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Nerve Growth Factor Attenuates Endoplasmic Reticulum Stress-Mediated Apoptosis *via* Suppression of Caspase-12 Activity

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Following endoplasmic reticulum (ER) stress, which occurs *via* inhibition of the glycosylation of newly synthesized proteins, caspase family proteins are activated to promote ER stress-mediated apoptosis. Here we report that nerve growth factor (NGF) suppressed the ER stress-mediated apoptosis in tunicamycin-treated PC12 cells through an extensive decrease of the caspase-3/-9/-12 activity. Detailed analysis of the mechanism underlying the NGF-mediated cell survival revealed that the activities of all seriate caspases were reduced through the phosphatidylinositol 3-kinase (PI3-K) signaling pathway induced by NGF. Moreover, we found that the activity of c-Jun N-terminal kinase (JNK) was not essential for the tunicamycin-induced apoptosis of PC12 cells. These results demonstrate that the inactivation of caspase-12 *via* the NGF-mediated PI3-K signaling pathway leads to inactivation of the caspase cascade including caspase-3 and -9.

Key words: apoptosis, caspases, ER stress, NGF, PI3-K.

Abbreviations: ER, endoplasmic reticulum; NGF, nerve growth factor; PI3-K, phosphatidylinositol 3-kinase; GRP, glucose-regulated protein; JNK, c-Jun N-terminal kinase; DMEM, Dulbecco's modified Eagle's medium; Tm, tunicamycin; PBS, phosphate-buffered saline; TUNEL, terminal deoxynucleotide transferase-mediated deoxyuridine triphosphate nick end-labeling; PMSF, phenylmethylsulfonyl fluoride.

The endoplasmic reticulum (ER) is as an organelle closely involved in the protein biosynthesis in the cell. A disturbance of this biosynthesis mechanism leads to the accumulation of unfolded proteins, and this phenomenon, known as ER stress, is thought to trigger ER stress-mediated apoptosis. In the intrinsic apoptotic signaling pathway, a complex of Apaf-1, dATP and cytochrome c released from mitochondria activates several caspases comprising a family of cysteine proteases (*1*). Caspase-9, as an initiator caspase, activates caspase-3 for the fragmentation of nuclear DNA via the degradation of DNA fragmentation factor (DFF45)/inhibitor of caspase-3-activated DNase (ICAD) and subsequent activation of DFF40/CAD (caspase-3-activated DNase) (*2*, *3*). A simple explanation of the phenotypic observations for ordinary apoptosis is provided by this mechanism. On the other hand, in the case of ER stress-mediated apoptosis, it is known that the caspase-12 activity induced by calpain or the Ire1/TNF receptor-associated factor 2 (TRAF2)/c-Jun N-terminal kinase (JNK) complex at the ER membrane plays a crucial role in the progression of apoptosis (*4*, *5*). A possible downstream mechanism in which caspase-12 directly activates caspase-9 by cleaving its inactive form (*6*) or JNK activated by ASK1 induces apoptosis (*7*, *8*) has also been reported.

In addition to the pro-apoptotic mechanism, anti-apoptotic or protective mechanisms are observed when ER stress occurs in the cell. For instance, the expression of chaperone proteins including glucose-regulated protein (GRP) 94 and 78 or abeyance of de novo protein biosynthesis, referred to as the unfolded protein response (UPR), *via* sensor proteins including Ire1, PERK (PKR-like ER kinase) and ATF6 (activating transcription factor 6) on the ER membrane (*9–12*), and degradation of unfolded proteins, also known as endoplasmic reticulum-associated degradation (ERAD), are involved in the protective process (*13*). However, the details of the mechanism underlying ER stress-mediated apoptosis and its protection remain obscure.

Besides intracellular protective mechanisms, some trophic factors, such as nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), insulin-like growth factor-I (IGF-I), hepatocyte growth factor (HGF), glial cell-derived neurotrophic factor (GDNF), ciliary neurotrophic factor (CNTF), and so on, can prevent the ordinary apoptosis (*14–17*). These factors activate phosphatidylinositol-3 kinase (PI3-K), which is a heterodimer composed of an 85 kD regulatory subunit (p85) and a 110 kD catalytic subunit (p110), after binding to their receptors. PI3-K-mediated signaling is a common anti-apoptotic pathway involving the phosphorylation of mitochondrial BAD (*18*), forkhead transcription factor (FKHR)(*19*), caspase-9 (*20*), or inhibitor of NF-kB kinase kinase (IKK) (*21*) by Akt, a molecule downstream of PI3-K. We recently showed that 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a neurotoxic agent, induces apoptosis in PC12 cells, and that NGF affords protection from this toxicity of MPTP *via* the PI3-K signaling path-

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way (*17*). Thus, we attempted to determine whether the PI3-K signaling pathway activated by NGF could also prevent tunicamycin (Tm)-induced ER stress or not. We observed that NGF promoted the survival of PC12 cells via the PI3-K signaling pathway during ER stress-mediated apoptosis induced by Tm, and this pathway prevented the activation of caspase-12, an ER stress-specific caspase, which lacks a possible phosphorylation site for Akt. Although JNK was activated by Tm, a specific inhibitor of JNK did not affect the viability of Tm-treated PC12 cells, indicating that JNK is not involved in the progression of Tm-induced apoptosis. Overall, we conclude that the NGF-mediated survival signaling pathway prevents ER stress-induced apoptosis through inactivation of caspase-12, a molecule in the apoptotic pathway upstream of caspase-9.

MATERIALS AND METHODS

Reagents—Tunicamycin, MPP^+, and antibody against caspase-12 (*22*) were purchased from Sigma. Other antibodies [phospho-Akt(473), Akt, phospho-p42/p44 MAPK, p42/p44 MAPK, phospho-JNK and JNK] were obtained from Cell Signaling Inc. Caspase inhibitors [carbobenzoxy-Asp-Glu-Val-Asp-fluoromethylketone (fmk) (DEVD), carbobenzoxy-Val-Ala-Asp-fmk (VAD), carbobenzoxy-Val-Glu-Ile-Asp-fmk (VEID), and carbobenzoxy-Ile-Glu-Thr-Asp-fmk (IETD)] were purchased from Calbiochem. Substrates coupled the fluorescent MCA (Ac-DEVD-MCA and Ac-LEHD-MCA) were obtained from Peptide Institute. NGF was a generous gift from Dr. Yamada (Osaka University).

Cell Culture—PC12 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% (v/v) precolostrum newborn calf serum (PNCS) and 5% (v/v) heat-inactivated horse serum (HS). For the measurement of cell viability, Hoechst 33258 staining, or TUNEL staining of PC12 cells, cells were seeded onto collagen-coated 96-well plates or 8-well chamber slides at 1×10^5 cells/cm^2. The next day, the medium was changed to serum-free DMEM (SF-DMEM), and tunicamycin, another inhibitor, or NGF was added at this time point if necessary. At 24 h after the addition of reagents, cell viability was measured or apoptotic cells were stained. We also measured the viability of cells exposed for a long time, and employed control cells at each time point to compare the data.

Measurement of Cell Viability—Viable cells were quantified by means of the alamar Blue™ (alamarblue) assay, which measures mitochondrial activity like the MTT assay (*17*). Briefly, cells were seeded into a 96-well plate and the medium was changed for the assay at an appropriate time. Then, the medium was changed again to SF-DMEM containing 5% (v/v) alamarblue solution and the cells were incubated for 1 h. The intensity of the fluorescence was determined with a spectrofluorometer with excitation at 560 nm and emission at 595 nm. Cell viability was defined as {(test sample count) – (blank count)/(untreated control count) – (blank count)} × 100.

LDH Release Assay—Apoptotic cells were quantitated by measurement of extracellular lactate dehydrogenase (LDH) activity using a LDH-Cytotoxicity Test (WAKO), as recommended by the manufacturer. Briefly, PC12 cells were seeded onto a 96-well plate, and tunicamycin, without or with caspase inhibitors, in 100 μl of SF-DMEM was used to treat the cells for 24 h. The control group was also cultured in SF-DMEM. Then, the SF-DMEM was recovered, and the LDH activity released was measured by adding reaction reagent comprising nitro blue tetrazolium (NBT), diaphorase, and nicotinamide adenine dinucleotide (NAD) to produce a colored diformazan. After 45 min, 100 μl of 1 M HCl was added to each sample to stop the reaction. Then, the absorbance, which reflects cell damage, was detected with a colorimeter (Viento, Dainippon) at 560 nm.

Hoechst 33258 Staining or TUNEL Staining—Apoptotic cells were stained by the terminal deoxynucleotide transferase-mediated deoxyuridine triphosphate nick end-labeling (TUNEL) method as previously described (*17*) or by the method described above with slight modification. Briefly, fixed and permeable cells were reacted with terminal deoxynucleotidyl transferase (TOYOBO) using biotinylated-TdT at 37°C for 1 h, and then with the FITC-conjugated avidin at 37°C for 1 h. For the Hoechst 33258 staining, the fixed cells were incubated with the 1 μg/ml of Hoechst 33258 dye in phosphate-buffered saline (PBS) for 30 min as described previously.

Immunoblot Analysis—PC12 cells were seeded in 6-well plates and the medium was changed to SF-DMEM for one night to reduce the level of phosphorylation in the cells. Then, the cells were lysed in a lysis buffer comprising 50 mM Tris-HCl (pH 7.8), 150 mM NaCl, 1% sodium dodecylsulfate (SDS), 1 mM ethylenediamine tetraacetic acid (EDTA), 1 mM phenylmethylsulfonyl fluoride (PMSF), 2 μg/ml of aprotinin and 1 mM Na_3VO_4 after treatment with NGF. The total lysate (20 μg per lane) was loaded on an SDS-PAGE gel, electrophoresed and blotted onto a nitro-cellulose membrane using a semi-dry blotter (Atto). The first antibody was loaded onto the membrane after the blocking with Block-Ace (Dainippon) and then the horseradish peroxidase-conjugated signal was enhanced using an ABC kit (Vector Laboratories). Bands were detected and visualized with a light capture system (Atto) using Immunostar reagents (WAKO). For reprobing, the membrane was vigorously shaken at 50°C in a buffer comprising 50 mM Tris-HCl (pH 6.7), 2% (w/v) SDS and 2-mercaptoethanol, and then blocked with Block-Ace. After subsequent antibody reaction, the bands were visualized as described above.

Quantification of Lipid Peroxidation—After exposure to MPP^+ or tunicamycin, the amount of lipid peroxide was determined using a Lipid Hydroxide Assay kit (Cayman Chemical). Briefly, PC12 cells were collected in lysis buffer comprising 10 mM Tris-HCl (pH 7.6), 150 mM NaCl and 1 mM PMSF on ice. Then, an ExtractR solution saturated with methanol and chloroform were added to each sample, and lipid peroxides were extracted from the chloroform layer after centrifugation. Chromogen (2.25 mM ferrous sulfate and a 1.5% methanolic solution of ammonium thiocyanate) was mixed with each sample, and the hydrophobic chloroform solutions were extracted after centrifugation. To determine the amount of lipid peroxide, tinctorial chloroform solutions were analyzed with a colorimeter (Viento, Dainippon) at 500 nm. The obtained absorbance values were calibrated as to the total amount of protein in the cells. The data were calcu-

lated as {(sample value) – (blank value)/(untreated sample value) – (blank value)} × 100.

Northern Blot Analysis—PC12 cells were seeded onto collagen-coated 6-cm-diameter dishes and the medium was changed to SF-DMEM supplemented with MPP^+ or tunicamycin, if necessary. Then, total mRNA was collected with ISOGEN (WAKO). The total RNA (20 μg) was electrophoresed on a 1% (w/v) agarose/2 M formaldehyde gel and then transferred to a nitrocellulose membrane (NEN Life Science). Hybridization was performed overnight at 65°C in 10% (v/v) Denhart solution (WAKO), 0.5% (w/v) SDS, 6× saline sodium citrate (SSC), and 100 μg/ml of salmon sperm DNA with a ^{32}P-GRP78 riboprobe. Then, the membrane was washed twice with 2× SSC containing 0.5% SDS at 65°C for 15 min and visualized by autoradiography (Kodak). To determine the RNA content, the membrane was rehybridized with a ^{32}P-28 S RNA riboprobe. The conditions for hybridization and washing were identical to those described above.

The ^{32}P-GRP78 riboprobe was generated with a random primer DNA labeling kit (TaKaRa), [α-^{32}P]dUTP and a linearized 377-bp fragment. The linearized fragment was obtained by RT-PCR from total RNA of tunicamycin-treated PC12 cells and subcloned into the pGEM-T Easy vector. The primer set used was: 5′-ACAAAACTGATTCCGAGGAACA-3′ and 5′-ATGCGCTCTTTGAGCTTTTTGT-3′. The ^{32}P-28 S RNA riboprobe was generated as described above with a 28 S RNA fragment (a gift from Dr. Kashihara).

Measurement of Caspase-3 and -9 Activities—Caspase-3 and -9 activities were measured using a fluorogenic peptide as described previously (*17*). Briefly, cells were collected and lysed in a lysis buffer comprising 10 mM Hepes-KOH (pH 7.4), 2 mM EDTA and 1 mM PMSF. After centrifugation, a volume of the supernatant was mixed with 2× ICE buffer comprising 20 mM Hepes-KOH (pH 7.4), 20% glycerol (v/v), 2 mM PMSF, 4 mM dithiothreitol and 50 μM Ac-DEVD-MCA for caspase-3 or 50 μM Ac-LEHD-MCA for caspase-9, followed by incubation for 1 h at 37°C. We confirmed that the enzyme activity of all samples increased linearly for at least two hours. After the addition of 200 μl of distilled water, fluorescence was detected using a spectrofluorometer (Dainippon). The excitation and emission wavelengths were 380 and 460 nm, respectively.

Statistical Evaluation—All values in the figures are means ± SEM. Student's *t*-test or one-way ANOVA was used to compare means between groups. A P value of <0.05 was considered statistically significant.

RESULTS

NGF Prevents Tunicamycin-Induced Apoptosis in PC12 Cells—In order to investigate the protective activity of NGF against ER stress-induced apoptosis, we examined the effect of NGF on the viability of tunicamycin (Tm)-treated PC12 cells. At first, we confirmed that Tm could induce ER stress and subsequently ER stress-mediated apoptosis. As shown in Fig. 1A, Tm dose-dependently reduced cell viability up to 1 μg/ml in 24 h. Tm also time-dependently reduced cell viability, and significantly induced the expression of GRP78 as an indicator of ER

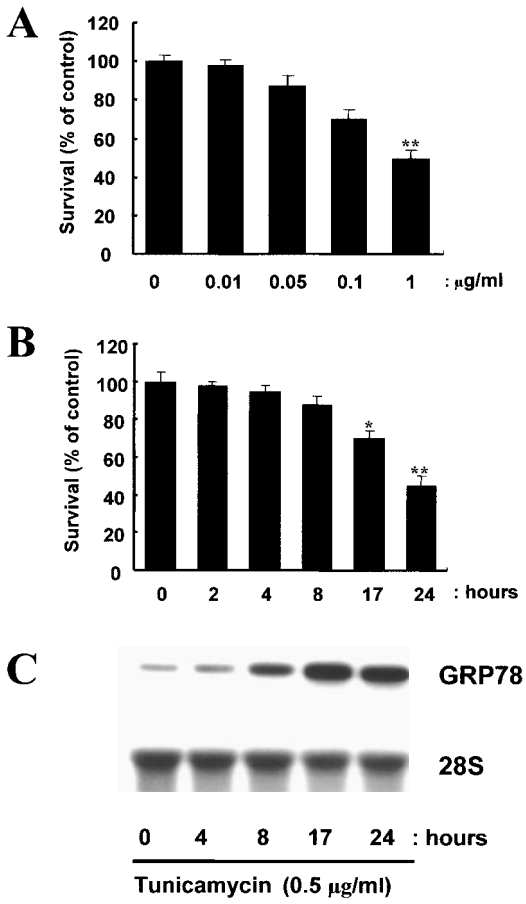

Fig. 1. **Induction of ER stress in tunicamycin-treated PC12 cells.** (A) PC12 cells were seeded as described under "MATERIALS AND METHODS," and tunicamycin (Tm) was added in serum-free DMEM for 24 h at the indicated concentrations. Viable cells were measured using alamarblue. Values are means ± SEM (n = 4), and statistical analysis was carried out with Student's t-test. 0 μg/ml *vs.* 1 μg/ml; $^{**}p < 0.01$. (B) Cells were seeded as described above, and 1 μg/ml of Tm was added for up to 24 h. Cell viability was measured as described above. Values are means ± SEM (n = 4), and statistical analysis was carried out with Student's t-test. 0 h *vs.* 17 h, $^{*}p < 0.05$; 0 h *vs.* 24 h, $^{**}p < 0.01$. (C) The expression of GRP78 or 28S rRNA was detected by Northern blot analysis with a radio-labeled probe as described under "MATERIALS AND METHODS." The specific bands were visualized by autoradiophy. The data are representive of two independent experiments.

stress (Fig. 1B and C). Then, we observed the morphological features of Tm-treated cells cultured in the presence of NGF. We found that PC12 cells died in an apoptotic manner judging from the results of TUNEL and Hoechst 33258 staining, and NGF clearly prevented this apoptosis, indicating that NGF attenuates the ER stress-induced apoptosis (Fig. 2). The protective effect of NGF was not complete judging from the observation that a small number of TUNEL- and Hoechst 33258-positive cells were observed even in the presence of NGF. For analysis of the NGF-mediated signaling pathway, we measured cell viability following treatment with LY294002 (LY) or PD98059 (PD), specific inhibitors of phosphatidylinositol 3-kinase (PI3-K) and upstream kinase (MEK1) of mitogen-activated kinases (MAPKs, also termed ERKs), respectively, together with Tm and NGF

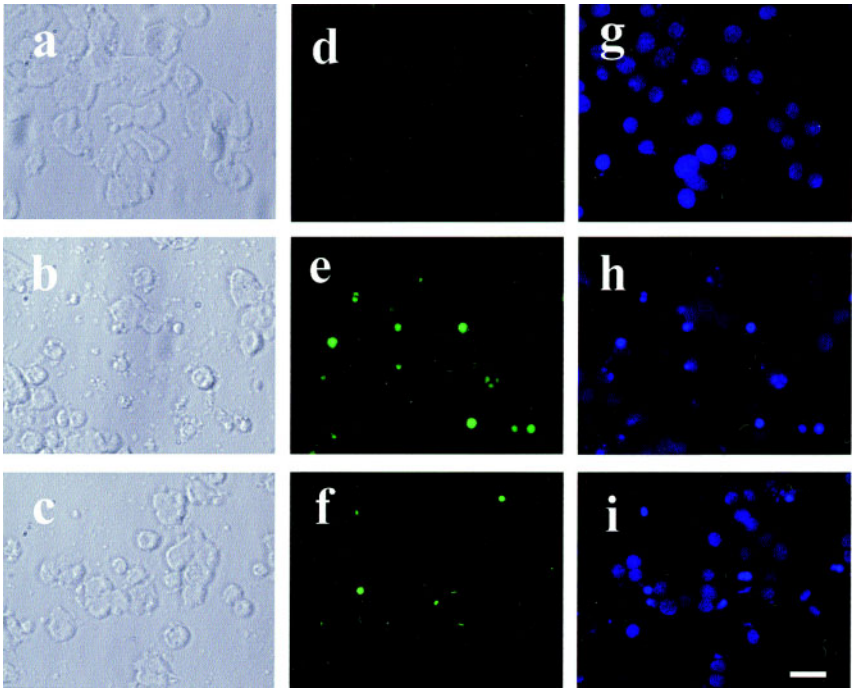

Fig. 2. **Apoptotic features of tunicamycin-treated PC12 cells.** Fragmentation of DNA in the nucleus and condensed chromatin were detected by TUNEL staining (d–f) and Hoechst 33258 staining (g–i), respectively, at 24 h after Tm treatment. The methods for staining were described under Materials and Methods. Untreated (a, d, g) or tunicamycin-treated cells in the absence (b, e, h) or presence of NGF (c, f, i) are shown. Apoptotic nuclei were detected and photographs were taken using a fluorescence microscopy system (Nikon). The bar denotes 20 μm.

(Fig. 3A). LY remarkably reduced the NGF-mediated cell survival while PD did not, indicating that PI3-K is essential for the NGF prevention of Tm-induced apoptosis. We confirmed that LY specifically inhibited the phosphorylation of Akt but not ERKs by using phosphorylated form-specific antibodies (Fig. 3B).

PI3-K Suppresses a Seriate Caspase Cascade—Next, we examined how the ER stress-mediated apoptosis was suppressed through the PI3-K signaling pathway by focusing on the caspase cascade using some caspase inhibitors. As shown in Fig. 4A and B, z-DEVD-fmk, an inhibitor of caspase-3, increased cell viability as did z-VAD-fmk, a general caspase inhibitor, however, z-VEID-fmk and z-IETD-fmk, inhibitors of caspase-6 and -8, respectively, did not, in the presence of Tm. The preventive effect of z-VAD-fmk was greater than that of z-DEVD-fmk. These results indicate that at least caspase-3 is involved in the Tm-induced apoptosis in PC12 cells. Other caspase(s) is/are also involved because the general inhibitor z-VAD-fmk strongly suppressed cell death. Then, to clarify whether or not the PI3-K signaling pathway suppresses the activity of a specific caspase, we measured the activities of caspase-3, -9 and -12, which are seriate components of the caspase cascade, using fluorogenic substrates or by immunoblotting. As shown in Fig. 5A and B, the caspase-3- and -9 activities, which were increased by Tm up to approximately 10-fold and approximately 1.8-fold, respectively, were reduced by NGF, and LY completely abolished the NGF effect. As shown in Fig. 5C, a notable increase of activated fragments of caspase-12 was caused by Tm treatment and the enhanced increase was reduced by NGF addition. LY prominently decreased the NGF effect. The activity of caspase-12 was only measured by immunoblot analysis due to a lack of a substrate for its enzymatic activity (Fig. 5C) (*22*). These lines of evidence indicate that the PI3-K signaling pathway suppresses ER stress-induced apoptosis by inhibiting caspase cascade including caspase-3, -9 and -12.

JNK Is Not Involved in Tm-Induced Apoptosis—c-Jun N-terminal kinase (JNK) is one of the components of the Ire1-TNF receptor associated factor 2 (TRAF2) complex (*4*, *5*). It is known that JNK in this complex induces apoptosis during ER stress as free JNK induces ordinary apoptosis. We observed significant activation of JNK up to 24 h on Tm treatment (Fig. 6A). With the indicated concentrations of a specific JNK inhibitor, SP600125, the activity of JNK decreased (Fig. 6B), although cell viability was not affected in the presence of SP600125 with 1 μg/ml Tm (Fig. 6C). These results suggest that JNK is not essential for the Tm-induced apoptosis of PC12 cells.

ROS Is Not Observed in Tm-Induced Apoptosis—In ordinary apoptosis, reactive oxygen species (ROS) are produced and mitochondrial dysfunction is induced with the release of cytochrome *c* (*23*). We then examined whether ROS were produced during Tm-induced apoptosis or not. As shown in Fig. 7A, Tm did not trigger the production of ROS, while 1-methyl-4-phenylpyridinium (MPP^+), a neurotoxin that induces ROS production in PC12 cells, did. *N*-Acetyl-L-cysteine (NAC), an antioxidant, only rescues cells injured by ROS. To confirm the involvement of ROS in MPP^+-induced but not Tm-induced apoptosis, the amount of lipid peroxide in MPP^+- or Tm-treated PC12 cells was determined. As shown in Fig. 7B, Tm did not increase the lipid peroxide level while MPP^+ did time-dependently. These results suggest that the production of ROS is not involved in the Tm-induced apoptosis of PC12 cells.

DISCUSSION

We focused on the protective effect of NGF toward ER stress-induced apoptosis. The mitochondrial dysfunction or permeabilization that has been reported to mediate

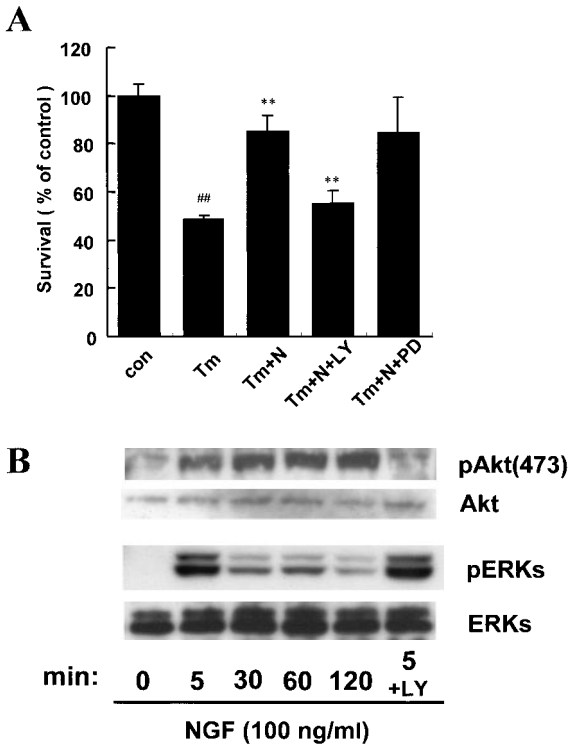

Fig. 3. **NGF-mediated cell survival *via* the PI3-K signaling pathway.** (A) PC12 cells were maintained and seeded as described under "MATERIALS AND METHODS." Tunicamycin (Tm) at 1 μg/ml and NGF (N) at 100 ng/ml were added or not (con) in the absence or presence of LY294002 (LY) or PD98059 (PD). Viable cells were measured after 24 h using alamarblue. Values are means ± SEM (n = 4), and statistical analysis was carried out with Student's t-test. con vs. Tm, $p < 0.01$; Tm *vs.* Tm+N, Tm+N vs. Tm+N+PD or LY, $^{**}p < 0.01$. (B) Cells treated with NGF in the absence or presence of LY294002 (LY) for the periods indicated were lysed in lysis buffer and then 20 μg of total protein per lane was used for SDS-PAGE. Then, immunoblotting was carried out using anti-phospho-Akt antibody [pAkt(473)], anti-Akt antibody (Akt), anti-phospho-p42/p44 MAPK antibody (pERKs), or anti-p42/p44 MAPK (ERKs) antibody as described under "MATERIALS AND METHODS." The bands were visualized with a light-capture system (Atto).

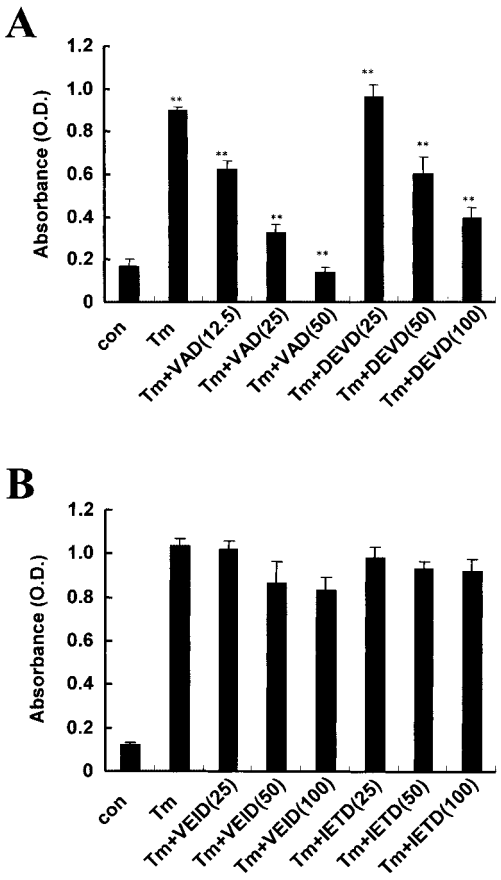

Fig. 4. **Involvement of caspases in tunicamycin-induced apoptosis.** (A, B) Caspase inhibitors (VAD: zVAD-fmk, DEVD: zDEVD-fmk, VEID: zVEID-fmk, IETD: zIETD-fmk) were added to PC12 cells at the indicated concentrations (μM) in the absence (con) or presence of tunicamycin (Tm) for 24 h. Viable cells were estimated at 24 h after the addition of reagents by means of LDH release assay as described under "MATERIALS AND METHODS." Values are means ± SEM (n = 4), and statistical analysis was carried out with one-way ANOVA among Tm-treated groups. $^{**}p < 0.01$, $p < 0.01$.

almost all cases of ordinary apoptosis triggers the activation of caspase-9 *via* the formation of an apoptosome complex of Apaf-1, dATP and released cytochrome c. This apoptosis can be arrested by phosphorylation of BAD (on the mitochondrial membrane), pro-caspase-9, FKHR or IKK through the PI3-K signaling pathway. Here we present new evidence that the NGF-mediated PI3-K signaling pathway can also suppress the activation of caspase-12 during Tm-induced apoptosis. Surprisingly, the amino acid sequence of caspase-12 does not include a potential phosphorylation site for Akt, although the PI3-K signaling pathway activates Akt. These results suggest that there are some target molecules for the PI3-K signaling pathway other than caspase-12. Caspase-12 forms a large complex during its activation on the ER membrane (*4*, *5*). We should focus on the proteins in this complex to identify new substrate(s) for Akt downstream of the PI3-K signaling pathway in further studies. We demonstrated that caspase-3, -9, and -12 were activated during Tm-induced apoptosis and that all these caspases were inactivated via the PI3-K signaling pathway induced by NGF. What is the major protective effector operating downstream of the PI3-K signaling pathway during ER stress-mediated apoptosis? (i) Caspase-9 is directly activated by caspase-12. (ii) Caspase-12 is considered to be important for ER stress from the reported observations on caspase-12 knock-out mice (*24*). (iii) We did not observe the release of cytochrome c (data not shown), indicating that the apoptosomes are not directly activated. Overall, we consider that the PI3-K signaling pathway suppresses ER stress mainly *via* some unknown inhibitory action(s) on caspase-12 activity. Detailed analyses to determine the caspases controlled by the PI3-K signaling pathway using knock-out mice defective in other relevant caspases and to detect the phosphorylation of pro-caspase-12 by other effectors are necessary. Jimbo *et al*. (*25*) recently reported that caspase-8 is involved in the progression of ER stress based on their observation of P19 embryonal carcinoma cells, while we could not observe any contribution of caspase-8 to the ER

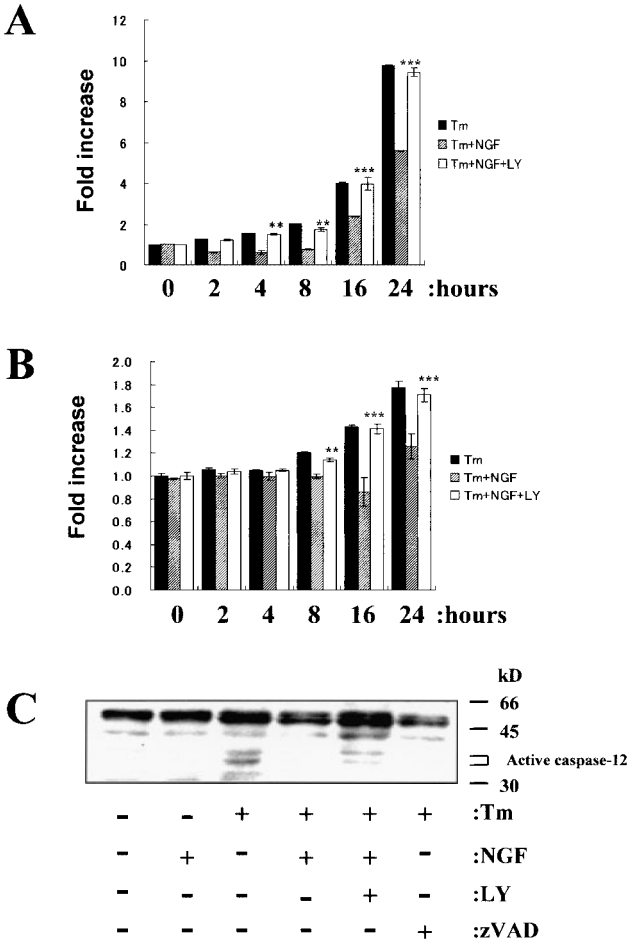

Fig. 5. **Suppression of caspase-3/-9/-12 activity by PI3-K.** (A, B) PC12 cells were cultured as described under "MATERIALS AND METHODS," and then the medium was changed to serum-free DMEM containing tunicamycin (Tm), Tm + 100 ng/ml NGF (NGF) or Tm + NGF + 5 μM LY294002 (LY) for the periods indicated under "MATERIALS AND METHODS." Then, cells were collected in the lysis buffer, and the activities of caspase-3 (A) and caspase-9 (B) were measured using specific fluorogenic substrates (A: 50 μM Ac-DEVD-MCA, B: 50 μM Ac-LEHD-MCA). Values are means ± SEM (n = 4), and statistical analysis was carried out with Student's t-test. 0 hour *vs.* indicated hour, $^{***}p < 0.001$, $^{**}p < 0.01$. (C) Cells were treated with or without Tm, NGF, LY or zVAD for 24 h and lysed, and then immunoblot analysis was performed using anti-caspase-12 antibody as described under "MATERIALS AND METHODS." + and – denote treated and untreated cells, respectively. The data are representive of two independent experiments.

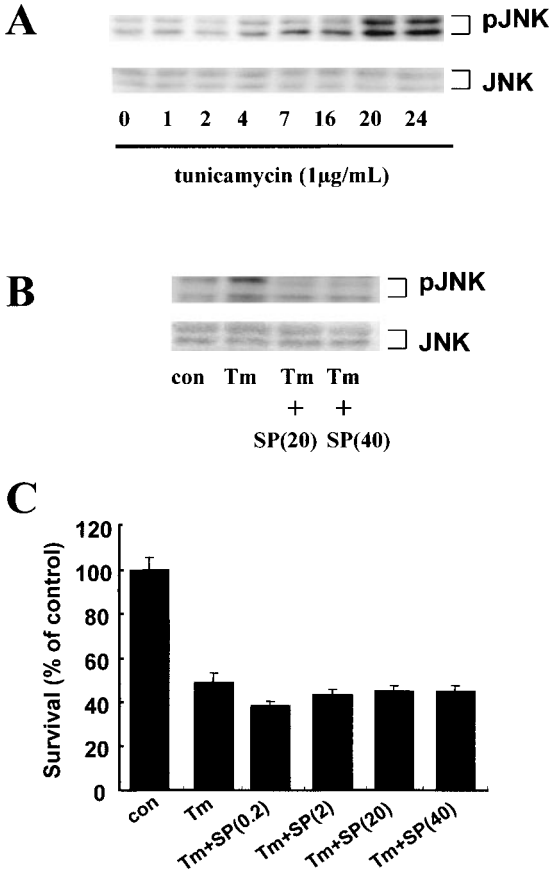

Fig. 6. **Activity of JNK in tunicamycin-induced apoptosis.** (A) PC12 cells were treated with 1 μg/ml of tunicamycin for the periods indicated (h). Lysates were prepared, and immunoblot analysis was carried out using anti-phospho-JNK antibody (pJNK), and then anti-JNK antibody (JNK) for reprobing. The specific bands were visualized with a light-capture system (Atto). (B) PC12 cells were treated in the absence (con) or presence of 1 μg/ml of tunicamycin (Tm) with or without SP600125 (SP: μM) for 24 h, and then immunoblot analysis was carried out as mentioned above. The same amount of protein used for detecting pJNK was subjected to SDS-PAGE for the detection of JNK. (C) SP600125 (SP) was added at the indicated concentrations (μM) for 24 h in the absence (con) or presence of 1 μg/ml of tunicamycin (Tm). Viable cells were measured using alamarblue. Values are means ± SEM (n = 4).

stress-induced apoptosis of PC12 cells and cultured cerebral cortical neurons (data not shown).

It is thought that the ubiquitine-proteasome pathway during ERAD plays a crucial role in preventing the accumulation of unfolded proteins. It has been reported that ubiquitine protein ligase facilitates prevention of p53-mediated apoptosis via the PI3-K signaling pathway in the ubiquitine-proteasome pathway (*26*). This protective system may be involved in NGF-mediated survival to prevent induction of the ER stress-mediated caspase cascade. We are currently examining this hypothesis.

We suggest that ROS are not produced during the ER stress-induced apoptosis in PC12 cells (Fig. 7A and B). We previously reported that 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) produced ROS and reduced cell viability, but did not induce the expression of GRP78, a marker of ER stress (*27*). MPTP is thought to induce ordinary apoptosis without ER stress in PC12 cells. From our present results, we consider that ER stress may involve some unknown intracellular mechanisms that differ from ordinary apoptosis.

We also suggest that JNK is not involved in Tm-induced apoptosis. Judging from those of our data, the activation of JNK is not essential to induce apoptosis, although the TRAF2/ASK1(apoptosis signal-regulating kinase 1)/JNK complex mediates apoptosis triggered by polyglutamine repeats (*8*). The latter observation was confirmed by analysis of ASK1 knock-out mice (*8*). However, it is not clear that all ER stress-mediated apoptosis occurs inevitably *via* this TRAF2/ASK1/JNK complex because there was evidence that ER stress is specifically reduced in cerebral cortex neurons or tubular proximal

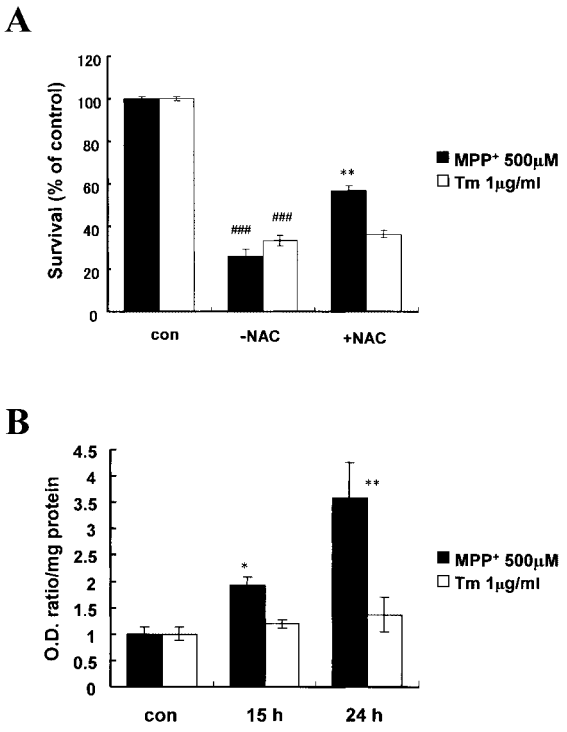

Fig. 7. **ROS are not increased in tunicamycin-treated PC12 cells.** (A) Viable cells treated with 500 μM MPP^+ or 1 μg/ml of tunicamycin (Tm) in the absence or presence of 2 mM N-Acetyl-L-cysteine (NAC) for 24 h were measured using alamarblue. Control cells (con) were cultured in SF-DMEM without either MPP^+ or Tm. –NAC represents untreated cells cultured in the absence of NAC with MPP^+ or Tm. +NAC represents treated cells cultured in the presence of NAC with MPP^+ or Tm. Values are means ± SEM ($n = 4$). Statistical analysis was carried out with Student's *t*-test. con v.s. untreated group, $p < 0.001$; –NAC with MPP^+ *vs.* +NAC with MPP^+, $^{**}p < 0.01$. (B) PC12 cells were treated with 500 μM MPP^+ or 1 μg/ml of tunicamycin (Tm) for the periods indicated. After the preparation of cell lysates, the quantity of peroxidized lipids was determined as described under "MATERIALS AND METHODS." The obtained values were normalized as to the quantity of protein in the cells. Values are means ± SEM ($n = 4$). Statistical analysis was carried out using Student's *t*-test. con *vs.* MPP^+-treated cells at 15 hours, $^*p < 0.05$; con *vs.* MPP^+-treated cells at 24 h, $^{**}p < 0.01$.

cells from caspase-12 knock-out mice (*24*). The action of the TRAF2/ASK1/JNK complex may be cell type-specific.

As found in many pathophysiological analyses, ER stress is crucial for progression of the diseases such as Alzheimer's disease (*28*), Parkinson's disease (*29*, *30*), and polyglutamine-mediated neurodegeneration (Hungtinton's or Machado-Joseph disease) (*31–33*) *via* the accumulation of unfolded proteins. Recent reports have also shown that type I diabetes progresses with ER stress-mediated apoptosis (*34*, *35*), and that the severity of Prelizaeus-Merbacher disease is modulated by UPR (*36*). The PI3-K signaling pathway may maintain the homeostatic balance of a cell for its survival. Thus, further detailed analyses of the neurodegenerative pathway and also the protective pathway may lead to important pathophysiological knowledge.

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