Genipin Suppresses A23187-Induced Cytotoxicity in Neuro2a Cells

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Genipin is an iridoid compound and an aglucon of geniposide isolated from *Gardenia fructus*. We have previously reported that genipin induces neurite outgrowth in PC12h and Neuro2a cells and protects against cytotoxicity induced by several conditions such as β-amyloid peptide, serum deprivation, and oxidative stress in rat primary hippocampal neurons and Neuro2a cells. In this paper, we examined the protective effect of genipin on A23187 (a calcium ionophore)-induced cytotoxicity in Neuro2a cells. A23187 induced cytotoxicity in concentration- and time-dependent manners as assayed by measurements of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetazolium bromide (MTT) reduction activity and lactate dehydrogenase (LDH) release. The cytotoxicity was significantly suppressed by genipin in a concentration-dependent manner. A23187 also significantly activated caspase3/7, which is known to be the critical mediator of apoptosis, after 1 h, and the cytotoxicity was clearly blocked by an inhibitor of caspase 3/7. Furthermore, A23187 induced the expression of immunoglobulin-binding protein/glucose-regulated protein of 78 kDa (BiP/GRP78) protein, which is an endoplasmic reticulum (ER) stress marker protein, and the expression was suppressed by genipin. These results suggest that genipin protects Neuro2a cells from A23187-induced cytotoxicity mediated by caspase 3/7 and ER stress. Therefore, genipin may be effective in preventing neurodegeneration observed in Alzheimer's disease and Parkinson's disease involving ER stress.

Key words A23187; genipin; neuroprotection; endoplasmic reticulum stress; Neuro2a cell

Genipin is an iridoid compound and the aglucon of geniposide isolated from an extract of *Gardenia fructus*. We have previously reported that genipin exerts neurotrophic activities in primary neurons and neuronal cell lines. Specifically, genipin induces neuronal differentiation in rat pheochromocytoma PC12h cells[1] and mouse neuroblastoma Neuro2a cells.[2] With respect to its mechanism of neurite outgrowth, we have suggested that genipin activates a nitric oxide (NO)-cyclic GMP (cGMP)-cGMP-dependent protein kinase (PKG) signaling pathway followed by the activation of extracellular signal-regulated kinase (ERK), a member of the mitogen-activated protein kinase (MAPK) family, in PC12h[3,4] and Neuro2a[5] cells. We have also reported that one of the target molecules of genipin *in vitro* is neuronal NO synthase (nNOS),[6—9] which suggests that nNOS plays a crucial role in neurotrophic activities of genipin. Moreover, we have also described the neuroprotective effects of genipin on cytotoxicity induced by β-amyloid peptide in rat primary hippocampal neurons,[10] and serum deprivation[11] and oxidative stress[12] in Neuro2a cells, and on trauma- and scopolamine-induced impairment of memory in mice.[13] Thus, genipin may be effective for treating neurodegeneration observed in Alzheimer's disease and Parkinson's disease.

These disorders are characterized by a loss of neurons in the brain and progressive increases in loss-of-function. It is known that the disruption of calcium homeostasis and the induction of oxidative stress and endoplasmic reticulum (ER) stress are involved in the onset of these disorders.[14—16] Therefore, we hypothesized that a reagent which protects neurons from such stresses or facilitates the neuronal differentiation of stem cells to supplement lost neurons may be a valuable clinical treatment for these disorders.

In this paper, we investigated whether genipin has a protective effect against the cytotoxicity induced by A23187 in Neuro2a cells. It is known that A23187, a calcium ionophore, induces ER stress by increasing the cytosolic calcium concentration.[14] Moreover, it is known that immunoglobulin-binding protein (BiP)/glucose-regulated protein of 78 kDa (GRP78) is one of the chaperones in the ER and that ER stress induces expression of BiP/GRP78 protein to prevent accumulation of abnormal proteins in ER.[17] First, we attempted to construct an experimental system for A23187-induced cytotoxicity in Neuro2a cells. Second, we tested the protective effect of genipin on the toxicity. Finally, we determined the involvement of ER stress in the toxicity and protective effect on the basis of BiP/GRP78 expression.

MATERIALS AND METHODS

Materials Genipin, transferrin, sodium pyruvate, and β-reduced nicotinamide adenine dinucleotide (NADH) were purchased from Wako Pure Chemical Industries, Ltd. (Japan). Insulin, progesterone, and alkaline phosphatase conjugated anti-mouse immunoglobulin (Ig)G antiserum were obtained from Sigma-Aldrich (U.S.A.). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Dojindo (Japan). Acetyl-L-aspartyl-L-glutamyl-L-valyl-L-aspart-1-aldehyde (Ac-DEVD-CHO) and acetyl-L-aspartyl-L-glutamyl-L-valyl-L-aspartic acid α-(4-methyl-coumaryl-7-amide) (Ac-DEVD-MCA) were obtained from Peptide Institute, Ind. (Japan). A23187 was obtained from Calbiochem (U.S.A.). Anti-BiP/GRP78 monoclonal antibody (clone 40) was purchased from BD Biosciences (U.S.A.). Genipin, A23187, Ac-DEVD-CHO, and Ac-DEVD-MCA were dissolved in dimethyl sulfoxide (DMSO). MTT was dissolved in phosphate-buffered saline (PBS) while sodium pyruvate was dissolved in distilled water.

Cell Culture Neuro2a cells were grown in Eagle's minimum essential medium (EMEM) containing 10% fetal bovine serum in 10% CO_2/90% humidified air at 37 °C.

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These cells were plated onto 48-well culture plates at a density of 2×10^3 cells/cm^2 for cytotoxicity assays or 100-mm culture dishes at subconfluent density for Western blot analysis. After 24 h of plating, the culture medium was replaced with serum-free EMEM supplemented with 5 μg/ml transferrin, 5 μg/ml insulin, and 20 nM progesterone to treat the cells with agents as specified in Results.

Cytotoxicity Assay Cytotoxicity was evaluated by measuring the activities of MTT reduction and lactate dehydrogenase (LDH) released from cytosol to culture medium as previously described.[12)]

Caspase 3 Activity Assay Caspase 3 activity was assayed as previously described.[12)] Briefly, the supernatant of the cell lysate for each treatment was incubated with Ac-DEVE-MCA (20 μM), a fluorogenic substrate for caspase 3. Next, cleavage of the substrate by caspase 3 was measured as the fluorescent intensity of 7-amino-4-methyl-coumarin (AMC) (excitation at 380 nm, emission at 460 nm). Data were normalized for the protein content of each supernatant and expressed as the relative value to the untreated group (time 0 h).

Western Blot Analysis Western blot analysis was carried out as previously described.[5)] Briefly, Neuro2a cell lysates were prepared and a portion (5 μg of protein) was separated on 10% sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The separated proteins were transferred to a polyvinylidene difluoride membrane and the membrane was hybridized with anti-BiP/GRP78 monoclonal antibody (1 : 5000 dilution). Alkaline phosphatase-conjugated anti-mouse IgG antiserum was used as the secondary antibody (1 : 10000 dilution). The immunoreactive signals were detected using a 5-bromo-4-chloro-3-indolyl-phosphate/nitroblue tetrazolium (BCIP/NBT) Phosphatase Substrate System detection kit (Kirdegaard and Perry Laboratories, U.S.A.). The blots shown are representative of three separate experiments.

Statistical Analysis The statistical significance of the differences between the mean values of the groups was analyzed using analysis of variance (ANOVA) and Scheffe's test as a *post hoc* test. When the p-value was <0.05, the difference was considered to be significant.

RESULTS

A23187-Induced Cytotoxicity As shown in Fig. 1A, A23187 (1—10 μM) significantly suppressed the MTT reduction activity of Neuro2a cells, in concentration- and time-dependent manners, measured after 2 and 4 h of treatment. In the LDH release assay, similarly, A23187 (1—10 μM) concentration- and time-dependently induced a significant increase in LDH activity (Fig. 1B). Cell damage by A23187 was observed at concentrations exceeding 5 μM after 2-h treatment and at concentrations above 1 μM after 4-h treatment in both assays.

Involvement of Caspase 3/7 in A23187-Induced Cytotoxicity We examined whether apoptosis is involved in A23187 (1 μM)-induced cytotoxicity. We measured the activity of caspase 3/7, the critical mediator of apoptosis, using Ac-DEVD-MCA as a fluorogenic substrate for caspase 3/7. A23187 significantly activated caspase 3/7 after 1-h treatment (Fig. 2A). In fact, an inhibitor of caspase 3/7, Ac-DEVD-CHO (5 and 10 μM), markedly depressed the A23187-induced decrease in MTT reduction activity (Fig. 2B) and increase in LDH release (Fig. 2C) after 2.5-h treatment.

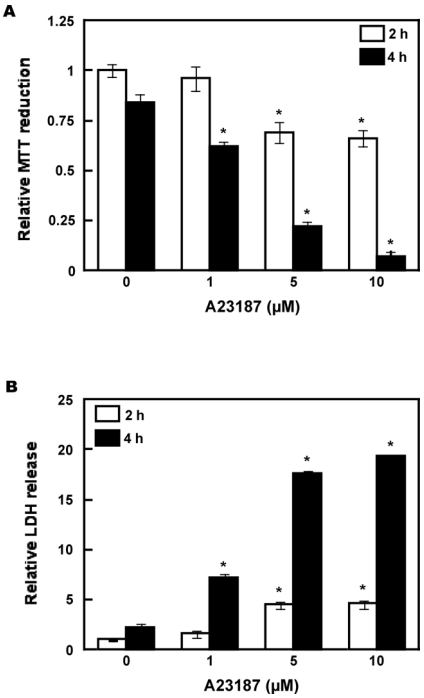

Fig. 1. Cytotoxic Effect of A23187 in Neuro2a Cells

Neuro2a cells were treated with A23187 (0—10 μM) for 2 (open column) and 4 h (closed column). Cytotoxicity was evaluated by MTT reduction activity (A) and released LDH activity (B). Values represent the mean±S.E.M. (n=4). $*p<0.01$ *vs.* 0 μM at 2 h.

Protective Effect of Genipin on A23187-Induced Cytotoxicity We next examined whether genipin is able to prevent the cytotoxicity induced by 1 μM A23187. Genipin (8 and 20 μM) significantly depressed the A23187-induced decrease in MTT reduction activity (Fig. 3A) and increase in LDH release (Fig. 3B) in concentration-dependent manners after 4-h treatment. Genipin at a concentration of 20 μM attenuated both the decrease and the increase by about 50%. As shown in Fig. 3C, the A23187-treated cells shrank considerably, but this was not the case in the presence of genipin.

Involvement of ER Stress in A23187-Induced Cytotoxicity BiP/GRP78 protein is an ER stress marker because its level of expression is increased in the presence of ER stress in order to protect the cells against ER dysfunction. Therefore, we tested whether expression of BiP/GRP78 protein was observed in A23187-treated cells. Treatment with 1 μM A23187 for 4 h induced BiP/GRP78 expression (Fig. 4, lane 3) compared to the control group (Fig. 4, lane 1). Moreover, the induction was partially blocked in the presence of 20 μM genipin (Fig. 4, lane 4). Treatment with genipin alone did not affect BiP/GRP78 expression (Fig. 4, lane 2).

DISCUSSION

We have previously demonstrated that genipin prevents cytotoxicity induced by several causes. For example, β-amyloid peptide-induced toxicity in rat primary hippocampal neurons[10)] and serum deprivation-, H_2O_2- and 6-hydroxydopa-

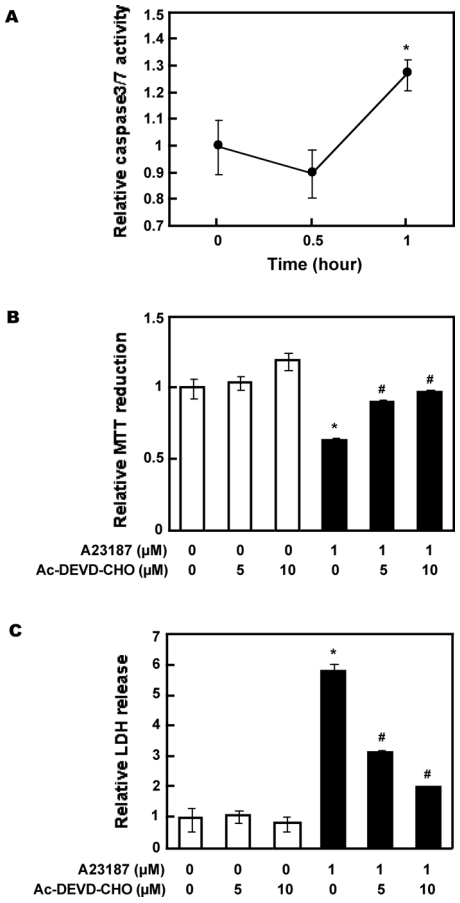

Fig. 2. Involvement of Caspase 3/7 in A23187-Induced Cytotoxicity in Neuro2a Cells

(A) Caspase 3/7 activation induced by A23187. Neuro2a cells were treated with 1 μM A23187 for 0—1 h. The cells were lysed and then incubated with 20 μM Ac-DEVD-MCA, a substrate for caspase 3/7. Cleavage of the substrate resulted in the emission of a fluorescent signal which represents caspase 3/7 activity. The activity was normalized with protein content in each sample and the activity in the 0 h treatment group. Values represent the mean±S.E.M. (n=6). $*p<0.05$ *vs.* 0 h. (B) and (C) Inhibition of A23187-induced cytotoxicity by a caspase 3/7 inhibitor. Neuro2a cells were treated with or without Ac-DEVD-CHO (5 or 10 μM) for 1 h before treatment with 1 μM A23187 for 2.5 h. Cytotoxicity was evaluated by MTT reduction activity (B) and released LDH activity (C). Values represent the mean±S.E.M. (n=4). $*p<0.01$ *vs.* A23187(0)/Ac-DEVD-CHO(0) and $\#p<0.01$ *vs.* A23187(1)/Ac-DEVD-CHO(0).

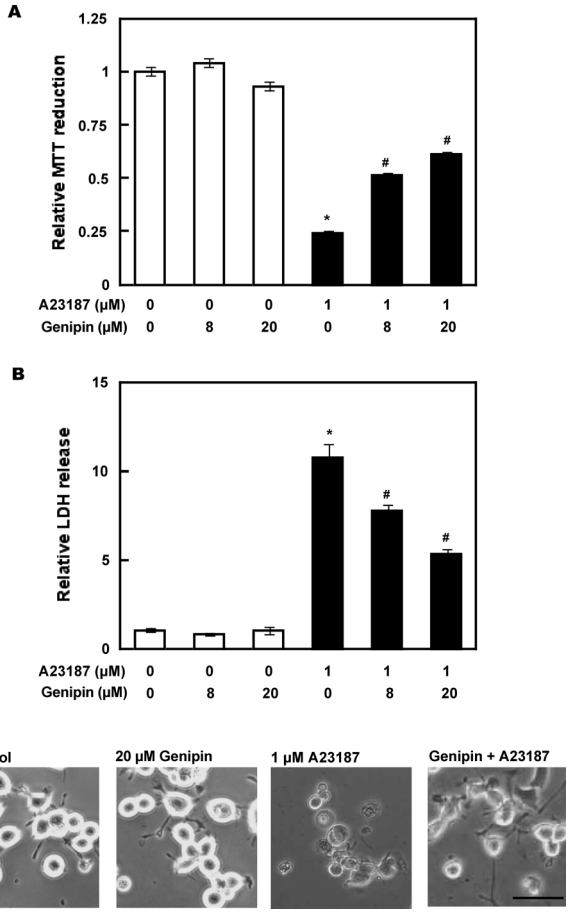

Fig. 3. Protective Effect of Genipin on A23187-Induced Cytotoxicity in Neuro2a Cells

(A, B) Evaluation by cytotoxicity assays. Neuro2a cells were treated with or without genipin (8 or 20 μM) for 1 h before treatment with 1 μM A23187 for 4 h. Cytotoxicity was evaluated by MTT reduction activity (A) and released LDH activity (B). Values represent the mean±S.E.M. (n=6). $*p<0.01$ *vs.* A23187(0)/genipin(0) and $\#p<0.01$ *vs.* A23187(1)/genipin(0). (C) Evaluation by morphological changes. Neuro2a cells were treated with or without 20 μM genipin for 1 h before treatment with 1 μM A23187 for 4 h, and then microphotographs were taken. Control represents cells treated without genipin and A23187. Scale bar=50 μm.

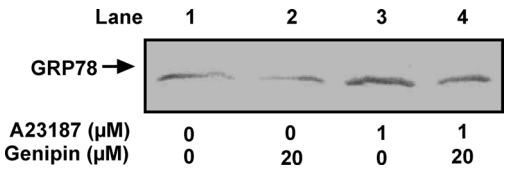

Fig. 4. Western Blot Analysis for Expression of BiP/GRP78 Protein in Neuro2a Cells

Neuro2a cells were treated with or without 20 μM genipin for 1 h before treatment with 1 μM A23187 for 4 h. Cell lysates corresponding to 5 μg of protein were subjected to 10% SDS-PAGE. The blot is representative of three independent experiments.

mine (6-OHDA)-induced toxicity in Neuro2a cells.[11,12] β-Amyloid peptide- and 6-OHDA-induced cytotoxicities are used as models of Alzheimer's disease and Parkinson's disease, respectively. Based on these findings, we believe that genipin may be a useful compound in the development of therapeutic agents for these diseases. To provide further support for this idea, we have investigated the effect of genipin on A23187-induced cytotoxicity in Neuro2a cells.

A23187 induced cytotoxicity in both MTT reduction and LDH assays. A23187 also activated caspase 3-like activity before a significant decrease in MTT reduction activity and increase in LDH release. This suggests that apoptosis is involved in A23187-induced cytotoxicity in Neuro2a cells. In fact, the cytotoxicity was considerably inhibited by an inhibitor of caspase 3/7. Genipin partially but significantly suppressed the cytotoxicity induced by A23187, suggesting that genipin does indeed exert an anti-apoptotic action. On the other hand, BiP/GRP78 is an indicator of ER stress response. This response occurs due to prevention of the accumulation of unfolded proteins in ER and induces up-regulation of ER chaperone expression such as BiP/GRP78 at the level of transcription.[18] A23187 clearly up-regulated the expression of BiP/GRP78 protein, suggesting that A23187 induces ER stress *via* disruption of calcium homeostasis in Neuro2a cells. Genipin, however, markedly suppressed the up-regulation, thus suggesting that genipin represses the induction of ER stress. That is, it seems that genipin exerts the protective effect at a site of upstream of caspase 3/7 activation. Unfortunately, the detailed mechanisms of the protection are still unclear.

We have previously reported that genipin induces neurite

outgrowth through the NO-cGMP-PKG signaling pathway in PC12h[3,4] and Neuro2a[5] cells and that genipin directly binds to nNOS followed by its activation *in vitro*.[6,9] Interestingly, Kitiphongspttana *et al.* reported that NO had a protective role during ER stress.[19] Therefore, it may be possible that genipin-produced NO contributes to the protection from A23187-induced cytotoxicity. In any case, we believe that genipin acts on a considerable distance upstream of the A23187-activated signaling pathway in order to prevent the development of ER stress, since genipin suppressed the expression of BiP/GRP78 in the presence of A23187. For example, genipin is suspected, based on the results of DNA microarray (data not shown), of inducing in advance several genes and/or proteins involved in the defense against oxidative or ER stress.

In summary, we have revealed here that genipin has a protective effect against A23187-induced cytotoxicity that is at least in part through the attenuation of ER stress loading in Neuro2a cells. Thus, the present findings suggest that genipin is effective at treating disorders involving ER stress, such as Alzheimer's disease and Parkinson's disease. A23187, however, induced disruption of calcium homeostasis, which causes many types of cellular stress, such as oxidative stress and ER stress. To clarify the mechanisms of the protective effect of genipin, further studies are needed using more specific model systems.

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