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Possible Involvement of Both Endoplasmic Reticulum- and Mitochondria-Dependent Pathways in Thapsigargin-Induced Apoptosis in Human Neuroblastoma SH-SY5Y Cells

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Abstract. Recently, it has been shown that endoplasmic reticulum (ER) stress causes apoptosis. However, the mechanism of the ER stress-dependent pathway is not fully understood. In human neuroblastoma SH-SY5Y cells, we detected a caspase-12-like protein that has a molecular mass (approximately 60 kDa) similar to that of mouse caspase-12. Thapsigargin, an inhibitor of ER-associated Ca^{2+}-ATPase, induced the degradation of caspase-12-like protein. In addition, the degradation of caspases-9 and -3, cleavage of poly(ADP-ribose) polymerase, DNA fragmentation, and cell death were also observed. Pretreatment with phorbol-12-myristate-13-acetate, which induces the expression of antiapoptotic Bcl-2, inhibited thapsigargin-induced degradation of caspases-9 and -3, but not caspase-12-like protein degradation. A caspase inhibitor, benzyloxycarbonyl-Val-Ala-Asp(OCH_3)-CH_2F, inhibited the degradation of caspase-12-like protein, but not that of caspases-9 and -3. These results suggest that thapsigargin may induce the activation of both ER- and mitochondria-dependent pathways in human SH-SY5Y cells.

Keywords: thapsigargin, endoplasmic reticulum stress, caspase, Bcl-2, human neuroblastoma SH-SY5Y cell

Introduction

Recent studies have indicated that prolonged dysfunction and/or stress in the endoplasmic reticulum (ER) may contribute to the pathogenesis and neurodegeneration in Alzheimer's and Parkinson's diseases (1 – 3). In vitro ER stress is caused by a variety of insults, such as Ca^{2+} ionophores, or the inhibition of protein folding, glycosylation, and trafficking (4, 5). An inducer of ER stress, thapsigargin, inhibits ER-associated Ca^{2+}-ATPase and disrupts Ca^{2+} homeostasis (6, 7). On the other hand, tunicamycin and brefeldin A are known to specifically inhibit *N*-glycosylation in ER and ER-Golgi transport, respectively (8). It has been reported that these chemical toxicants cause ER stress-dependent apoptotic cell death through the activation of ER-specific caspase-12 (8). Although a specific inhibitor is not yet available, a non-selective caspase inhibitor, such as benzyloxycarbonyl-Val-Ala-Asp(OCH_3)-CH_2F (z-VAD.fmk), has been reported to inhibit caspase-12 (8).

The antiapoptotic protein Bcl-2 is well known to inhibit the release of apoptogenic cytochrome *c* from mitochondria into the cytosol (9, 10), suggesting that Bcl-2 may participate in stabilizing mitochondria (11). The protein level of Bcl-2 may be upregulated in brains of patients with Alzheimer's disease (12) as well as in those with Parkinson's disease (13). Such upregulation of Bcl-2 protein may represent a compensatory response of remaining neurons to protect themselves from oxidative stress and subsequent apoptosis (14, 15). In addition, Bcl-2 protein induces neuronal differentiation (16) and promotes the regeneration of severed axons in mammalian neurons (17). Thus, Bcl-2 plays a role in both antiapoptosis and neuronal regeneration. In human SH-SY5Y cells, pretreatment with an activator of protein kinase C (PKC), such as phorbol-12-myristate-13-acetate (PMA), or an antiparkinsonian drug, such as pramipexole, induced Bcl-2 expression and inhibited apoptotic cell death caused by 1-methyl-4-phenylpyridinium ion (MPP^+), a mitochondrial complex I inhibitor

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(18 – 20). The disorder caused by the misfolding and aggregation of proteins has recently been referred to as conformational disease, including Alzheimer's, Parkinson's, and polyglutamine diseases. (21 – 24). Thus, since ER stress-induced neurodegeneration may participate in the pathogenesis of such diseases, studies of ER stress-induced apoptosis in neuron-like human cell lines may help to elucidate the neuroprotective strategy. In the present study, we examined the effects of PMA-pretreatment and z-VAD.fmk on ER stress-induced cell death in human neuroblastoma SH-SY5Y cells.

Materials and Methods

Materials

Thapsigargin, tunicamycin, brefeldin A, PMA, and purified cytochrome *c* (horse heart) were purchased from Sigma (St. Louis, MO, USA). MPP^+ was obtained from Research Biochemicals International (Natick, MA, USA); staurosporine was from Kyowa Hakko Kogyo (Tokyo); 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) was from Dojindo (Kumamoto); and 2'-deoxyadenosine 5'-triphosphate (dATP) was from Amersham Pharmacia Biotech (Buckinghamshire, UK). The caspase inhibitor z-VAD.fmk was from Peptide Institute (Osaka).

Primary antibodies included mouse monoclonal antibodies to Lys-Asp-Glu-Leu (KDEL) (StressGen, Victoria, BC, Canada) and Bcl-2 (Dako, Copenhagen, Denmark), and rabbit polyclonal antibodies to human caspase-9 and proenzyme/active fragments of caspase-3 (PharMingen, San Diego, CA, USA), poly(ADP-ribose) polymerase (PARP) (Upstate Biotechnology, Lake Placid, NY, USA), and Bcl-2 and mouse caspase-12 (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The enhanced chemiluminescent detection system kit (ECL kit) was from Amersham Pharmacia Biotech, and the Bradford protein assay was from BioRad Laboratories (Hercules, CA, USA).

Cell culture and treatment

Human neuroblastoma SH-SY5Y cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin and kept at 37°C in humidified 5% CO_2 / 95% air. The cells were pretreated with or without PMA (100 nM) or staurosporine (10 nM). After pretreatment, cells were washed 3 times with DMEM and then further treated with thapsigargin (at 1 – 100 nM), A23187 (0.3 μM), tunicamycin (1 μg/ml), or brefeldin A (0.1 μg/ml) in the presence or absence of z-VAD.fmk (100 μM) for 0 – 3 days. Treated cells were subjected to a cell-survival assay and DNA fragmentation, immunocytochemical, and immunoblotting analyses.

MTT assay

We performed an MTT assay as an index of surviving cells. In living cells, MTT is converted to formazan, which has a specific absorption maximum. After SH-SY5Y cells were treated, the culture medium was changed to a medium containing 5 mg/ml MTT, and the cells were incubated for an additional 4 h. They were then mixed thoroughly with an equal volume of isopropanol/0.04 M HCl. After centrifugation at 10,000 $\times g$ for 5 min, the absorbance of the supernatant was measured at 570 nm.

Assay of DNA fragmentation

After approximately 2×10^6 cells were treated in 60-mm diameter dishes, the cells were scraped from the dishes using a rubber policeman, centrifuged at 800 $\times g$ for 10 min, and resuspended in 100 μl of lysis buffer: 10 mM Tris-HCl (pH 7.4) containing 10 mM EDTA and 0.5% Triton X-100. After incubation for 10 min at 4°C, lysates were centrifuged at 15,000 $\times g$ for 30 min. The supernatants were supplemented with 2 μl of RNase A (20 mg/ml) and then incubated at 37°C for 1 h. Next, 2 μl of proteinase K (20 mg/ml) was added and incubation was continued at 37°C for 1 h. Twenty μl of 5 M NaCl and 120 μl of isopropanol were added and the mixture was held overnight at –20°C. After centrifugation at 15,000 $\times g$ for 20 min, DNA pellets were resuspended in 10 mM Tris-HCl (pH 7.4) containing 1 mM EDTA. The DNA fragments thus obtained were electrophoretically separated on 2% agarose gel for 90 min at 50 V. The gel was stained with ethidium bromide and photographed under UV transillumination. A 100 base-pair ladder was used as a DNA size marker (Amersham Pharmacia Biotech).

Immunoblot analysis

After treatment, cell lysates were subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and then immunoblotting using antibodies against Bcl-2 (diluted 1:500), KDEL (1:1000), PARP (1:600), human caspases-9 (1:1000) and -3 (1:1500), and mouse caspase-12 (1:500). For semi-quantitative analysis, the bands of these proteins on radiographic films were scanned with a CCD color scanner (DuoScan; AGFA, Leverkusen, Germany) and then analyzed. Densitometric analysis was performed using the public domain NIH Image 1.56 program (written by Wayne Rasband at the U.S. National Institutes of Health and available from the Internet by anonymous ftp from zippy.nimh.nih.gov.).

Confocal immunocytochemistry using antibodies against KDEL and Bcl-2

After pretreatment with vehicle or PMA and further treatment with vehicle or thapsigargin, SH-SY5Y cells were immediately fixed and then incubated for 4 days at 4°C with both mouse anti-KDEL antibody and rabbit anti-Bcl-2 antibody. The labeled primary antibodies were detected by FITC-anti-mouse IgG antibody and rhodamine-anti-rabbit IgG antibody, and their fluorescence was obtained with a laser scanning confocal microscope LSM410 (Carl Zeiss, Jena, Germany).

Preparation and induction of a cell-free caspase system

Cytosolic extract was prepared as previously described (25, 26). To initiate caspase activation, an aliquot of the cytosolic extract (50 μg of protein) was incubated with thapsigargin or 1 mM dATP and 1 μM cytochrome *c* at 30°C for 4 h. Treated extracts were subjected to assay for immunoblotting with antibodies against caspases-12 (1:500), -9 (1:1000), and -3 (1:1500).

Statistical evaluation

The results of the MTT assay and the densitometric analysis for immunoblotting are given as the mean ± S.E.M. The significance of differences was determined by an analysis of variance (ANOVA). Further statistical analysis for *post hoc* comparisons was performed using the Bonferroni/Dunn test (StatView; Abacus Concepts, Berkeley, CA, USA).

Results

Thapsigargin-induced ER stress, caspase activation, and cell death

ER stress induces 78-kDa and 94-kDa glucose-regulated proteins (GRP78 and GRP94, respectively) through a signaling pathway of the unfolded protein response (4). Since GRP78 and GRP94 contain the carboxyl terminal sequence Lys-Asp-Glu-Leu (KDEL), which is an ER-retrieval signal, we used anti-KDEL antibody to detect GRP78 and GRP94. Although GRP78 and GRP94 were constitutively expressed in human neuroblastoma SH-SY5Y cells, treatment with ER stress inducers such as thapsigargin (30 nM), tunicamycin (1 μg/ml), and brefeldin A (0.1 μg/ml), but not MPP^+ (1 mM) (an inhibitor of mitochondrial complex I), significantly enhanced protein levels of GRP78 and GRP94 after 12 h (Fig. 1). In the immunocytochemical analysis, although immunoreactivity for anti-KDEL antibody (which includes immunoreactivity for both GRP78 and GRP94) was not detected in vehicle-treated cells (Fig. 2A), treatment with thapsigargin for 12 h markedly increased KDEL immunoreactivity in the cytoplasm

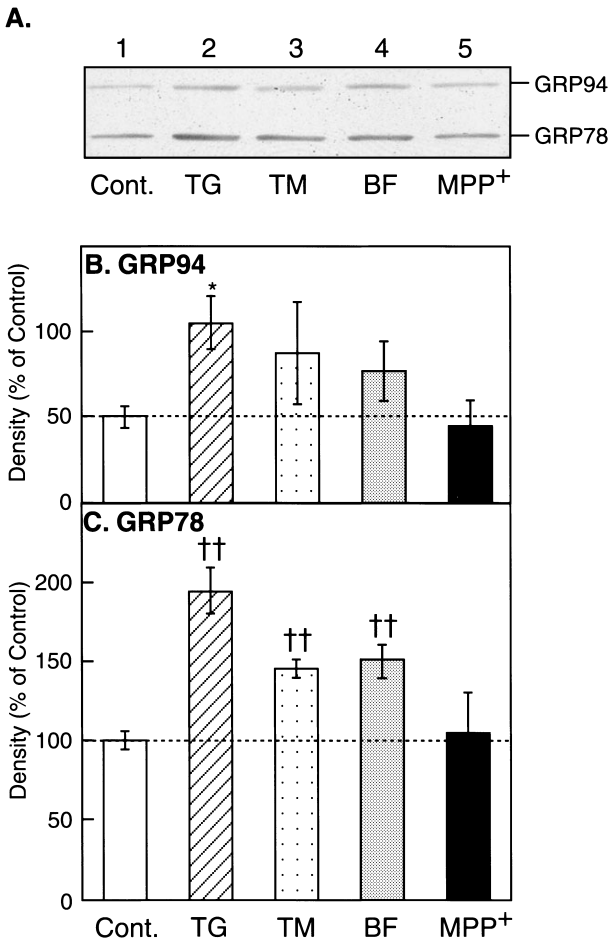

Fig. 1. Expression of ER chaperones GRP78/94 induced by ER stress. Human SH-SY5Y cells were incubated with vehicle (Cont.), 30 nM thapsigargin (TG), 1 μg/ml tunicamycin (TM), 0.1 μg/ml brefeldin A (BF), or 1 mM MPP^+ for 12 h. After treatment, cells were scraped, and cell lysates were prepared. Each sample (10 μg of protein/lane) was subjected to immunoblot analysis (A) with anti-KDEL antibody, and protein bands of 94 kDa (GRP94, B) and 78 kDa (GRP78, C) were assessed. The density of the GRP78 protein band in the vehicle control (Cont.) was taken as 100%. Each value is the mean ± S.E.M. (%) of three determinations. Significance (*post hoc* comparisons by the Bonferroni/Dunn test): $*P<0.05$ vs the level of GRP94 in the control; $^{\dagger\dagger}P<0.01$ vs the level of GRP78 in the control.

(probably ER) (Fig. 2B). These results suggest that thapsigargin, tunicamycin, and brefeldin A cause ER stress, while MPP^+ does not, in human SH-SY5Y cells.

Subsequently, treatment with thapsigargin caused cell death in a concentration- and time-dependent manner (Fig. 3: A and B), accompanied by DNA fragmentation (Fig. 4D, lane 2). Anti-mouse caspase-12 antibody recognized the 60-kDa protein band in human SH-SY5Y cells and the density of this band decreased after treatment with thapsigargin (Fig. 3C). The level of procaspase-9 (48 kDa) was also decreased by thapsigargin

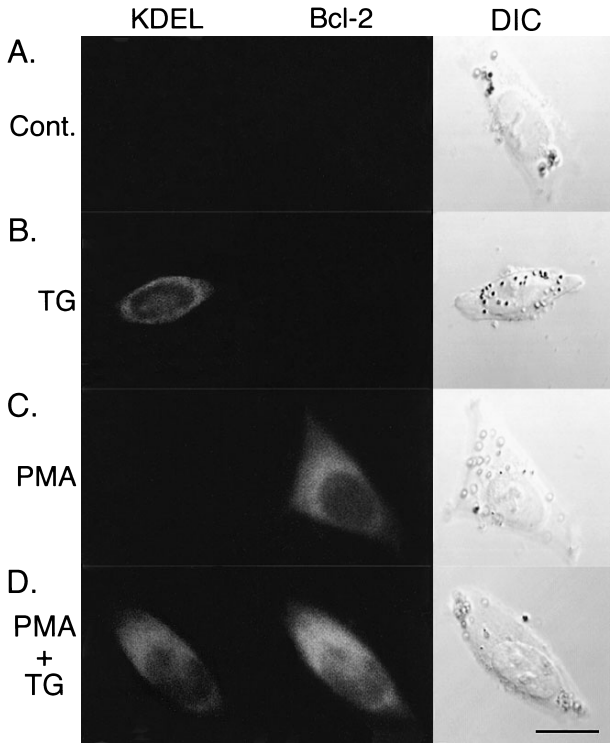

Fig. 2. Immunoreactivities for KDEL and Bcl-2 in SH-SY5Y cells. SH-SY5Y cells were pretreated for 4 days with vehicle (A, B) or 100 nM PMA (C, D). After pretreatment, cells were washed in PBS and further treated for 12 h with vehicle (A, C) or 30 nM thapsigargin (TG) (B, D). Subsequently, cells were fixed and co-incubated with mouse anti-KDEL antibody and rabbit anti-Bcl-2 antibody. Images of the cells (difference interference contrast, DIC) were obtained by confocal laser microscopy. These data represent three independent experiments with similar results. Scale bar, 20 μm.

(Fig. 3D). Using an antibody against the proenzyme and active fragments of caspase-3, the amount of 32-kDa procaspase-3 was decreased and 20/17-kDa active fragments cleaved from 32-kDa procaspase-3 were detected after 1 day of treatment with thapsigargin (Fig. 3E). In addition, thapsigargin-induced cleavage of PARP into an 85-kDa fragment was detected by an antibody against the cleavage site of PARP (Fig. 3F). Thus, the activation of caspase-12-like protein, caspases-9, and -3 may involve in the apoptotic cell death caused by thapsigargin-induced ER stress.

PMA-induced Bcl-2 expression protects against thapsigargin-induced apoptosis

We previously found that an activator of PKC (such as PMA) and anti-parkinsonian drugs (such as pramipexole and talipexole) induce Bcl-2 expression in SH-SY5Y cells (20). Although only small amounts of Bcl-2 protein were detected in untreated samples, pretreatment with 100 nM PMA for 4 days significantly enhanced the protein level of Bcl-2 (Fig. 4: A and B). In contrast, 10 nM staurosporine (a PKC inhibitor) decreased the constitutive expression of Bcl-2 and also inhibited PMA-induced Bcl-2 expression (Fig. 4: A and B). Concerning immunocytochemical analysis, although Bcl-2 immunoreactivity was not detected in vehicle-treated cells (Fig. 2A), pretreatment with PMA for 4 days markedly increased Bcl-2 immunoreactivity in the cytoplasm (Fig. 2C). Although thapsigargin alone did not induce the expression of Bcl-2, immunoreactivities for both KDEL and Bcl-2 were markedly detected in cells that were pretreated with PMA before the application of thapsigargin (Fig. 2D).

Pretreatment with PMA significantly inhibited cell death and DNA fragmentation induced by thapsigargin (Fig. 4: C and D), similar to that induced by A23187, a calcium ionophore. However, cell death induced by tunicamycin or brefeldin A was not inhibited by pretreatment with PMA (Fig. 4C). On the other hand, the mitochondrial dysfunction induced by MPP^+ (an inhibitor of complex I) and sodium azide (NaN_3, an inhibitor of complex IV) caused cell death that was also inhibited by pretreatment with PMA (Fig. 4C).

Pretreatment with PMA inhibits caspases-9 and -3, but not caspase-12-like protein

Although pretreatment with PMA inhibited thapsigargin-induced cell death, the thapsigargin-induced increase in KDEL immunoreactivity was unaffected (Fig. 2: B and D), suggesting that PMA-induced Bcl-2 expression may not inhibit thapsigargin-induced ER stress. Thapsigargin-induced cell death was accompanied by the degradation of caspase-12-like protein, procaspases-9 and -3, and PARP (Fig. 3: C – F). Therefore, to clarify the mechanism of PMA-induced neuroprotection, we examined the effects of pretreatment with PMA on the degradation of these proenzymes. Pretreatment with PMA significantly inhibited thapsigargin-induced degradation of PARP and procaspases-9 and -3, but did not change the level of caspase-12-like protein (Fig. 5). Thus, pretreatment with PMA may inhibit mitochondria-dependent caspases-9 and -3, but not caspase-12-like protein, in human SH-SY5Y cells.

Thapsigargin does not directly affect caspases in a cell-free system

We further examined whether thapsigargin directly affects caspases in a cell-free system. The cytosolic extract from SH-SY5Y cells used in this experiment was rich in apoptotic protease-activating factor-1 (Apaf-1), while cytochrome *c* was undetectable (data not shown). Treatment with thapsigargin at 30 nM – 3 μM for 4 h did not induce the cleavage of caspase-12-like protein and

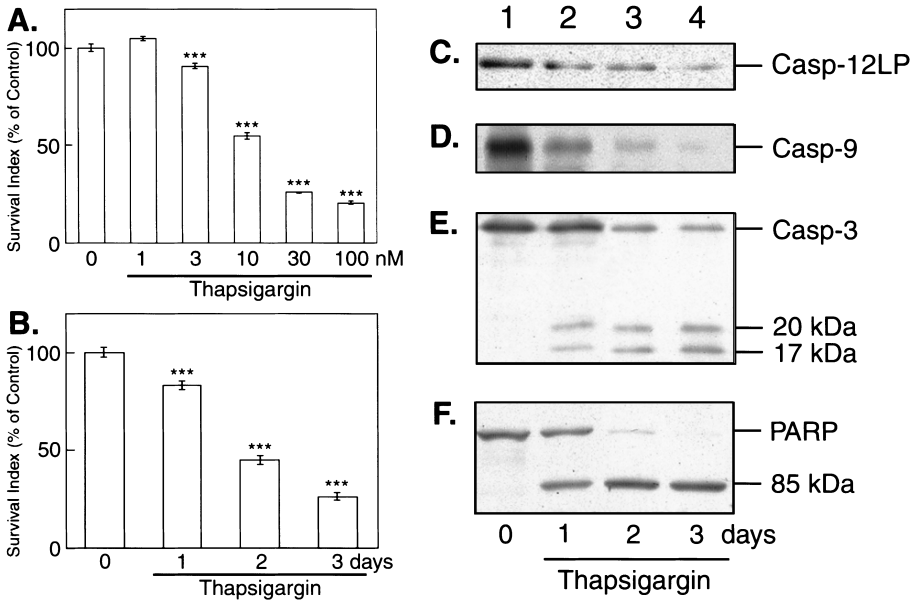

Fig. 3. Thapsigargin-induced degradation of caspases or PARP, and cell death. SH-SY5Y cells were incubated with vehicle or thapsigargin (at 1 – 100 nM in A or at 30 nM in B – F) for 3 days (A) or 1 – 3 days (B – F). A and B: Cell survival was measured by the MTT assay. Each value is the mean ± S.E.M. (%) of three determinations. Significance: ***P<0.001 vs the level in the vehicle treatment. C – F: the degradation of caspase-12-like protein (Casp-12LP), caspases-9, -3 (Casp-9, -3), and PARP and cell survival were examined. Each sample (10 μg of protein/lane) was subjected to immunoblot analysis with antibodies against mouse caspase-12 (C), human caspases-9 (D), -3 (E), and PARP (F).

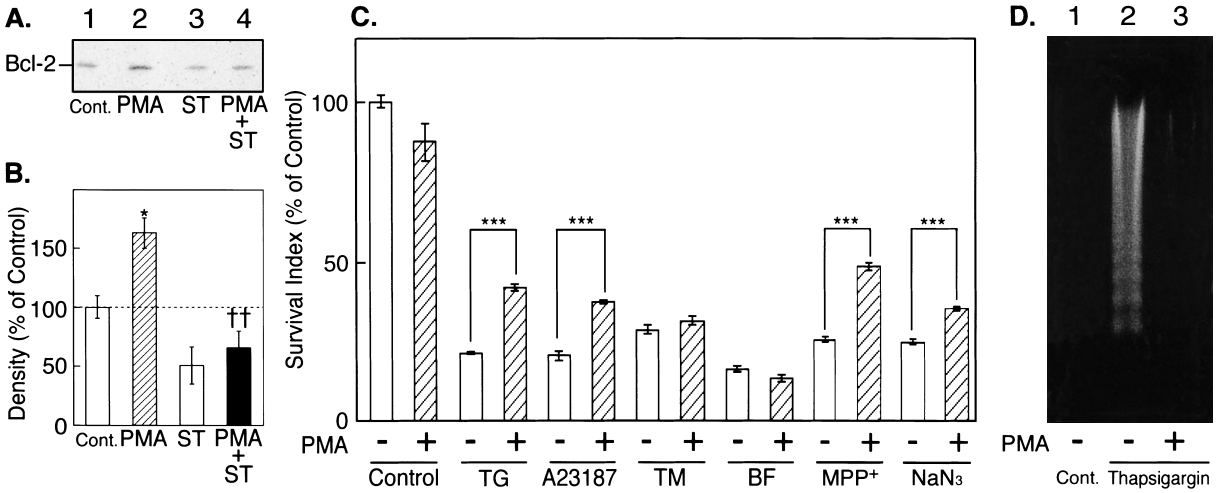

Fig. 4. PMA-induced Bcl-2 expression and protection against apoptotic cell death. SH-SY5Y cells were pretreated with 100 nM PMA and/or 10 nM staurosporine (ST) for 4 days. After pretreatment, cells were washed in DMEM media and further treated with 30 nM thapsigargin (TG), 0.3 μM A23187, 1 μg/ml tunicamycin (TM), 0.1 μg/ml brefeldin A (BF), 1 mM MPP^+, or 1 mM sodium azide (NaN_3) for 3 days. Bcl-2 expression, cell survival, and DNA fragmentation were then examined. A and B: Bcl-2 expression. Each value is the mean ± S.E.M. (%) of three determinations. Significance: *P<0.05 vs the level in the vehicle treatment (Cont.); $^{\dagger\dagger}P$<0.01 vs the level in PMA treatment. C: Pretreatment with PMA significantly inhibited cell death induced by thapsigargin, A23187, MPP^+, or NaN_3, but not that induced by TM or BF. ***P<0.001 vs the level in ER stress without pretreatment with PMA (white column). D: DNA fragmentation.

caspases-9 and -3 (Fig. 6A). Recently, cytochrome *c* and dATP (or ATP) have been identified as the stimulants for Apaf-1 (27, 28). Addition of 1 mM dATP and 1 μM cytochrome *c* markedly induced the cleavage of caspases-9 and -3 and also degradation of caspase-12-like protein (Fig. 6A). These results indicate that thapsigargin does

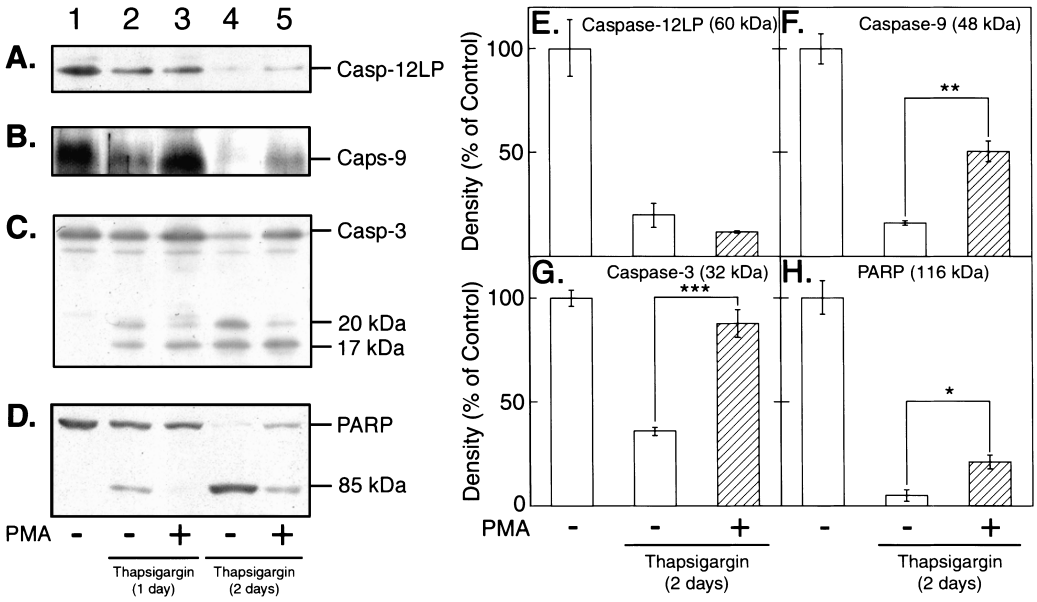

Fig. 5. Inhibition of thapsigargin-induced degradation of caspases-9, -3, or PARP by pretreatment with PMA. Each sample was subjected to immunoblot analysis with antibodies against mouse caspases-12 (A), human caspases-9 (B), -3 (C), and PARP (D). E – H, The densities of protein bands in caspase-12-like protein (Casp-12LP, 60 kDa), caspases-9 (Casp-9, 48 kDa), -3 (Casp-3, 32 kDa), and PARP (116 kDa) are given as the mean ± S.E.M. (%) of three determinations, with the density of each pro-enzyme in the vehicle-treatment considered to be 100%. Significance: **P*<0.05, ***P*<0.01; ****P*<0.001 vs the level in treatment with thapsigargin alone.

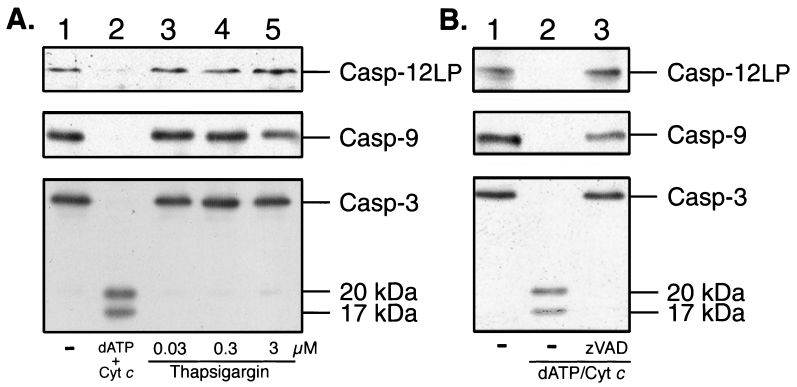

Fig. 6. Effects of thapsigargin and caspase inhibitors on caspase activation in an in vitro cell-free system. The cytosolic extract from untreated SH-SY5Y cells was treated for 4 h with vehicle (–), thapsigargin (A), or 1 mM dATP plus 1 μM cytochrome *c* (dATP/Cyt *c*) in the presence of vehicle (–) or 100 μM z-VAD.fmk (zVAD, B). Each sample was subjected to immunoblot analysis with antibodies against mouse caspase-12 and human caspases-9 and -3.

not directly affect these caspases. On the other hand, the in vitro cleavage of caspases-9 and -3 and caspase-12-like protein by dATP *plus* cytochrome *c* was completely inhibited by a non-selective caspase inhibitor, z-VAD.fmk (Fig. 6B).

Inhibition of thapsigargin-induced apoptosis by z-VAD.fmk

Based on these results with z-VAD.fmk, we examined the effect of this inhibitor on thapsigargin-induced cell death. Although z-VAD.fmk at 100 μM did not affect cell viability, simultaneous treatment with this inhibitor significantly suppressed thapsigargin-induced cell death (Fig. 7A). In addition, thapsigargin-induced DNA fragmentation was also markedly inhibited by z-VAD.fmk (Fig. 7B). Thus, simultaneous treatment with z-VAD.fmk inhibited thapsigargin-induced apoptosis, like a PMA pretreatment.

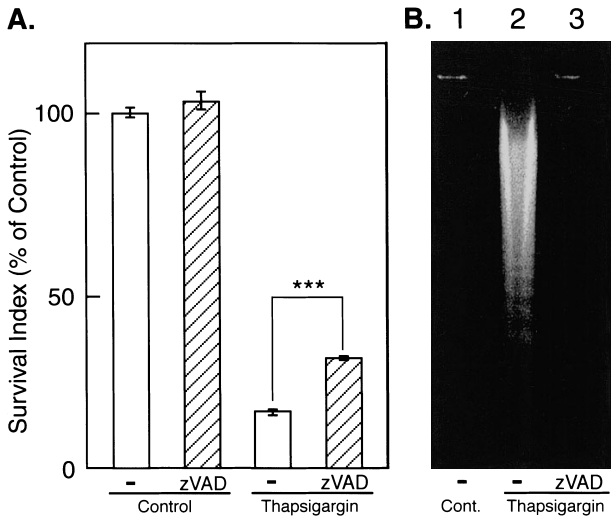

Fig. 7. Protective effect of caspase inhibitor against thapsigargin-induced apoptosis. SH-SY5Y cells were treated for 3 days with 30 nM thapsigargin in the presence or absence of 100 μM z-VAD.fmk (zVAD). Cell survival and DNA fragmentation were then examined. A: Cell survival. Each value is the mean ± S.E.M. (%) of three determinations. Significance: ***P<0.001 vs the treatment with thapsigargin alone. B: DNA fragmentation.

z-VAD.fmk inhibits caspase-12-like protein, but not caspases-9 and -3

To clarify the mechanism of caspase inhibitor-induced neuroprotection, we examined the effect of z-VAD.fmk on the degradation of proenzymes. In contrast to pretreatment with PMA, simultaneous treatment with z-VAD.fmk significantly inhibited thapsigargin-induced degradation of caspase-12-like protein and PARP, but did not change the levels of procaspases-9 and -3 (Fig. 8). Thus, although z-VAD.fmk inhibited caspase-12-like protein and caspases-9 and -3 in the in vitro cell-free system (Fig. 6B), it only inhibited caspase-12-like protein, but not caspases-9 and -3, in cultured SH-SY5Y cells (Fig. 8).

Discussion

Recent studies have suggested that mouse caspase-12 is an ER-specific caspase that participates in ER stress-induced apoptosis (8, 26). In addition, a 60-kDa of caspase-12-like protein was recognized by anti-mouse caspase-12 antibody in human-derived cell lines, such as HeLa cells, A549 lung carcinoma cells, 293T embryonic kidney cells, and Jurkat E6 T-lymphoma cells (8, 26, 29). Surprisingly, a recent study has reported that a caspase-12-homologous gene in the human chromosome 11q22.3 contains several mutations which may preclude the expression of a full-length protein (30). In the present study using human neuroblastoma SH-SY5Y cells, we also detected a caspase-12-like protein that

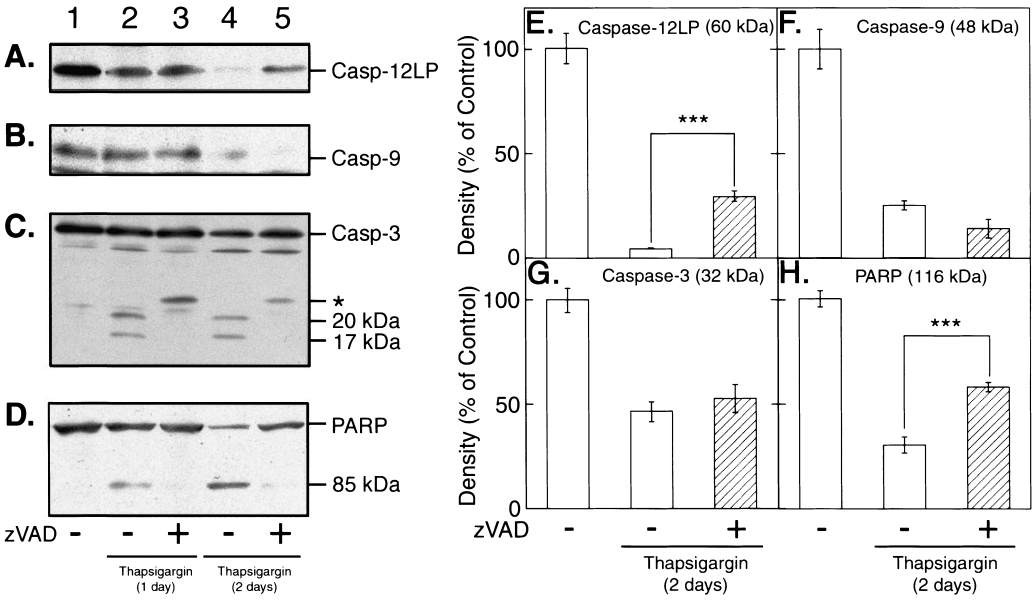

Fig. 8. Inhibition of the thapsigargin-induced degradation of caspase-12-like protein and PARP by z-VAD.fmk. Each sample was subjected to immunoblot analysis with antibodies against mouse caspase-12 (A), human caspases-9 (B), caspase-3 (C), and PARP (D). In C, asterisk (*) donates a 22 kDa-cleavage product in the presence of z-VAD.fmk (zVAD). E – H, The densities of protein bands in caspase-12-like protein (Casp-12LP, 60 kDa), caspase-9 (Casp-9, 48 kDa), caspuse-3 (Casp-3, 32 kDa), and PARP (116 kDa) are given as the mean ± S.E.M. (%) of three determinations, with the density of each pro-enzyme in the vehicle-treatment considered to be 100%. Significance: ***P<0.001 vs the level in treatment with thapsigargin alone.

has a molecular mass (approximately 60-kDa) similar to that of mouse caspase-12 and was degraded by thapsigargin, an inhibitor of ER-associated Ca^{2+} ATPase. Thus, although a human ortholog for mouse caspase-12 has not yet been clear (31), another and/or unknown member(s) of the caspase family, which is recognized by anti-mouse caspase-12 antibody, may function in the ER stress-dependent apoptosis in human cells (26, 32, 33).

In this study, thapsigargin also induced the degradation of procaspases-9 and -3 and caused apoptosis in human SH-SY5Y cells. Therefore, we further examined the effects of Bcl-2 overexpressed by a PKC activator PMA and the nonselective caspase inhibitor z-VAD.fmk on thapsigargin-induced apoptosis. Pretreatment with PMA inhibited the apoptotic cell death induced by thapsigargin or A23187, which disrupts Ca^{2+} homeostasis. However, pretreatment with PMA did not inhibit cell death induced by tunicamycin (an inhibitor of *N*-glycosylation in ER) or brefeldin A (an inhibitor of ER-Golgi transport). Thus, PMA-induced Bcl-2 expression may inhibit cell death caused by Ca^{2+}-sensitive ER stress, but not that caused by other ER stresses in human SH-SY5Y cells. Cell death induced by mitochondrial dysfunction, such as that caused by MPP^+ or NaN_3, was also inhibited by pretreatment with PMA. Interestingly, recent papers indicated that ER-mediated facilitating Ca^{2+} spikes, which are mediated by the inositol-3-phosphate receptors in the ER, trigger opening of the mitochondrial permeability transition pore and, in turn, the release of cytochrome *c* (34, 35). Based on these observations, we speculate that ER stress induced by the disruption of Ca^{2+} homeostasis, but not by the inhibition of glycosylation and ER-Golgi transport, may be associated with mitochondrial dysfunction.

More recent studies have suggested that caspase-12-like protein is activated by an Apaf-1/caspase-9-dependent pathway through caspase-7 activation (26, 36, 37) and that mouse caspase-12 reversely activates caspase-9 (32, 33). Thus, mouse caspase-12 or human caspase-12-like protein may interact with caspase-9. Therefore, it is considered that caspases-9 and -3 may be activated by a mitochondria-dependent cytochrome *c*/Apaf-1 pathway (26, 37) and by an Apaf-1-independent ER-caspase-12 pathway (32, 33). In the present study, cytochrome *c* and dATP induced the degradation of caspase-12-like protein in a cell-free system, suggesting that caspase-12-like protein may be degraded by Apaf-1/caspase-9 in vitro. In contrast, z-VAD.fmk did not inhibit the thapsigargin-induced degradation of procasases-9 and -3 in cultured SH-SY5Y cells. Overexpression of Bcl-2 by PMA pretreatment did not reduce the degradation of caspase-12-like protein. In addition, we have recently found that rotenone, a specific mitochondrial complex I inhibitor, also induced the degradation of procaspase-9 and caspase-12-like protein which was inhibited by PMA and z-VAD.fmk, respectively (38). These results suggest that Bcl-2 overexpression and z-VAD.fmk predominantly inhibit mitochondria-dependent caspase-9 and ER-dependent caspase-12-like protein, respectively, in human SH-SY5Y cells. Alternatively, caspase-12-like protein may not interact with caspase-9 in living human SH-SY5Y cells, although caspase-12-like protein may crosstalk with caspase-9 in vitro. As described above, there is a possibility that Ca^{2+}-sensitive ER stress and mitochondrial dysfunction may interact with each other. Therefore, we presume that the disruption of Ca^{2+} homeostasis may be intermediate between ER stress and mitochondrial dysfunction at the upstream of the crosstalk between caspase-12-like protein and caspase-9 in human SH-SY5Y cells.

In conclusion, thapsigargin induced the degradation of caspase-12-like protein, procaspases-9 and -3, and PARP. Pretreatment with PMA inhibited caspases-9 and -3 and then protected against DNA fragmentation and cell death. On the other hand, z-VAD.fmk predominantly inhibited caspase-12-like protein rather than caspases-9 and -3. These results suggest that ER stress induced by thapsigargin, but not by tunicamycin and brefeldin A, may induce the activation of both ER-dependent caspase-12-like protein and mitochondria-dependent caspases-9 and -3 in human SH-SY5Y cells.

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