Neuroprotective Action of Genipin on Tunicamycin-Induced Cytotoxicity in Neuro2a Cells

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Accumulation of unfolding or misfolded proteins within the lumen of the endoplasmic reticulum (ER) triggers ER stress, and sustained ER stress ultimately leads to cell death. Both of these events are involved in the activation of glucose-regulated protein of 78 kDa (GRP78, also known as Bip), CCAAT/enhancer-binding protein (C/EBP) homologous protein (CHOP, also known as growth arrest and DNA damage-inducible gene 153 (GADD153)), and caspase-I2. ER stress has been shown to be involved in neurodegenerative disorders, such as Alzheimer, Parkinson, and polyglutamine diseases. We previously showed that genipin, a natural iridoid compound, has a protective effect against amyloid-β (Aβ)-induced cytotoxicity. Here, we studied the protective effects of genipin on cytotoxicity induced in Neuro2a cells by the specific ER stress inducer tunicamycin (TM). TM treatment significantly reduced cell viability in a dose-dependent manner. Genipin dramatically rescued the cells against TM-induced cell death. In addition, genipin suppressed ER stress-induced upregulation of CHOP and GRP78. These data suggest that genipin is effective at protecting against neurodegenerative disorders.

Key words genipin; endoplasmic reticulum stress; neuroprotection; tunicamycin; Neuro2a cell

The endoplasmic reticulum (ER) is an important subcellular compartment implicated in posttranslational protein processing and transport. Several proteins are translated into the lumen of the ER where posttranslational modification, folding, and oligomerization occurs. Accumulation of unfolding or misfolded proteins within the lumen of the ER induces ER stress. During ER stress, multiple mechanisms appear to contribute to apoptosis. These include the activation of double-stranded RNA-activated protein kinase (PKR)-like ER kinase (PERK), glucose-regulated protein of 78 kDa (GRP78, also known as Bip), and CCAAT/enhancer-binding protein (C/EBP) homologous protein (CHOP, also known as growth arrest and DNA damage-inducible gene 153 (GADD153)).1–3) In addition, caspase-12 is specifically involved in apoptosis that results from stress in the ER.4,5) ER and oxidative stress have been shown to be involved in neurodegenerative disorders, such as Alzheimer disease (AD), Parkinson disease (PD), and polyglutamine disease.6–8) Many researchers have carried out studies from several different approaches to elucidate the mechanisms of these disorders, however, the exact details of the mechanisms remain unclear.

Nerve growth factor and brain-derived neurotrophic factor, a member of the neurotrophin family, regulate several functions of the nervous system, such as development, survival, and regeneration. In vitro and in vivo studies indicate that neurotrophins have neuroprotective effects and rescue neurons from various insults.9,10) However, they cannot be used as a medical treatment because of their inability to cross the blood–brain barrier and vulnerability to hydrolytic enzymes. Therefore, we have attempted to identify natural lipophilic compounds with endogenous neurotrophic factor-like activity to induce neuronal differentiation, survival, and regeneration in plants traditionally used as anti-ammnestic or anti-inflammatory medications.

Iridoids are known to possess various biological activities such as anticancer, antimicrobial, choleretic, hemodynamic, and hepatoprotective effects.10–14) We showed previously that iridoid compounds such as geniposide, gardenoside, catalpol, aucubin, and their hydrolysates have neurotrophicogenic activity in PC12h cells.15) In particular, genipin, the aglycon of geniposide, potently induces neurite outgrowth. Furthermore, we have shown that genipin has protective effects against amyloid-β (Aβ)-induced cytotoxicity.16,17) 6-hydroxydopamine (6-OHDA), and hydrogen peroxide (H₂O₂).18) Thus, it is believed that the protective effects of genipin may be useful in the treatment of neurodegenerative disorders.

In this study, we have examined the possibility that genipin has a neuroprotective effect on cytotoxicity induced by tunicamycin (TM). TM, a specific ER stress inducer, has also been proposed to be a putative neurotoxic factor. Therefore, we used TM to examine the neuroprotective effect of genipin on the cytotoxicity in a model of other neurodegenerative disorders.

MATERIALS AND METHODS

Materials Neuro2a cells (mouse neuroblastoma) were purchased from RIKEN Cell Bank (Tokyo, Japan). Eagle’s minimum essential medium (EMEM) was purchased from GIBCO (Grand Island, U.S.A.) and fetal bovine serum (FBS) from Equitech-Bio, Inc. (Kerrville, TX, U.S.A.). Kanamycin was purchased from Meiji Seika (Tokyo, Japan). Genipin was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) and dissolved in dimethylsulfoxide (DMSO). TM was obtained from Sigma Aldrich (St. Louis, U.S.A.) and also dissolved in DMSO. A CellTiter 96® AQueous One Solution Cell Proliferation Assay kit was purchased from Promega (Madison, U.S.A.) and a Sepasol RNA I super kit was obtained from Nacalai Tesque (Kyoto, Japan). Oligonucleotides with different base sequences were all obtained from GENSET KK (Kyoto, Japan). A Takara RNA...
PCR Kit (AMV) was obtained from Takara Bio, Inc. (Shiga, Japan). Rat monoclonal anti-caspase-12 antibody, alkaline phosphatase conjugated goat anti-rat immunoglobulin G (IgG), alkaline phosphatase conjugated goat anti-mouse IgG, and alkaline phosphatase conjugated goat anti-rabbit IgG antiseraums were obtained from Sigma Aldrich. Rabbit polyclonal anti-CHOP, anti-PERK, and anti-phosphorylated-PERK (p-PERK) antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Mouse monoclonal anti-BiP/GRP78 antibody was obtained from BD Biosciences (Franklin Lakes, NJ, U.S.A.). A nitro tetrazolium-bromo-4-chloro-3-indolyl-phosphate (BCIP/NBT) Phosphatase Substrate System detection kit was obtained from Kirkegaard & Perry Laboratories (Gaithersburg, MD, U.S.A.). Other chemicals used were all of the highest purity commercially available.

**Cell Culture** Neuro2a cells were grown in EMEM containing 10% FBS, 10 mM NaHCO3, 2 mM l-glutamine, and 125 μg/ml kanamycin at 37 °C with 10% CO2. Neuro2a cells were prepared prior to every experiment as follows. Neuro2a cells were plated onto 48-well plates for cell viability assay at a density of 0.5 × 104 cells/well. For reverse transcription polymerase chain reaction (RT-PCR) and Western blotting analysis, the cells were placed into 50 ml tissue culture flasks at subconfluent density. After 24 h of culture, the medium was replaced with serum-free EMEM supplemented with TIP (5 μg/ml transferrin, 5 μg/ml insulin, 20 nm progesterone).

**Cell Viability Assay** The cell viability of Neuro2a cells was evaluated by measuring the activity of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) reduction using a CellTiter 96® AQueous One Solution Cell Proliferation Assay kit. Twenty microliters of reagent was pipetted into each well of 96-well plates containing the samples in 100 μl of culture medium. The plates were incubated for 1 h at 37 °C with 10% CO2, and then the absorbance was recorded at 490 nm using a 96-well plate reader.

**RT-PCR** Total RNA was isolated from Neuro2a cells from one flask in 1 ml of Sepasol reagent and quantified by spectrophotometry at 260 nm. To amplify a 356 bp fragment of CHOP, the sense primer was 5’-CTGCCCT-TTCACCTTGGAGAC-3’ and the antisense primer was 5’-CGATCAAATGGAAGCCCTTA-3’. These primers flank the 90—109 sense and 445—426 antisense bases of the GRP78 cDNA sequence (GenBank access: AJ002387). To amplify a 252 bp fragment of PERK, the sense primer was 5’-AACCTTGGCATTGTTGAAAGG-3’ and the antisense primer was 5’-CCCTGGTTGCTAGCCGTA-3’. These primers flank the 539—558 sense and 1010—991 antisense bases of the GAPDH cDNA sequence (GenBank access: M32599). Five hundred nanograms of total RNA was transcribed with AMV Reverse Transcriptase XL (0.25 U/μl) using 0.125 μM of Oligo dT-primer (Takara RNA PCR Kit (AMV) Ver. 3). Two microliters of the reverse transcribed product for CHOP, GRP78, or PERK was amplified in a thermal gene cycler (GeneAmp PCR System 9700; Eppendorf, Foster City, CA, U.S.A.) using 0.2 μM of each sense and antisense primer, and Takara Ex Taq HS DNA polymerase (final 0.5 U) in a final volume of 20 μl. After an initial denaturation at 94 °C for 3 min, amplification was performed with 25 (CHOP, GRP78) or 30 (PERK) cycles as follows: 94 °C for 30 s (denaturation), 58 °C for 30 s (annealing), and 72 °C for 1 min (extension). PCR products were analyzed by restriction assays and fractionated on 2% agarose gel. Upon completion of electrophoresis, ethidium bromide-stained PCR products were photographed with a Kurabo ChemiStage camera. Densitometric analysis was carried out with the software NIH image and the densitometric values of various bands were divided by the density values of their respective GAPDH bands and normalized with respect to arbitrary units of the control group.

**Western Botting Analysis** Neuro2a cells from one flask were isolated and then sonicated in lysis buffer containing 50 mM Tris-HCl (pH 7.4), 0.1 mM (p-amidinophenyl)-methanesulfonyl fluoride, 1 mM dithiothreitol, 0.5 mM EGTA (pH 8.0), and inhibitor cocktail. Supernatants (5 μg protein) were loaded on a SDS polyacrylamide gel (10%) and analyzed. Protein concentrations were measured by the Bradford method18) using γ-globulin as a standard. After transferring to a polyvinylidene difluoride (PVDF) membrane, protein bands were reacted with anti-caspase-12 antibody (dilution, 1:1000), anti-CHOP antibody, anti-PERK antibody, anti-p-PERK antibody (dilution, 1:200), or anti-BiP/GRP78 antibody (dilution, 1:10000). After washing, the filter was reacted with anti-rat IgG antibody, anti-mouse IgG antibody (dilution, 1:10000), or anti-rabbit IgG antibody (dilution, 1:1000). To detect signals, samples were visualized with a BCIP/NBT Phosphatase Substrate System detection kit.

**Statistical Analysis** Statistically significant differences between groups were estimated by analysis of variance (ANOVA) followed by Scheffe’s test. A value of p<0.05 was considered to indicate a significant difference.

**RESULTS**

**TM Induced Cytotoxicity** TM, thapsigargin (TG), and brefeldin A (BFA) are the most commonly used pharmacologic agents to experimentally induce ER stress.19,20 TM inhibits N-linked glycosylation; TG disrupts ER Ca2⁺ stores, and BFA causes disassembly of the Golgi apparatus. To construct the ER stress model, we examined TM-induced cytotoxicity in Neuro2a cells. As shown in Fig. 1, TM significantly suppressed the MTS reduction activity of Neuro2a cells, in a dose-dependent manner, measured after 24 h of treatment. Significant decreases in MTS reduction were observed by treatment with 0.1 (Fig. 1B), 1, 3, and 5 (Fig. 1C) μg/ml of TM and the decreases were 63, 80, 82, and 77%, respectively, compared with control (Figs. 1A, E).
Protective Effect of Genipin on the TM-Induced Cytotoxicity

As shown in Fig. 1B, a low dose of 0.1 μg/ml TM induced morphologically significant cell damage. However, 20 μM genipin treatment inhibited the cell damage (Fig. 1D). In MTS assay, genipin restored the inhibition of the MTS reduction activity in a dose-dependent manner. Genipin demonstrated a significant neuroprotective effect at a dose of 10 μM (Fig. 1F). Protective effect of genipin on MTS reduction assay: Vertical bars in each histogram represent means±S.E.M. (n=8). *p<0.01, **p<0.05 compared to each conditions (E: control; F: TM (0.1 μg/ml); G: each TM). Scale bar=200 μm.

Genipin Suppresses the Induction of CHOP and GRP78 mRNA, But Not PERK mRNA

The mRNA levels of CHOP, GRP78, and PERK under TM-induced ER stress in Neuro2a cells were analyzed. After pretreatment with genipin or DMSO, Neuro2a cells were treated by TM and the expression levels of CHOP, GRP78, and PERK were measured. In the treatment of genipin alone, the mRNA levels of CHOP, GRP78, and PERK were not changed (data not shown). RT-PCR revealed that TM treatment resulted in increases in CHOP and GRP78 mRNA and the increases were significantly suppressed in the presence of genipin, although the expression of PERK mRNA was not changed by the TM treatment and/or genipin treatment (Fig. 2).

Discussion

The details of the mechanisms of various neurodegener-
ative disorders are still unclear. Therefore, we have been investigating whether genipin has a preventive effect on the cytotoxicity in models of various neurodegenerative diseases. We have recently described the neuroprotective activity of genipin in Aβ-induced cytotoxicity, as a model of AD, in rat primary cultured hippocampal neurons, and in 6-OHDA- and H2O2-induced cytotoxicity, as a model of PD, in Neuro2a cells.

In this study, genipin was found to exert a neuroprotective effect on TM-induced cytotoxicity in Neuro2a cells. TM is the most used pharmaceutical agent to experimentally induce ER stress by inhibiting N-linked glycosylation of protein. As a consequence, the accumulation of unfolding or misfolded proteins within the lumen of the ER induces ER stress. A recent study has shown that neurodegenerative disorders such as AD and PD are involved in the disruption of ER function. The present data revealed that TM significantly inhibited MTS reduction activity in Neuro2a cells, indicating TM has cytotoxicity (Fig. 1). We therefore decided to examine the possibility that genipin has a neuroprotective effect on cytotoxicity induced by TM. TM-induced cytotoxicity in Neuro2a cells was significantly suppressed by genipin pretreatment (Fig. 1). A significant neuroprotective effect of genipin was observed at genipin doses of 10 μM and above (Fig. 1). Similarly, genipin demonstrated a significant protective effect against TG-/BFA-induced cytotoxicity in Neuro2a cells (data not shown).

Next, we investigated the mRNA and protein expression of ER stress-related molecules in Neuro2a cells with TM (0.1 μg/ml) in the presence (20 μM) and absence (DMSO) of genipin. RT-PCR revealed that the mRNA levels of CHOP and GRP78 under TM-induced ER stress in Neuro2a cells were significantly increased, and were significantly suppressed in the presence of genipin. However, the induction of PERK mRNA was not affected (Fig. 2B). In Western blotting analysis, similarly, the protein levels of CHOP and GRP78 under TM-induced ER stress were significantly suppressed in the presence of genipin (Fig. 3B). These results suggest that genipin exerted not only neurotrophic activity but also neuroprotective activity. A recent study has reported that caspase-12 knock-out mice not only show resistance to ER stress but also death caused by Aβ protein, which is considered to play a central role in the occurrence of neuronal death in AD. This suggested the possibility that ER stress or caspase-12, or both, are involved in the process of neuronal death in AD. We showed that genipin significantly suppressed the protein level of caspase-12 in TM-induced ER stress (Fig. 3B). As has been previously reported, ER stress induces up-regulation of caspase-12 (full-length), and genipin might rescue the stress and might be useful for the prevention and/or treatment of neuronal damage observed in the brain of patients with neurodegenerative disorders. A caspase-12-homologous gene is known to be located on human chromosome 11q22.2, but the gene is interrupted by frame shift and a premature stop codon, and also has amino acid substitution in the critical site for caspase activity. Therefore, human caspase-12 appears to have been lost, and the caspases that substitute for caspase-12 to be activated specifically by ER stress have not been identified in humans thus far. A recent study reported that human caspase-4 located within the caspase-1/interleukin-1β converting enzyme genes cluster shows characteristics that are similar to mouse caspase-12. Furthermore, caspase-4 has been shown to function in ER stress-induced apoptosis in several cell lines, including SK-N-SH and HeLa cells, and caspase-4 seems to be responsible for cell death after Aβ treatment. Thus, Aβ-induced cell death might be mediated by caspase-4 as well as caspase-12.

The present findings strongly suggest that genipin attenuates cytotoxicity induced by TM in Neuro2a cells. Hence, we postulate that genipin most likely acts as a new neurotrophic factor-like compound with both neurotogenic and neuroprotective effects. Although the exact mechanisms of neurodegenerative disorders such as AD and PD are not yet known, it has been proposed that the main causes of these diseases are ER stress and oxidative stress. However, the present study was based on in vitro studies. Therefore, it is still unclear whether genipin can act as a neurotrophic drug on ER stress-induced neuronal diseases such as AD and PD. Further studies are now underway to clarify the effects in vivo studies and the mechanisms of these effects of genipin.

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REFERENCES