variety of conditions that disturb protein folding in the ER, and prolonged ER stress can lead to the destruction of cells by apoptosis. The alleviation of ER stress and the protection of cells from ER stress-induced apoptosis are required for the development of therapeutics for ER stress-related diseases. Furthermore, the biopharmaceutical industry requires solutions to ER stress in mammalian cell cultures that severely affects the production quantity and quality of biological products. In this study, we demonstrate for the first time that SP1 is an inhibitor of ER stress-mediated apoptosis and acts as an upstream regulator in ER stress responses by suppressing ER Ca^{2+} release and increases in GRP78 expression. In light of its ER stress-induced anti-apoptotic activities, SP1 can offer new solutions to combat ER stress in cell cultures that can be widely applied in the medicine and biopharmaceutical industries.

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