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Tunicamycin-Induced ER Stress Upregulates the Expression of Mitochondrial HtrA2 and Promotes Apoptosis Through the Cytosolic Release of HtrA2

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Recent studies provide some evidence that the HtrA2 protein is intimately associated with the pathogenesis of neurodegenerative disorders and that endoplasmic reticulum (ER) quality control and ER stress-associated cell death play critical roles in neuronal cell death. However, little is known about the intimate relationship between HtrA2 and ER stress-associated cellular responses. In the present study, we have demonstrated that the HtrA2 protein level was gradually and significantly increased by up to 10-fold in the mitochondria under tunicamycin (Tm)-induced ER stress, which eventually promoted cell death through the release of HtrA2 into the cytoplasm. Using an ecdysone-inducible mammalian expression system, we demonstrate that the extent of cell death in 293-HtrA2 cells was approximately 20 times higher under Tm-induced ER stress, indicating that the increase in the HtrA2 protein level in the mitochondria itself is necessary but not sufficient for the promotion of cell death. Taken together, these results suggest that HtrA2 may serve as a mediator of ER stress-induced apoptosis and ER-mitochondrial cross-talk in some cellular processes.

Keywords: Apoptosis, tunicamycin, HtrA2, ER stress

The endoplasmic reticulum (ER) is an essential intracellular organelle responsible for the quality control of secretory, transmembrane, and ER-resident proteins in order to facilitate proper protein folding and maturation within the ER lumen and the transport of the native proteins to their final destinations [16, 30]. Multiple conditions that adversely affect the ER protein folding process, including nutrient deprivation, disturbances in the intracellular redox equilibrium and concentration of calcium ion, and the failure of post-translational modifications, such as *N*-linked glycosylation, lead to the accumulation of unfolded or misfolded proteins in the ER, thereby causing ER stress [8]. Cells subsequently react to the stress on the ER and activate a series of signal transduction cascades, collectively termed the ER stress response or unfolded protein response (UPR), as self-defense mechanisms [15, 34, 38].

The intrinsic pathway of the UPR consists of tripartite signals that involve general translational attenuation, the transcriptional induction of ER chaperone genes, such as binding protein (BIP)/78-kDa glucose-regulated protein (GRP78), and the activation of ER-associated degradation (ERAD) [15, 34, 38, 45]. These cellular responses enable the cells to reduce the unfolded or misfolded protein load in the ER, thereby alleviating ER stress and promoting cell survival [16, 34, 38]. However, severe or prolonged ER stress leads to the induction of the apoptotic cell death pathway *via* mitochondria-dependent or -independent mechanisms [22, 27].

Several lines of evidence suggest that ER-mediated apoptosis may play a critical role in the pathogenesis of several neurodegenerative disorders [18, 28]. Genetic studies have demonstrated that *mnd*2 (motor neuron degeneration) mice with a substitution of cysteine for the active serine at residue 276 of the HtrA2 protease domain exhibited progressive loss of striatal neurons, which eventually led to motor dysfunction [6, 35]. A previous study using an HtrA2 deficiency mouse model also revealed some neurological phenotypes with selective loss of striatal neurons [14]. Moreover, the substitution of serine for glycine at residue 399 (G399S) of HtrA2 has been identified in some patients with Parkinson's disease (PD), but not in healthy control subjects [21]. These studies provide some evidence that HtrA2 is intimately associated with the pathogenesis of neurodegenerative disorders, such as PD.

Recent reports have shown that ER stress can affect not only the ER, but also the mitochondria [10, 12]. ER stress

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induced by the ER Ca^{2+}-ATPase inhibitor thapsigargin was reported to induce a Bax-dependent apoptotic pathway by modulating the cytosolic release of mitochondrial proapoptotic molecules, such as cytochrome *c*, Smac/DIABLO, and HtrA2 [43]. In the case of HtrA2, the subcellular localization of HtrA2 is critical to the execution of its dual functions: the regulation of mitochondria biogenesis under normal conditions, and the induction of apoptosis *via* caspase- or serine protease-dependent pathways in response to various cellular stresses, such as staurosporine, anti-Fas antibodies, TRAIL, UV irradiation, or recombinant BID [11, 13, 20, 36, 40, 42]. These studies raised the possibility that HtrA2 may play a role in the cross-talk between the ER and mitochondria during ER stress-mediated apoptosis; however, the relationship between HtrA2 and ER stress has not yet been identified.

In the present study, we have demonstrated that tunicamycin (Tm)-induced ER stress not only increases the level of the HtrA2 protein in the mitochondria, but it also causes the cytosolic release of the resulting HtrA2 protein, and both events are necessary for ER stress-mediated apoptosis. Our study therefore suggests that HtrA2 may play a role in regulating the cross-talk between the ER and mitochondria and that it may serve as an important mediator of cell death induced by ER stress.

Materials and Methods

Chemicals and Antibodies

All chemicals were purchased from Sigma Chemical Co. (St Louis, MO, U.S.A.), unless otherwise stated. The antibodies (Abs) used for the immunoblot (IB) analyses were anti-HtrA2 (Koma Biotech., Korea), anti-GRP78 (Santa Cruz Biotech., Santa Cruz, CA, U.S.A.), anti-Hsp60 (Santa Cruz Biotech.), anti-cytochrome *c* (Santa Cruz Biotech.), anti-beta actin (Sigma), and anti-FLAG-M2 (Sigma).

Mammalian Cell Culture and Tunicamycin Treatment

Human embryonic kidney (HEK) 293T and 293 cells were purchased from American Type Culture Collection (ATCC, Manassas, VA, U.S.A.) and maintained in DMEM (Invitrogen, Carlsbad, CA, U.S.A.) supplemented with 8% heat-inactivated FBS (Invitrogen) and penicillin (100 U/ml)/streptomycin (100 μg/ml) and grown at 37°C in a humidified 5% CO_2 atmosphere [26]. For the ER stress experiments, 1.5×10^6 cells were plated on 100-mm culture dishes, and after 24 h the cells were treated with the indicated concentrations of Tm (100 μg/ml of stock solution dissolved in DMSO) (Calbiochem, La Jolla, CA, U.S.A.) for 18 h.

Immunoblot (IB) Analysis

HEK293T cells exposed to Tm were lysed in RIPA buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% SDS, 1% Triton X-100, 1% sodium deoxycholate) containing 0.02 mM PMSF, 0.1 mM NaF, 10 μg/ml aprotinin, and 0.01 mM sodium orthovanadate. Cell lysates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotted with the indicated Abs, and the antigen-antibody complex was detected with an enhanced chemiluminescent (ECL) immunoblotting system as described by the manufacturer (Amersham Pharmacia Biotech, Piscataway, NJ, U.S.A.) [25]. Band intensities were quantified by densitometric analysis using the TINA program 2.0 (Strauben, Hardt, Germany) to assess the relative level of the target protein.

Cell Death Assay

HEK293T cells were plated at a density of 1×10^5 cells/well on 6-well plates and incubated in the presence of various concentrations of Tm. After incubation for 24 h, detached and adherent cells were stained with 0.4% trypan blue solution (Invitrogen) and then all nonviable cells that were stained with the dye were counted along with the number of viable cells, which excluded the dye, in a hemocytometer under an optical microscope. At least three independent experiments were performed in triplicate.

Subcellular Fractionation

HEK293T cells were plated at a density of 1×10^5 cells/100-mm dish and treated with various concentrations of Tm for 24 h. Cells were detached with trypsin/EDTA (Invitrogen), washed once with PBS, and suspended in 400 μl of digitonin lysis buffer (0.02% digitonin, 250 mM sucrose, 20 mM HEPES pH 7.4, 5 mM $MgCl_2$, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 10 mM Tris-HCl, pH 7.4) containing 1 mM PMSF, 10 μg/ml aprotinin, and 10 μg/ml leupeptin. After 10 min, cells were passed through a 27-gauge needle 10 times. The lysates were centrifuged at 900 ×*g* for 2 min at 4°C to separate the pellet nuclei and unbroken whole cells. The supernatant was then separated into a cytosolic fraction (supernatant) and mitochondrial fraction (pellet) by centrifugation at 14,000 rpm for 10 min. The mitochondrial fraction was suspended in 50 ml of RIPA buffer on ice for 30 min, and cellular debris were removed by centrifugation at 12,000 rpm for 15 min.

Generation of Stable Cell Lines that Inducibly Express Human HtrA2

The pF-HtrA2 (wt) plasmid encoding full-length human HtrA2 with a C-terminal FLAG tag was digested with the restriction enzymes EcoRI and XhoI and subcloned into the ecdysone-inducible eukaryotic expression vector pIND (Invitrogen). The resulting pIND-HtrA2 (wt) plasmid and the pVgRXR plasmid encoding a heterodimer of the ecdysone receptor (VgEcR) and the retinoid X-receptor (RXR) (Invitrogen) were transfected into HEK293 cells using the lipofectAMINE (Invitrogen) reagent. G418 (AG Scientific Inc., San Diego, CA, U.S.A.) and zeocin (Invitrogen) were used to screen for potential positive clones, and the clonal cell line, termed 293-HtrA2, was established from the clones that expressed the highest level of inducible HtrA2 induced by treatment with 5 μM ponasterone A (Pon) for 24 h. The selected cell line was maintained in DMEM supplemented with 8% FBS containing 400 μg/ml of G418 and 50 μg/ml of zeocin.

Results and Discussion

ER Stress Increases the HtrA2 Protein Level and Leads to the Induction of Cell Death

Previous studies have shown that serine proteases are involved in the mediation of cell death induced by various

stimuli, such as ER stress and IL-3 deprivation, in a caspase-independent manner [4, 5, 29, 37]. These results raise the possibility that serine proteases with proapoptotic activity may be potential candidates for the promotion of apoptosis through a caspase-independent and serine protease-dependent mechanism. To investigate whether the serine protease HtrA2 is associated with the ER stress pathway, HEK293T cells were treated with various concentrations of Tm, a potent inhibitor of *N*-linked glycosylation and an ER-stress inducible reagent, for 24 h, and total cell lysates were analyzed by an IB assay with the indicated Abs (Fig. 1A). We observed that the HtrA2 protein level was increased by approximately 4-fold at Tm concentrations of 2–3 μg/ml, compared with an untreated control. The ER stress-responsive protein GRP78, a known target gene of the URP against ER stress, was rapidly increased at 1–2 μg/ml of Tm, but slightly decreased thereafter. Numerous studies have demonstrated that the induction of GRP78 is an early cellular response used to neutralize damaged or unfolded proteins and a defense mechanism used to protect cells against ER stress through the UPR, which is coordinately regulated by three proximal ER stress sensors, IRE1, PERK, and ATF6 [1, 19, 28, 33, 44]. Nevertheless, we could not readily exclude the possibility that prolonged ER stress may contribute to the induction of apoptosis rather than cell survival through the downregulation of genes involved in the protection against ER stress, such as chaperones [16, 34, 38].

On the other hand, we can speculate that the upregulation of the HtrA2 protein that was preceded by the induction of GRP78 may play a role in modulating cell survival or death against Tm-induced ER stress, but the decision between cell survival and death may depend on the subcellular localization of HtrA2. To assess whether the increase in the HtrA2 protein level contributes to the final outcome (cell survival or death) of cells treated with

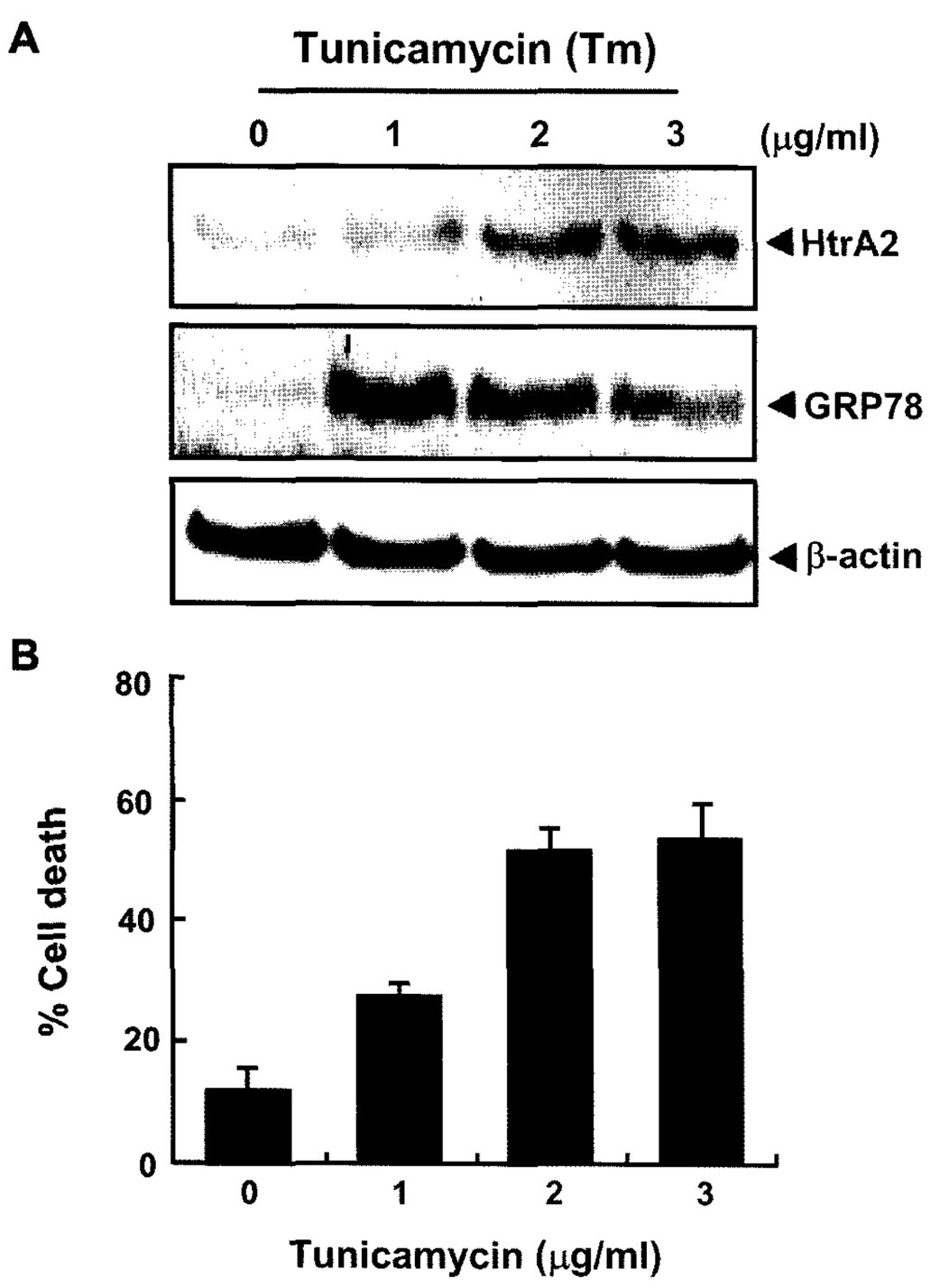

Fig. 1. ER stress elevates the expression levels of HtrA2 and triggers cell death.

A. HtrA2 protein levels were elevated after treatment with tunicamycin (Tm), an ER stress-inducing agent or a potent inhibitor of *N*-linked glycosylation. HEK293 cells were treated with Tm at various concentrations (0–3 μg/ml) for 24 h, and whole-cell lysates were prepared and analyzed by IB with anti-HtrA2 Ab. The blot was stripped and reprobed with anti-GRP78 Ab in order to detect high levels of GRP78 expression as an indicator of ER stress and the UPR, and with anti-β-actin Ab in order to monitor the equality of loading. The data are representative of three independent experiments. **B**. ER stress-induced cell death. HEK293T cells were treated with 0 to 3 μg/ml of Tm for 18 h. Detached and adherent cells were collected and stained with 0.4% trypan blue. Surviving and dead cells were quantified by microscopically counting the number of trypan blue-positive (dead) cells from the total number of cells, and the percentage of cell death was defined as the number of dead cells per total number of cells. Data represent the mean and error bars of three independent experiments with samples, and the error bars indicate the standard error of the mean.

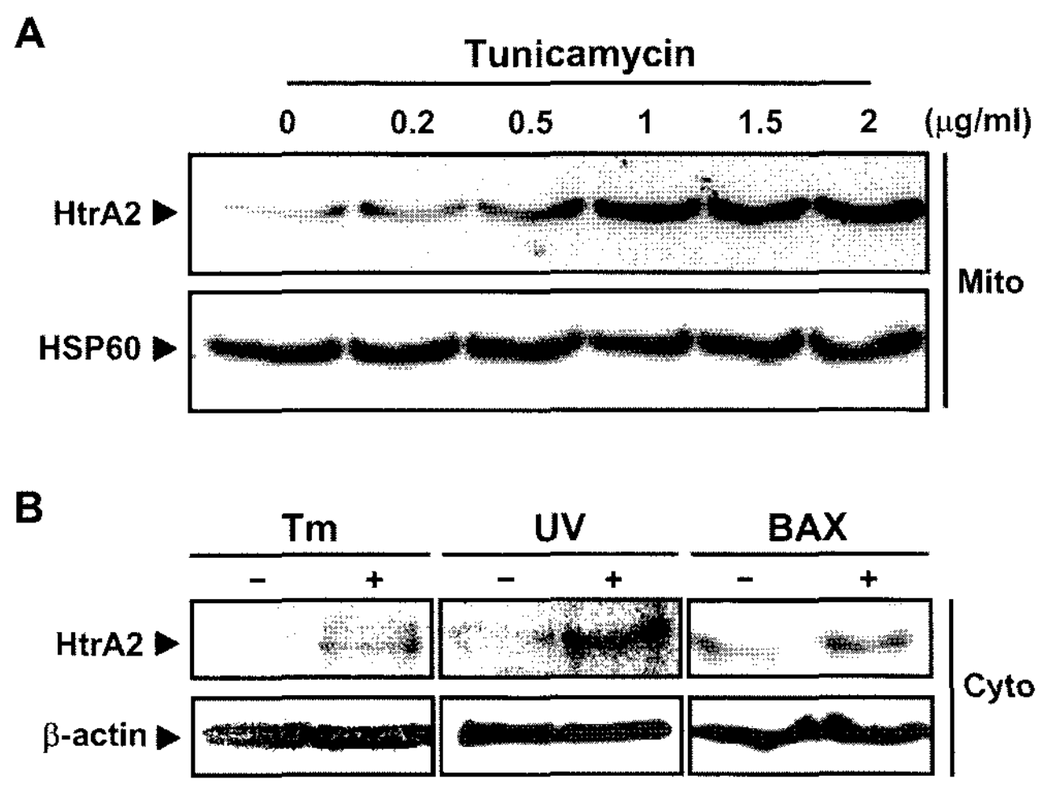

Fig. 2. ER stress-induced increase in mitochondrial HtrA2 and its release into the cytosol.

A. The induction of HtrA2 in the mitochondria following an increase in Tm concentration. After exposure to Tm (0–2 μg/ml) for 24 h, HEK293T cells were fractionated into the mitochondrial fraction (Mito), and the mitochondrial protein levels were analyzed by IB with anti-HtrA2 or anti-HSP60 Abs. **B**. The release of HtrA2 from the mitochondria into the cytosol in response to various stimuli. HEK293T cells were transfected with Bax (12 h) as an indicator of HtrA2 release or exposure to Tm (2 μg/ml, 18 h) or UV irradiation (100 J/m^2, 1 min), followed by fractionation into the cytosol. The cytosolic fraction (Cyto) was analyzed by IB with anti-HtrA2 Ab.

Tm, we counted the number of live and dead cells, and cell death was expressed as a ratio of dead to total cells (Fig. 1B). After 1 μg/ml of Tm treatment, the cells showed an approximately 3-fold increase in the extent of cell death when compared with the untreated cells. Cell death was significantly increased by approximately 5-fold in Tm-treated cells (at 2 μg/ml and 3 μg/ml) when compared with the untreated control cells. The result indicates that cell death increased along with the increase in the HtrA2 protein level in response to Tm-induced ER stress.

Upregulation of HtrA2 in the Mitochondria Under ER Stress

A number of studies have shown that HtrA2 is predominantly localized in the mitochondria [11, 32, 36, 39]. To investigate whether the increase in the HtrA2 protein level is associated with its localization in the mitochondria, cells were fractioned using a digitonin-based subcellular fractionation method, and the mitochondrial fraction was analyzed by an IB assay with Abs specific for HtrA2, HSP60 as a mitochondrial marker, and β-actin as a cytosolic marker (Fig. 2). As the concentration of Tm increased, the level of the HtrA2 protein gradually and significantly increased by up to 10-fold (at 2 μg/ml) in the mitochondria of the Tm-treated cells in comparison with that of the untreated control (Fig. 2A). The results of a previous study also suggest that HtrA2 was increased in response to Tm, although the findings of the previous study showed that HtrA2 was localized in the nucleus [9]. The result therefore suggests that the increase in the HtrA2 protein level in the mitochondria might be associated with ER stress-induced cell death.

Mitochondrial Release of HtrA2 into the Cytoplasm in Response to ER Stress

Mitochondria contain several proapoptotic molecules, such as HtrA2, Smac, and cytochrome *c*, that trigger apoptotic cell death when released into the cytoplasm in response to apoptotic stimuli [3, 7, 23, 31]. To elucidate whether the apparent cell death observed in Tm-treated cells (Fig. 1B) is closely related to the release of HtrA2 from the mitochondria into the cytosol, the cytosolic fraction was analyzed by IB assay after treatment with 2 μg/ml of Tm for 18 h (Fig. 2B). We found that HtrA2 was released from the mitochondria into the cytosol in response to Tm-induced stress, which is similar to the mitochondrial release of HtrA2 in response to other apoptotic stimuli, such as ultraviolet (UV) irradiation (100 J/m^2, 12 h) [36, 37] and Bax overexpression (Fig. 2B) [17, 43]. The results indicate that the increase in mitochondrial HtrA2 may be one of the key factors in ER stress-induced cell death through the release of HtrA2 into the cytoplasm, although we could not define the physiological role of the increase in HtrA2 in the mitochondria in response to ER stress.

ER Stress-Induced Cell Death is Intimately Associated with the Mitochondrial Release and Increased Expression of HtrA2

To determine whether the increase in HtrA2 is directly involved in ER stress-induced cell death, we established a stable cell line highly expressing full-length HtrA2 using an ecdysone-inducible mammalian expression system and designated 293-HtrA2 cells, because this system is designed for tightly regulated expression of the target gene after treatment with the steroid hormone ecdysone analog, Pon [24]. The pattern of HtrA2 expression was analyzed by an IB assay with anti-HtrA2 and anti-FLAG Abs (Fig. 3). The 50-kDa full-length and 36-kDa mature forms of HtrA2 were detected at a ratio of approximately 1 to 30 in the 293-HtrA2 cell line; therefore, endogenous full-length HtrA2 is hardly detectable in mammalian cells during routine experiments. We observed 10-fold higher levels of mature HtrA2 from the Pon-treated 293-HtrA2 cell line as compared with the untreated control cell line (Fig. 3B). The result indicates that we could further analyze whether the increase in HtrA2 directly influences ER-induced cell death by artificially manipulating the expression levels of HtrA2. The percentage of cell death was approximately

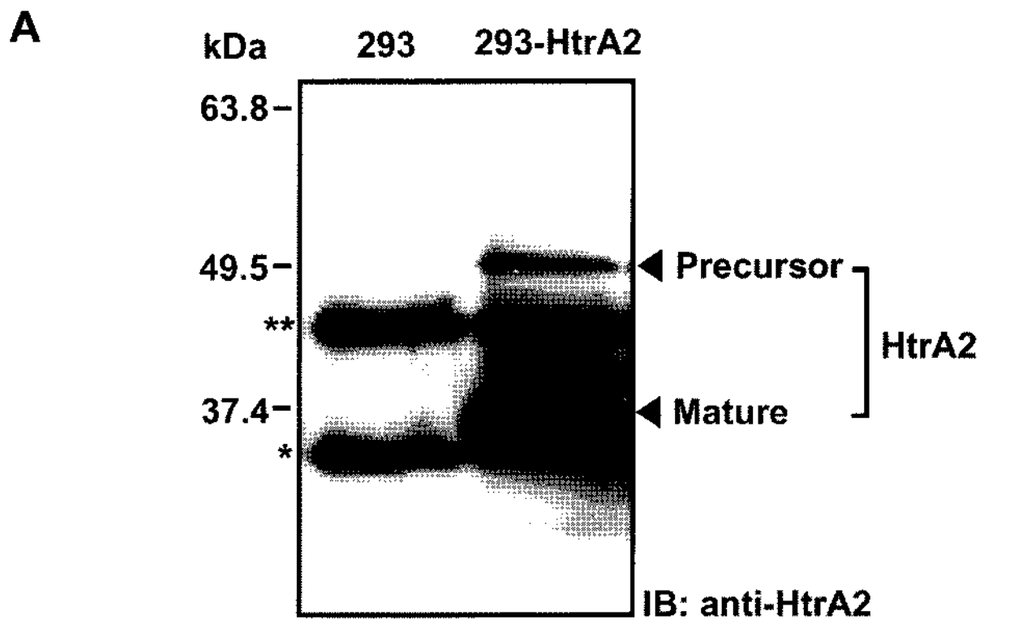

Fig. 3. Characterization of a ponasterone A (Pon)-inducible cell line expressing wild-type precursor HtrA2.

A. Inducible stable cell lines, 293 (stably transfected with the pIND plasmid) and 293-HtrA2 (stably transfected with the pIND-HtrA2-FLAG plasmid), were analyzed by IB with anti-HtrA2 Ab after induction with 5 μM Pon for 24 h. Asterisks, * and **, indicate endogenous HtrA2 and a nonspecific band, respectively. **B**. Pon-inducible expression of HtrA2. Stable cell lines were treated without (−) or with (+) 5 μM Pon for 24 h, and the expression level of Pon-induced HtrA2 was analyzed by IB with anti-FLAG Ab.

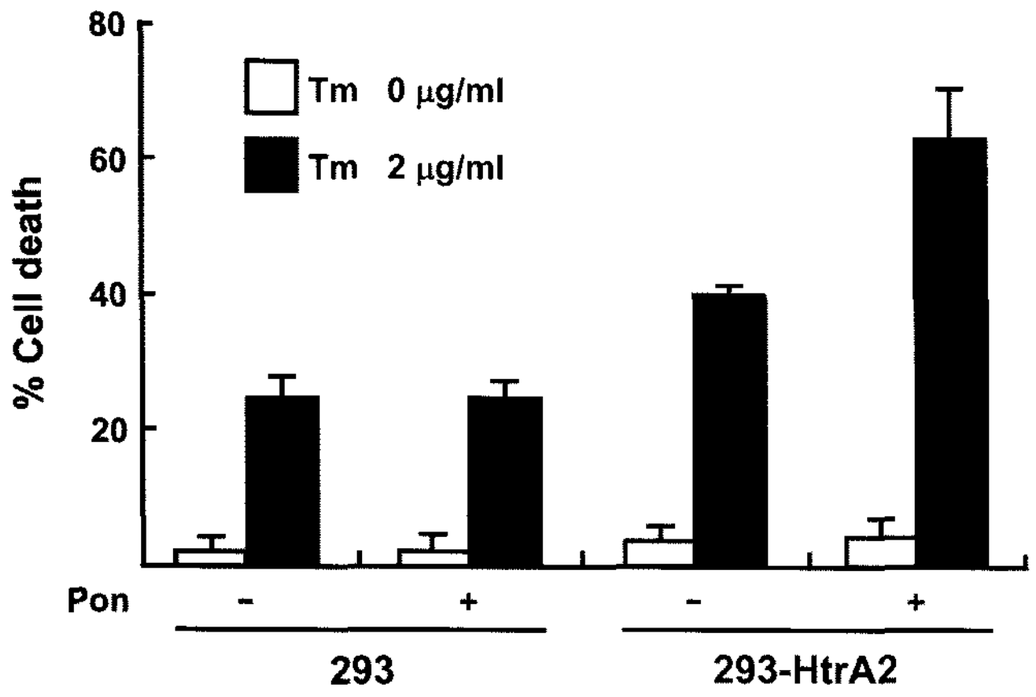

Fig. 4. HtrA2-mediated cell death in response to ER stress.
Stable cell lines were plated at a density of 1×10^5 in six-well plates, cultured in the absence (–) or presence (+) of 5 μM Pon, and treated without or with Tm for 24 h. Cell death was assessed by trypan blue exclusion assay. Data are presented as the percentage of surviving cells (mean±SD; n=3).

20% in the Tm-treated parental HEK293 cells, regardless of whether or not the cells were exposed to Pon (5 μM) (Fig. 4). No significant difference in the extent of cell death was observed between Pon-free (–) and Pon-exposed (+) 293-HtrA2 cell lines, which showed almost basal levels of cell death. The result indicates that the increase in the HtrA2 protein level itself in the mitochondria had negligible effects on cell death. Under Tm-induced ER stress, the percentage of cell death in the 293-HtrA2 cells was 40% in the absence of Pon, whereas the percentage of cell death was more than 60% in the 293-HtrA2 cells in which HtrA2 overexpression was induced by treatment with Pon. Recent studies have shown that ER stress induces apoptosis through mitochondrial membrane permeabilization *via* a Bax-dependent pathway, and thus the loss of Bax blocks the ER stress-induced release of mitochondrial proapoptotic molecules into the cytosol [2]. Our data therefore provide more supportive evidence that HtrA2 is involved in the regulation of ER stress-induced cell death events by increasing the expression level of HtrA2 in the mitochondria along with the cytosolic release of mitochondrial HtrA2 in response to Tm-induced ER stress. Several lines of evidence suggest that HtrA2 serine protease has two different major roles depending on its subcellular localization: the maintenance of mitochondrial homeostasis under normal physiological conditions, and the execution of apoptosis through the mitochondrial release of HtrA2 into the cytosol under pathological conditions [14, 21, 36, 40]. We also speculate that there is a possibility that the mitochondrial increase in the HtrA2 protein level may serve as an early cellular defense strategy against ER stress, thus further studies are necessary for the detailed elucidation of the molecular mechanisms by which HtrA2 regulates ER stress-induced cell death and plays a significant role in cellular defense mechanisms.

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