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神経系小胞体ストレスと誘導型アポトーシスの
ニコチンとニューロトロフィンによる防御機構

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Abstract: Neuronal degenerative diseases including Alzheimer's, Parkinson's and polyglutamine diseases are considered to involve endoplasmic reticulum (ER) stress, which leads to ER stress-mediated apoptosis of some neurons. In the process of these diseases, it has been reported that unfolded proteins are accumulated in the ER and this step is critical for progression of neuronal apoptosis through the caspase cascade involving an ER stress-specific caspase, caspase-12. During ER stress, chaperone proteins including glucose-regulated protein 78 (GRP78) are expressed to fold these unfolded proteins as an unfolded protein response (UPR). In cultured cells, ER stress can be induced by tunicamycin (Tm) or thapsigargin (Tg).

We report that nerve growth factor (NGF), a well-known member of the neurotrophin family, suppressed Tm-, Tg- and 2-deoxy-glucose (2DG)-induced ER stress-mediated apoptosis in PC12 cells as a model neuron. NGF prevented the progression of ER stress-induced apoptosis by the suppression of activation of caspase-12 via the PI3-kinase-Akt signaling pathway. NGF up-regulated the ER stress-stimulated expression of GRP78, but this up-regulation of GRP78 by NGF did not contribute to the protective effect of NGF on ER stress-induced apoptosis.

We found that nicotine protected against Tm-induced ER stress-mediated apoptosis but not Tg-induced ER stress-mediated apoptosis in PC12 cells. We also found that the expression of GRP78 was suppressed by nicotine in Tm-treated PC12 cells. Interestingly, the expression of GRP78 was not changed by nicotine in Tg-treated cells. Moreover, nicotine reduced the activation of caspase-12 in Tm-treated cells, but not in Tg-treated cells. These results suggest that nicotine prevents Tm-induced ER stress-mediated apoptosis by attenuating Tm-induced ER stress itself, probably by the clearance of accumulated unfolded proteins from the ER.

1. Introduction

Recently it is known that some neurons in patients of neurodegenerative diseases including Alzheimer's, Parkinson's and polyglutamine diseases are considered to be exposed to ER stress and consequently undergo apoptotic cell death [1-8]. The sources of ER stress are genetic or acquired after birth. Whether ER stress is induced by genetic or acquired effectors, neurons die through the apoptotic signaling specific to ER stress, but not through the well-known ordinary apoptotic signaling. In cultured cells, ER stress can be chemically induced by tunicamycin (Tm), thapsigargin (Tg) or 2-deoxy-glucose (2DG). Tm induces ER stress by inhibiting the glycosylation of newly synthesized proteins and Tg induces ER stress by inhibiting Ca^{2+}-ATPase which maintains the homeostasis of Ca^{2+} in the ER [9-10]. 2DG induces ER stress by depriving cultured cells of glucose.

These compounds also lead to ER stress-mediated apoptosis via an intracellular mechanism involving the caspase cascade [9-10]. At the same time, chaperone proteins including GRP78 are up-regulated during Tm- and Tg-induced ER stress [11-12]. Thus, these compounds activate the same intracellular signals involving ATF6, Ire1 and PERK to induce GRP78 expression, and trigger ER stress-mediated apoptosis when ER stress is severe. During ER stress-induced apoptosis of rat or mouse neurons, caspase-12 localized in ER is specifically activated [13-14]. After the activation of caspase-12, caspase-9 is activated without the release of cytochrome c from mitochondria, and then caspase cascade downstream of caspase-9 is activated as the ordinary apoptosis. Therefore, there exist two apoptotic signalings, the ordinary apoptotic singanling and the ER stress-induced apoptotic signaling. The latter ER stress-induced apoptotic signaling is specifically mediated by the ER stress-specific caspase-12. On the other hand, neurotrophins including NGF (nerve growth factor) and BDNF (brain-derived neurotrophic factor) are known to suppress ordinary apoptosis [15]. It is noteworthy whether neurotrophins can also suppress ER stress-specific apoptosis or not.

In this report, we describe the protective singnaling mechanism by which NGF suppresses Tm-induced, Tg-induced and 2DG-induced ER stress-mediated apoptosis in PC12 cells as a model neuron. NGF prevents the progression of ER stress-induced apoptosis by the suppression of activation of caspase-12 via the PI3-kinase-Akt signaling pathway. But NGF does not attenuate ER stress itself. NGF up-regulated the ER stress-stimulated expression of GRP78, but this up-regulation of GRP78 by NGF did not contribute to the protective effect of NGF on ER stress-induced apoptosis in PC12 cell. Next we describe that nicotine suppresses Tm-induced ER stress-mediated apoptosis, but not Tg-induced ER stress-mediated apoptosis. Nicotine prevents Tm-induced ER stress-mediated apoptosis via the attenuation of Tm-induced ER stress itself by a mechanism which does not work in Tg-induced ER stress.

2. NGF prevents 2DG-induced ER stress-mediated apoptosis via the PI3-kinase-Akt signaling pathway.

We observed that NGF prevented 2DG-induced ER stress-mediated apoptosis as well as Tm-induced and Tg-induced ER stress-mediated apoptosis in PC12 cells (Fig.1). The protective effect of NGF on ER
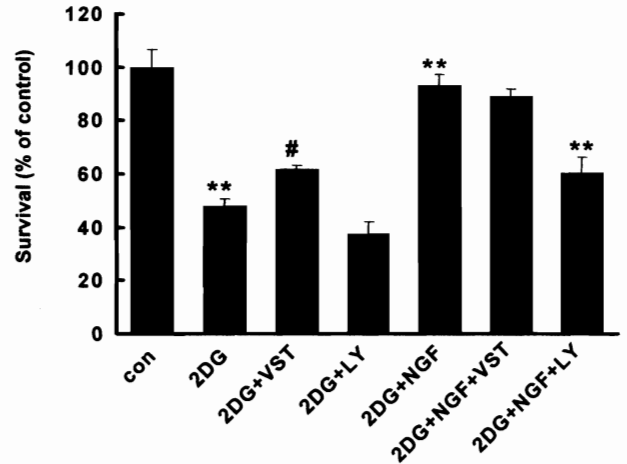
Fig.1 NGF has a survival-promoting effect on 2DG-induced ER stress-mediated apoptosis and VST does not alter the increased cell viability in the presence of NGF.

PC12 cells were maintained with DMEM containing 10% (v/v) fetal bovine serum. Cells were plated onto collagen-coated 96-well plates. The next day, the medium was changed to SF-DMEM (con) or SF-DMEM plus 24.5 mM 2DG in the absence (2DG) or presence (2DG+NGF) of 100 ng/ml NGF (NGF). 3.3 μM VST (VST) or 5 μM LY294002 (LY) was also added if necessary (2DG+VST, 2DG+LY, 2DG+NGF+VST and 2DG+NGF+LY). Then, cells were incubated for 2 days. Viable cells were measured using the alamarblue assay. Values are means±SEM (n=4) and statistical analysis was carried out with one-way ANOVA; **$P<0.01$. We also used Student's t-test to compare the significance between 2DG and 2DG+VST; #$P<0.05$.

stress-mediated apoptosis was decreased by the treatment with LY294002, a specific inhibitor of phosphatidylinositol 3-kinase (PI3-kinase). These results indicate that NGF prevented 2DG-induced ER stress-mediated apoptosis by the suppression of

activation of caspase-12 via the PI3-kinase-Akt signaling pathway.

3. NGF up-regulates the ER stress-stimulated expression of GRP78, but this up-regulation of GRP78 by NGF does not contribute to the protective effect of NGF on ER stress-induced apoptosis in PC12 cell.

We also found that 2DG increased the expression of GRP78 (Fig.2) and NGF up-regulated the 2DG-stimulated expression of GRP78 (Fig.3). On
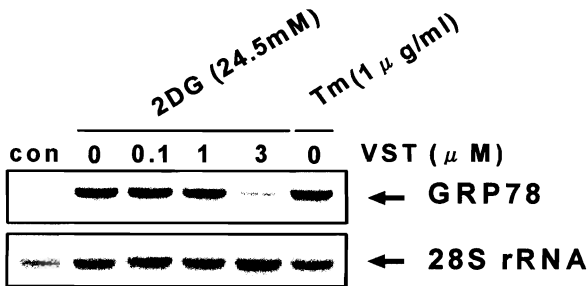
Fig.2 2DG-induced GRP78 expression and its suppression by VST in PC12 cells.

PC12 cells were cultured, and then 24.5 mM 2-deoxy-D-glucose (2DG) in the absence or presence of 0.1, 1.1 and 3.3 μM versipelostain (VST) was added to serum-free DMEM (SF-DMEM) (con). 1 μg/ml tunicamycin (Tm) was also added as a positive control. Cells were maintained for 8 hours. Then, total RNA was collected with ISOGEN. Total RNA was electrophoresed on a 1% (w/v) agarose/2 M formaldehyde gel and transferred onto a membrane. Hybridization was performed overnight at 65 °C in 10% Denhart solution, 0.5% (w/v) SDS, 6×saline-sodium citrate (SSC) and 100 μg/ml salmon sperm DNA with a radiolabeled GRP78 probe. The membrane was washed twice with 2×SSC containing 0.5% SDS at 65 °C for 30 minutes, and visualized by Fuji BAS2000. To estimate the RNA content, the membrane was rehybridized with a radiolabeled 28 S rRNA probe. The radiolabeled GRP78 probe was generated with a random primer DNA labeling kit (TaKaRa), [α-32P]dCTP and a linearized 377-bp cDNA fragment. The cDNA fragment was obtained by PCR with a cloned GRP78 cDNA-containing plasmid. The set of primers were 5'-ACAAAACTGATTCCGAGGAACA-3' and 5'-ATGCGCTCTTTGAGCTTTTTGT-3'.

the other hand, versiperlostatin (VST), which has an ability to down-regulate GRP78 expression through a
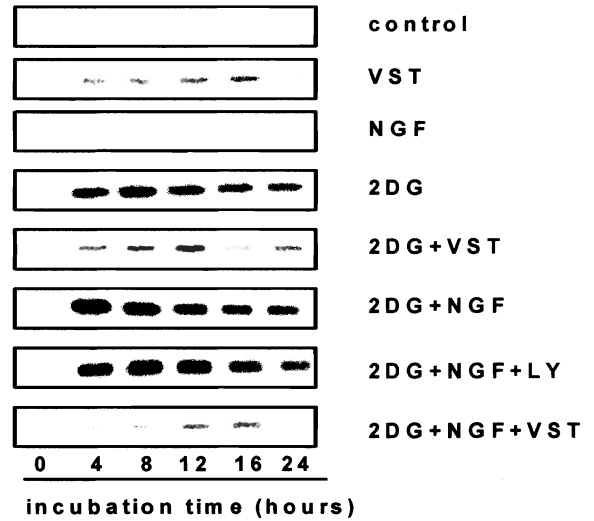
Fig.3 Suppression of GRP78 expression by VST in 2DG+NGF-treated PC12 cells.

PC12 cells were maintained and seeded onto a 6-cm diameter dish. The next day, SF-DMEM (control), 3.3 μM VST (VST), 100 ng/ml NGF (NGF), 24.5 mM 2DG (2DG), 2DG+VST, 2DG+NGF, 2DG+NGF+5 μM LY (2DG+NGF+LY) and 2DG+NGF+VST were added, and cells were cultured for 24 hours. Then, cells were lysed with ISOGEN, and the isolated total RNA was subjected to Northern blotting as described in Fig.2.

reduced production of a spliced XBP-1 mRNA and therefore is a specific inhibitor of GRP78 expression (Figs.2 and 3), increased the cell viability in the presence of 2DG alone, but did not increased that in the presence of 2DG plus NGF (Fig.1). That is to say, VST had no effect on the protective effect of NGF on 2DG-induced ER stress-mediated apoptosis. These results suggest that the up-regulation of GRP78 by NGF does not contribute to the protective effect of NGF on ER stress-induced apoptosis in PC12 cell, and that NGF prevents the progression of ER stress-induced apoptosis, but does not attenuate ER stress itself.

4. Nicotine protects against Tm-induced ER stress-mediated apoptosis but not Tg-induced ER stress-mediated apoptosis in PC12 cells.

Chronic nicotine treatment blocks arachidonic acid-, glutamate-, β-amyloid-, and tunicamycin-induced neurotoxicity [16, 17]. Nicotine also promotes cell survival of spinal cord motorneurons [18]. Many nicotinic acetylcholine receptor (nAChR) subtypes, for instance, α4β2 and α7, have been reported to be involved in these protective effects of nicotine [19-23]. The calcium influx, the activation of the PI3-kinase/Akt-mediated pathway, and the induction of Bcl-2 have been suggested to play important roles in nicotine-induced neuroprotective effects in various cells (19, 21, 22, 24-27).

We examined whether nicotine has a survival-promoting effect on cells treated with Tm and those treated with Tg. As a result, we found that nicotine prevented Tm-induced ER stress-mediated apoptosis in PC12 cells, whereas nicotine had no significant effect on Tg-induced ER stress-mediated apoptosis (Fig. 4) [28].

5. The expression of GRP78 is suppressed by nicotine in Tm-treated PC12 cells, but not in Tg-treated cells.

To investigate the prevention of progression of ER stress-mediated apoptosis by nicotine, we measured the activation of caspase-12 known as the ER stress-specific caspase. We previously reported that caspase-12 was activated during both Tm- and Tg-induced apoptosis, and that zVAD-fmk, a general caspase inhibitor, suppressed the activation of caspase-12 induced by both Tm and Tg to the control level. As shown in Fig. 5, the activation of caspase-12 was decreased by nicotine in Tm-treated cells, but not in Tg-treated cells. These results indicate that nicotine prevents the progression of only Tm-induced ER stress-mediated apoptosis.

To elucidate the molecular mechanism of nicotine prevension, we analyzed the expression of GRP78 during 24 hours after the addition of nicotine to Tm- and Tg-treated cells, by immunoblotting. Interestingly, nicotine suppressed the expression of GRP78 in Tm-treated cells, however, the expression of GRP78 was not altered by nicotine in Tg-treated cells (Fig.6). These data indicate that Tm-induced ER stress-mediated apoptosis is suppressed by nicotine

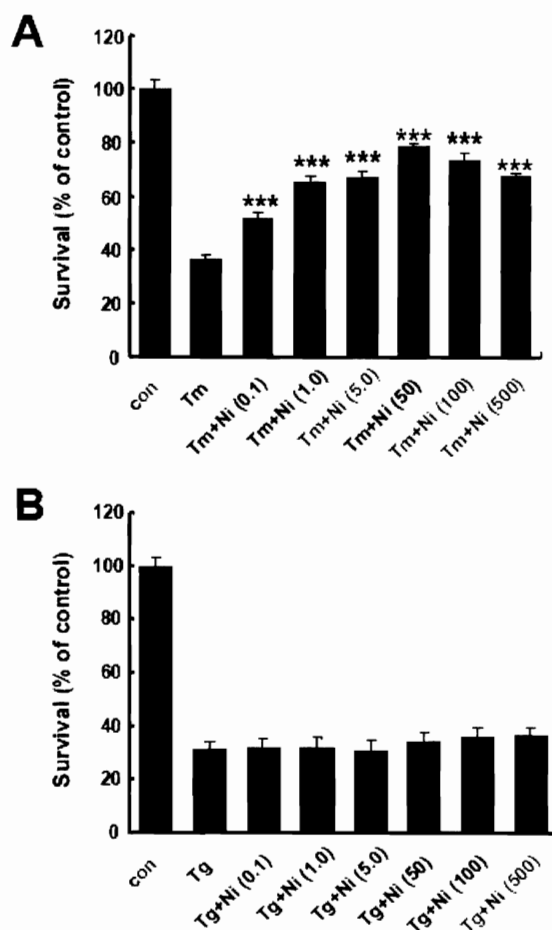through the attenuation of Tm-induced ER stress itself.

Fig.4 Survival-promoting effect of nicotine on Tm-induced ER stress-mediated apoptosis.

A and B, PC12 cells were cultured as described in Experimental Procedures, and 1 μg/ml tunicamycin (Tm) or 0.3μM thapsigargin (Tg) in the absence (con) or presence of nicotine (Ni) was added to serum-free DMEM. The final concentrations of nicotine are shown as μM in parentheses. PC12 cells were maintained for 24 hours, and viable cells were quantified using the MTT assay. Values are means±SEM (n=4) and statistical analysis was carried out with one-way ANOVA. ***P<0.001

We consider that this effect of nicotine may be achieved by the clearance of accumulated unfolded proteins in the ER, however, the mechanism of nicotine action only in Tm-treated cells is still unkown. It has been reported that p97/VCP is important for the clearance of accumulated unfolded proteins during ER

stress [29]. Thus, p97/VCP may be a key molecule in the nicotine prevention of Tm-induced ER stress.
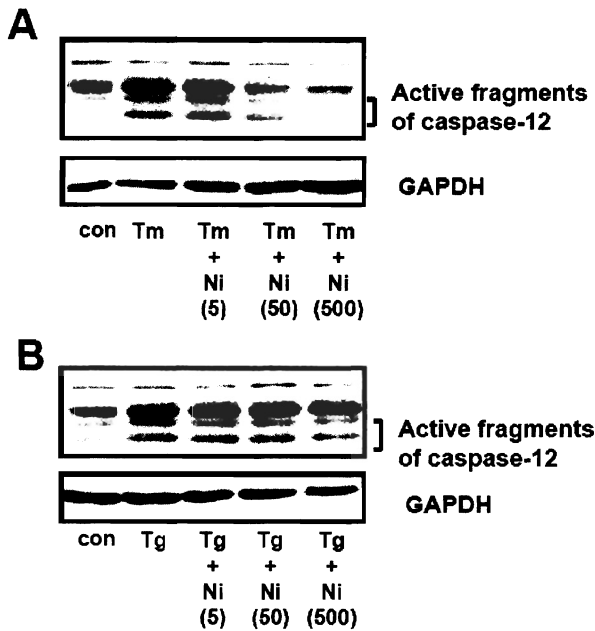
GRP78 expression. Moreover, XBP-1, which is

A

Active fragments of caspase-12

GAPDH

con Tm Tm + Ni (5) Tm + Ni (50) Tm + Ni (500)

B

Active fragments of caspase-12

GAPDH

con Tg Tg + Ni (5) Tg + Ni (50) Tg + Ni (500)

A

GRP78

Actin

con Tm Tm + Ni (5) Tm + Ni (50) Tm + Ni (500)

B

GRP78

Actin

con Tg Tg + Ni (5) Tg + Ni (50) Tg + Ni (500)

Fig.5 Nicotine suppresses activation of caspase-12 in Tm-treated cells.

A and B, PC12 cells untreated (con) or treated with 1 μg/ml tunicamycin (Tm) or 0.3 μM thapsigargin (Tg), or 1 μg/ml Tm or 0.3 μM Tg in the presence of nicotine (Ni) for 24 hours were lysed in lysis buffer as described in Experimental Procedures. The final concentrations of Ni are shown as μM in parentheses. Twenty μg of total protein per lane was used for SDS-PAGE. Then, immunoblotting was carried out using anti-caspase-12 antibody (Sigma) and anti-GAPDH antibody (Chemicon) as described in Experimental Procedures. The bands were visualized with a light capture system (Atto) and the location of active fragments of caspase-12 was shown on the right.
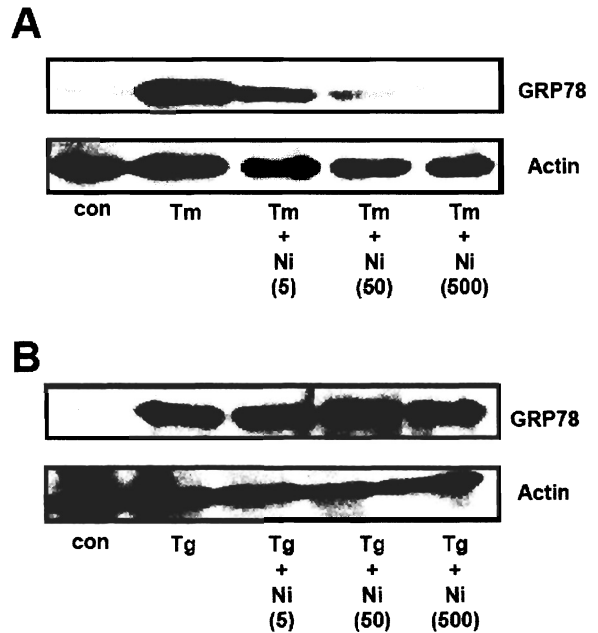
Fig.6 Nicotine suppresses GRP78 expression in Tm-treated cells.

A and B, PC12 cells untreated (con) or treated with 1 μg/ml tunicamycin (Tm) or 0.3 μM thapsigargin (Tg) , or 1 μg/ml Tm or 0.3 μM Tg in the presence of nicotine (Ni) for 24 hours were lysed in lysis buffer as described in Fig.2. The final concentrations of Ni are shown as μM in parentheses. Immunoblotting was carried out using anti-GRP78 antibody (SantaCruz) or anti-GAPDH antibody (Chemicon). The bands were visualized with a light capture system (Atto) after treatment with SuperSignal West Femto (Pierce).

6. Nicotine prevents Tm-induced ER stress-mediated apoptosis by attenuating Tm-induced ER stress itself by a mechanism which does not work in Tg-induced ER stress.

The GRP78 gene has the cis-element ERSE (ER stress response element) in the promoter region. During ER stress, cleaved ATF6 works as a transcriptional factor binding to the ERSE to induce

generated by translation of XBP-1 mRNA spliced by Ire1, also binds to the ERSE to induce GRP78 expression. There may be a possibility that the suppression of GRP78 expression by nicotine is achieved through the suppression of these two pathways. However, we consider that the two pathways may not contribute to the suppression of GRP78 expression by nicotine, because both Tm and Tg induced GRP78 expression through the two pathways and the suppression of GRP78 expression by nicotine was not observed in Tg-treated cells. Therefore, we suggest that nicotine prevents

Tm-induced ER stress through the clearance of accumulated unfolded proteins by the ejection of unfolded proteins from the ER and the degradation of unfolded proteins via the ubiquitin-proteasome system. As shown in Fig.5 , the activation of caspase-12 which should occur after the accumulation of unfolded proteins was also suppressed by nicotine in addition to the suppression of GRP78 expression. Tg is an inhibitor of Ca^{2+}-ATPase which located on the ER membrane and disturbs the homeostasis of Ca^{2+} in the ER. If nicotine affects Ca^{2+}-mediated cell survival mechanism in the ER, it is possible to explain why nicotine has not a survival-promoting effect on Tg-induced ER stress.

We consider that the effect of nicotine on ER stress itself may be very useful in the therapy of neuronal degenerative diseases including Alzheimer's, Parkinson's and polyglutamine-related diseases. It is well-known that the nicotinic acetylcholine receptors are ion channels. On the other hand, it has been reported that Akt, which is known as a survival-promoting kinase, is involved in the nicotine-mediated signaling pathway through the nicotinic acetylcholine receptors [30]. We consider that we should focus on the signaling pathway induced by nicotine in relation with Akt to clarify the mechanism of survival-promoting effect of nicotine on Tm-induced ER stress.

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