Neuro2a Cell Death Induced by 6-Hydroxydopamine is Attenuated by Genipin

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(Received June 25, 2008; Accepted September 10, 2008; Published online September 29, 2008)

We have previously reported that genipin, a natural iridoid compound, shows neuritogenic activity in cultured rat pheochromocytoma PC12h and mouse neuroblastoma Neuro2a cells. 6-Hydroxydopamine (6-OHDA) is a dopaminergic neurotoxin putatively involved in the pathogenesis of Parkinson's disease (PD). Here, we studied the protective effects of genipin on 6-OHDA-induced cytotoxicity in Neuro2a cells. 6-OHDA treatment markedly reduced Neuro2a cell viability in a concentration-dependent manner causing DNA condensation and fragmentation. Genipin significantly protected the cells against the 6-OHDA-induced cytotoxicity. Genipin also protected the cells against hydrogen peroxide (H_2O_2)-induced cytotoxicity. It is known that 6-OHDA is rapidly and non-enzymatically oxidized by molecular oxygen to form H_2O_2 and the corresponding *p*-quinone. These data suggest that genipin is effective at protecting against neurodegeneration that involves oxidative stress, such as PD.

Key words —— 6-hydroxydopamine, hydrogen peroxide, genipin, neuroprotection, Neuro2a cell

INTRODUCTION

Neurodegenerative diseases, such as Alzheimer's disease (AD) and Parkinson's disease (PD), are often associated with the loss of neurons as a result of deposition of their respective characteristic proteins.[1,2] It is known that oxidative stress is involved in the onset of these diseases.[3,4] To develop new medicines for these diseases, many researchers have been energetically carrying out studies from several angles. We have attempted to search for compounds with endogenous neurotrophic factor-like activity to induce neuronal differentiation, survival, and regeneration. Endogenous neurotrophic factors, such as nerve growth factor (NGF) and brain-derived neurotrophic factor, are polypeptides and as such are only very slightly permeable through the blood-brain barrier, indicating that their administration is limited as a medical treatment. Therefore, we have been screening natural lipophilic compounds in plants traditionally used as anti-amnestic or anti-inflammatory medications as effective substitutes for endogenous neurotrophic factors.

Genipin is an irioid compound and the aglucon of geniposide isolated from *Gardenia fructus*. We have previously reported that genipin markedly induces neurite outgrowth through a nitric oxide (NO)-guanosine 3′,5′-cyclic monophosphate (cGMP)-cGMP-dependent protein kinase (PKG) pathway followed by extracellular signal-regulated kinase (ERK) activation in rat pheochromocytoma PC12h[5–7] and mouse neuroblastoma Neuro2a cells.[8] This signaling pathway was observed in the neurite outgrowth process induced by NGF in PC12h cells. Moreover, we have suggested that a molecular target of genipin is neuronal NO synthase (nNOS) *in vitro*[9,10] and by computer simulation.[11] Genipin not only induces neuronal differentiation but also protects against cell death induced by β amyloid peptide,[12] a major deposit in the brain of AD patients, and serum deprivation[13] in cultured cells.

In the present study, we have examined the possibility that genipin has a neuroprotective effect on cytotoxicity induced by 6-hydroxydopamine (6-OHDA). 6-OHDA is a neurotoxin widely used to selectively destroy catecholaminergic systems[14,15] by oxidative stress induced by its auto-oxidation products, such as hydrogen peroxide (H_2O_2).[16] 6-OHDA has also been proposed as a putative neuro-

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toxic factor in the pathogenesis of PD.[17,18] Therefore, we used 6-OHDA to examine the neuroprotective effect of genipin on the cytotoxicity in a model of PD, another neurodegenerative disease.

MATERIALS AND METHODS

Materials —— Genipin, H_2O_2, Hoechst 33258, trypan blue, transferrin, and β-NADH were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Insulin, progesterone and 6-OHDA were obtained from Sigma-Aldrich (St. Louis, MO, U.S.A.). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Dojindo (Kumamoto, Japan). Acetyl-Asp-Glu-Val-Asp-α-(4-methyl-coumaryl-7-amide) (Ac-DEVD-MCA) was purchased from Peptide Institute (Osaka, Japan). Genipin and Ac-DEVD-MCA were dissolved in dimethyl sulfoxide (DMSO), and 6-OHDA was dissolved in distilled water. Hoechst 33258, trypan blue and MTT were dissolved in phosphate-buffered saline (PBS).

Cell Culture —— Neuro2a cells were cultured as described previously.[8] Briefly, the cells were grown in Eagle's minimum essential medium (EMEM) containing 10 % (v/v) fetal bovine serum in 10 % CO_2/90 % humidified air at 37°C. The cells were plated onto 48-well culture plates at a density of 1.5×10^4 cell/cm^2. After 24 hr of culture, 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 the results.

Cytotoxicity Assay —— Cytotoxicity was evaluated by measuring the activities of MTT reduction and released lactate dehydrogenase (LDH), and by staining with trypan blue. For MTT reduction activity, the cells were treated with 0.25 mg/ml MTT at 37°C for 45 min. The medium was then removed and the reduction product, MTT-formazan, was solubilized with DMSO. The absorption at 550 nm of each sample solution was measured as MTT reduction activity of the cells. For released LDH activity, the culture medium was subjected to the conventional rate assay. The supernatant after centrifugation (1000 rpm) of each culture medium was added to working solution (88 μg/ml β-NADH, 1 mM sodium pyruvate, 0.1 M phosphate buffer, pH 7.5), and then the production of β-NAD$^+$ was immediately measured at 340 nm for 1 min. The data for activities of MTT reduction and released LDH are expressed in terms of the relative activity in relation to each control group. For staining with trypan blue, the cells were exposed to 0.4 % (w/v) trypan blue at room temperature for 5 min and then were observed with a light microscope.

Hoechst 33258 Staining —— Neuro2a cells cultured onto 35-mm plastic dishes were fixed with 0.1 % (v/v) glutaraldehyde in PBS at room temperature for 30 min. After fixation, the cells were exposed to 0.2 mM Hoechst 33258 in PBS at room temperature for 5 min while protected from light. The cells were then observed by fluorescence microscopy.

Caspase-3 Activity Assay —— Neuro2a cells were rinsed with cold PBS and lysed in lysis buffer (50 mM Tris, 1 mM EDTA, 1 mM dithiothreitol, pH 7.5). Each lysate was sonicated and centrifuged at 1300 rpm for 5 min at 4°C. The supernatant was incubated with a specific fluorogenic substrate for caspase-3/7, Ac-DEVD-MCA (20 μM), in lysis buffer at 37°C for 1 hr while protected from light. The reaction was stopped by the addition of 2.5 mM monoiodoacetic acid. Cleavage of the substrate by caspase-3 was measured as the fluorescent intensity of 7-amino-4-methyl-coumann (AMC) using a fluorescence spectrophotometer (excitation at 380 nm, emission at 460 nm). Data were normalized for the protein content of each sample and expressed as the relative value to the untreated group.

Statistical Analysis —— Statistically significant differences between groups were estimated by analysis of variance (ANOVA) followed by Scheffe's test. A $p < 0.01$ was considered to indicate a significant difference.

RESULTS

6-OHDA-Induced Cytotoxicity

As shown in Fig. 1A, 6-OHDA (10 and 20 μM) induced a significant reduction in the MTT reduction activity of Neuro2a cells, in a concentration-dependent manner, measured after 6 and 24 hr of treatment. In the LDH release assay, 6-OHDA (10 and 20 μM) induced a significant increase in LDH activity after 24 hr-treatment but not after 6 hr-treatment in Neuro2a cells (Fig. 1B). Moreover, the cytotoxicity induced by treatment with 10 μM 6-OHDA for 24 hr was accompanied by DNA condensation and fragmentation as shown in Hoechst

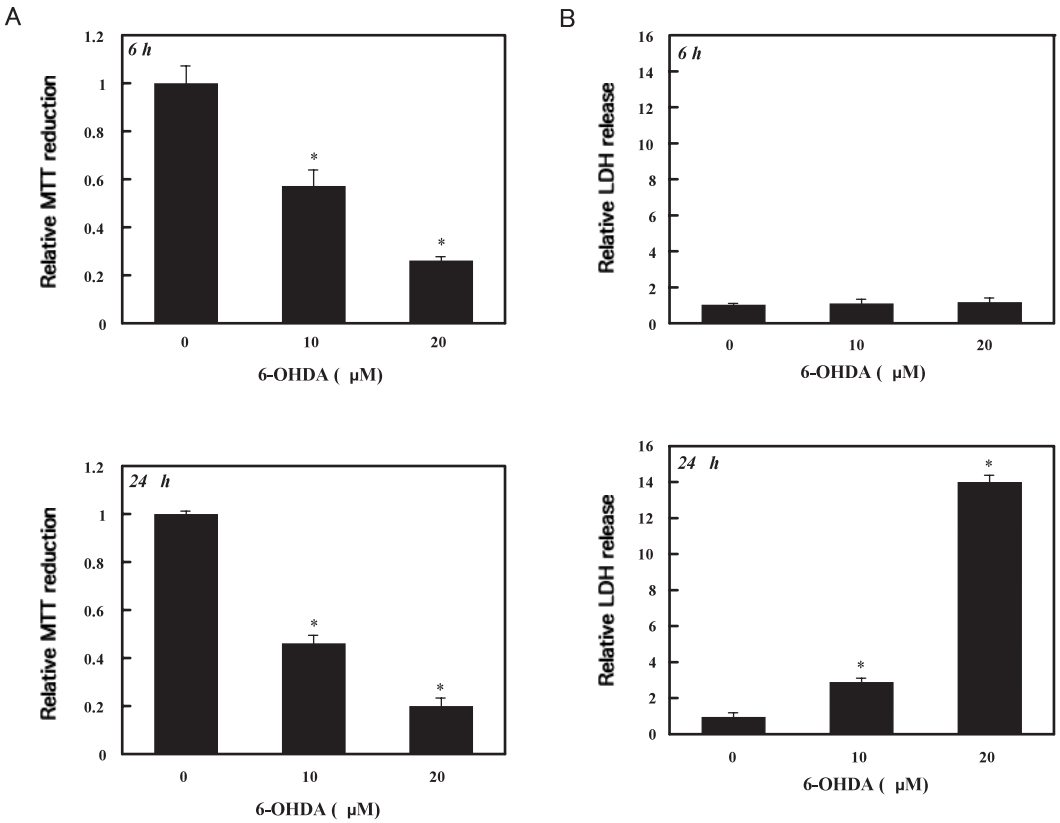

Fig. 1. The Cytotoxic Effect of 6-OHDA in Neuro2a Cells

Neuro2a cells were treated with 6-OHDA (0–20 μM) for 6 and 24 hr. Cytotoxicity was evaluated by MTT reduction (A) and LDH release (B). Values represent the mean ± S.E. (SEM, $n = 4$). $^{*}p < 0.01$ vs. 0 μM.

33258 staining (Fig. 2).

H_2O_2-Induced Cytotoxicity

It is known that 6-OHDA is rapidly and non-enzymatically oxidized by molecular oxygen to form H_2O_2 and the corresponding *p*-quinone.[19] Therefore, we tested whether H_2O_2 is involved in the cytotoxicity in Neuro2a cells. H_2O_2 induced cytotoxicity in both the MTT reduction (Fig. 3A) and LDH assays (Fig. 3B) in a concentration-dependent manner after 24 hr-treatment. Additionally, the cytotoxicity was followed by DNA condensation and fragmentation at a low concentration of 5 μM (Fig. 4A). Since DNA condensation and fragmentation are markers of apoptosis, we further analyzed caspase-3 activity, the mediator of apoptosis. Caspase-3 was significantly activated by 24 hr-treatment with H_2O_2 in a concentration-dependent manner (Fig. 4B). This significant activation was observed in cells treated with H_2O_2 at concentrations which did not induce significant LDH release (Fig. 3B).

Protective Effect of Genipin on 6-OHDA-Induced Cytotoxicity

Trypan blue exclusion assay was used to detect cytotoxicity. Genipin (2.5 μM), added to the cells 1 hr before treatment with 6-OHDA (20 μM), clearly reduced the frequency of trypan blue-positive cells (Fig. 5A). Although 6-OHDA induced cell shrinkage in almost all cells, genipin inhibited it and also induced neurite outgrowth (Fig. 5A). This protective effect of genipin was evaluated by LDH release assay. Genipin significantly reduced LDH release induced by 24 hr-treatment with 6-OHDA in a concentration-dependent manner (Fig. 5B).

Protective Effect of Genipin on H_2O_2-Induced Cytotoxicity

Genipin (5 and 10 μM), added to the cells simultaneously with H_2O_2 (10 μM), reduced LDH release induced by 24 hr-treatment with H_2O_2 (Fig. 6). Genipin at the concentration of 10 μM significantly reduced LDH release.

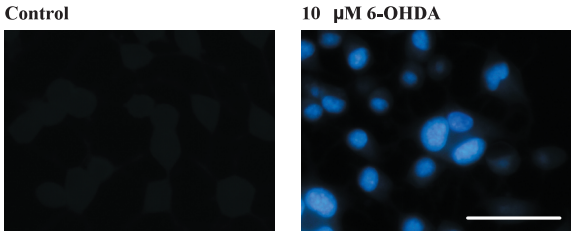

Fig. 2. 6-OHDA-Induced Condensation and Fragmentation of Nucleus

Neuro2a cells were treated with or without (Control) 6-OHDA (10 μM) for 24 hr. Cell nuclei were stained with Hoechst 33258 and observed under a fluorescence microscope. Scale bar = 50 μm.

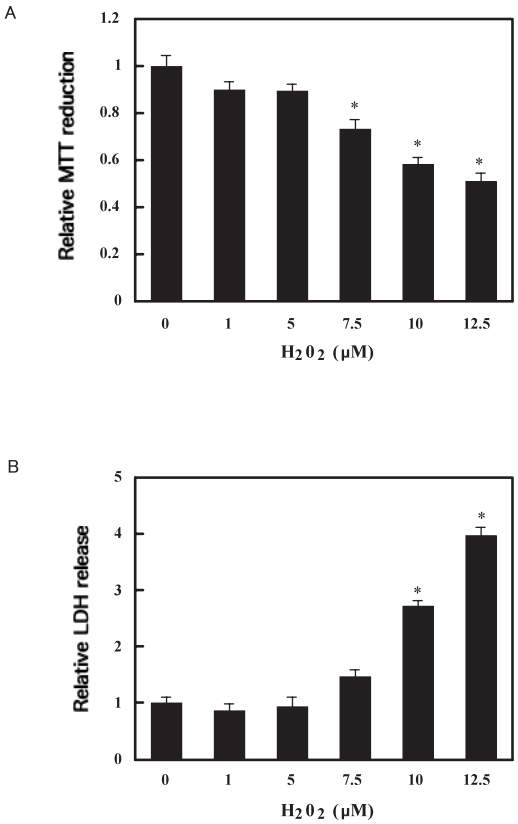

Fig. 3. The Cytotoxic Effect of H_2O_2 in Neuro2a Cells

Neuro2a cells were treated with H_2O_2 (0–12.5 μM) for 24 hr. Cytotoxicity was evaluated by MTT reduction (A) and LDH release (B). Values represent the mean ± S.E. ($n = 7$). $^*p < 0.01$ vs. 0 μM.

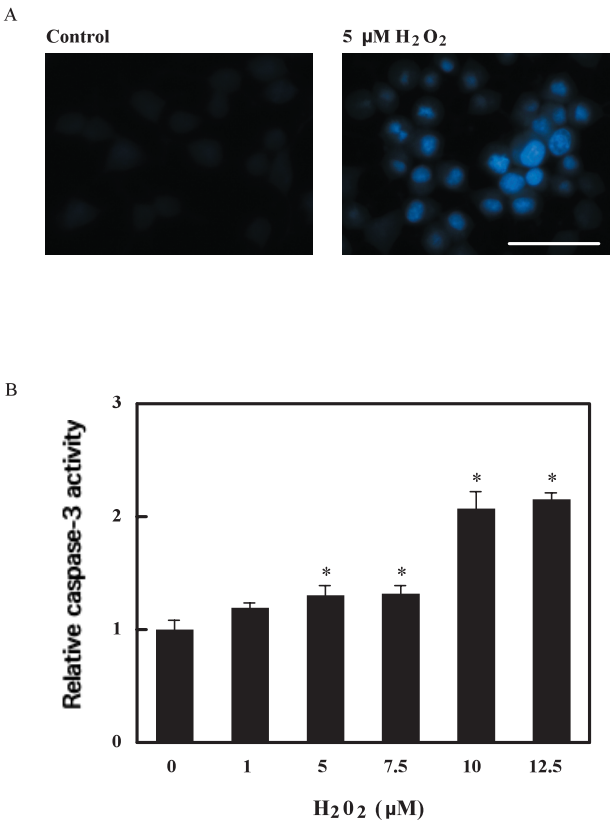

Fig. 4. Involvement of Apoptosis in H_2O_2-Induced Cytotoxicity in Neuro2a Cells

Neuro2a cells were treated with H_2O_2 (0–15 μM) for 24 hr. (A) Cell nuclei were stained with Hoechst 33258 and observed under a fluorescence microscope. Control indicates cells treated without H_2O_2. Scale bar = 50 μm. (B) Neuro2a cells were lysed and then incubated with Ac-DEVD-MCA (20 μM), a substrate for caspase-3/7. Cleavage of the substrate emitted a fluorescent signal which represents caspase-3 activity. The activity was normalized with protein content in each sample and the activity in the 0 μM H_2O_2-treated group. Values represent the mean ± S.E. ($n = 8$). $^*p < 0.01$ vs. 0 μM.

DISCUSSION

In the present study, genipin was found to exert a neuroprotective effect on 6-OHDA-induced cytotoxicity in Neuro2a cells. Moreover, genipin also attenuated the H_2O_2-induced cytotoxicity in these cells.

We have been investigating whether genipin is effective at attenuating cytotoxicity in models of various neurodegenerative diseases. Since we have previously reported that genipin is effective at protecting β amyloid-induced cytotoxicity, a model of AD, in rat primary cultured hippocampal neurons,[12] in this study we evaluated the protective effect of genipin on a model of PD using 6-OHDA. In Neuro2a cells, 6-OHDA induced a significant decrease in MTT reduction and increase in LDH release, indicating the cytotoxicity of 6-OHDA. This cytotoxicity was accompanied by DNA fragmentation, indicating that apoptosis was induced by 6-OHDA in Neuro2a cells. It has been previously reported that 6-OHDA is transported into the intracellular space through catecholamine transporters such as dopamine and L-dopa.[20] It has also been reported that Neuro2a cells possess a system for L-dopa uptake.[21] It has been suggested that the cy-

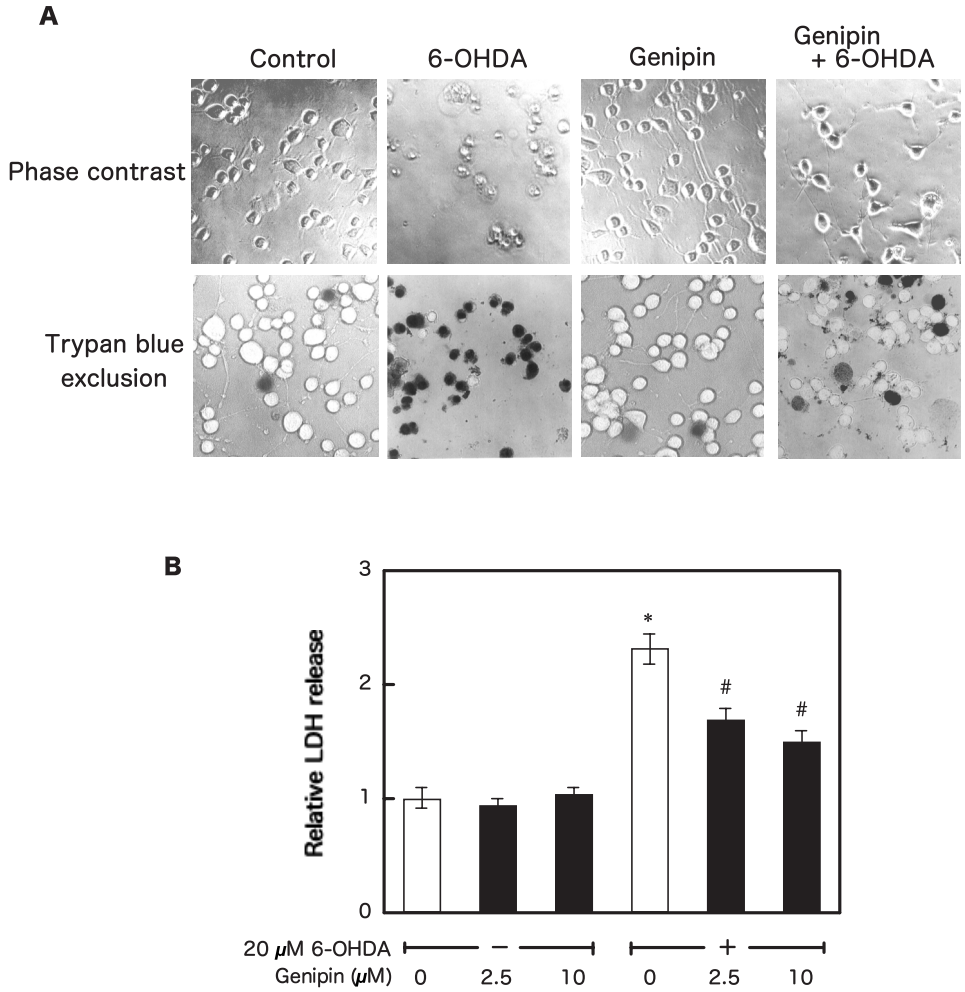

Fig. 5. Protective Effect of Genipin on Cytotoxicity Induced by 6-OHDA

(A) Neuro2a cells were treated with or without 2.5 μM genipin for 1 hr before treatment with or without 20 μM 6-OHDA for 24 hr. The upper panels are images under a phase-contrast microscope and the lower panels are images of trypan blue staining. Each image shows the treatment with vehicle alone (Control), 6-OHDA alone, genipin alone or genipin plus 6-OHDA as indicated. Scale bar = 50 μm. (B) Neuro2a cells were treated with or without genipin (2.5 or 10 μM) for 1 hr before treatment with (+) or without (−) 20 μM 6-OHDA for 24 hr. Cytotoxicity was evaluated by LDH release. Values represent the mean ± S.E. ($n = 6$). $^{*}p < 0.01$ vs. 6-OHDA (−)/Genipin (0) and $^{\#}p < 0.01$ vs. 6-OHDA (+)/Genipin (0).

totoxicity is mediated by the intracellular action of 6-OHDA, even though further investigation is necessary.

Next, we explored whether H_2O_2, a product of 6-OHDA auto-oxidation, might mediate the cytotoxicity in Neuro2a cells. H_2O_2 significantly induced a decrease in MTT reduction and an increase in LDH release with DNA condensation and fragmentation. Interestingly, H_2O_2 also activated caspase-3 at concentrations which did not induce significant LDH release, indicating that apoptosis is involved in the H_2O_2-induced cytotoxicity. In regard to 6-OHDA-treated cells, we failed to detect activation of the enzyme because of interference with the fluorescence of the substrate by 6-OHDA degradation products.

We believe that genipin exerted not only neuritogenic activity but also neuroprotective activity. Genipin significantly inhibited both 6-OHDA- and H_2O_2-induced cytotoxicity. These results suggest that genipin may be effective at preventing or protecting against neuronal damage observed in the brain of patients with PD. In addition, genipin will most likely demonstrate a broad spectrum of protective effects on apoptosis induced by oxidative stress and not limited to neurodegenerative diseases. In fact, it has been reported that genipin suppresses Fas-induced apoptosis in mouse primary liver cells.[22] However, the mechanism of the protective effect of genipin on 6-OHDA- and H_2O_2-induced cytotoxicity remains unclear. It has been reported that 6-OHDA-induced apoptosis is prevented

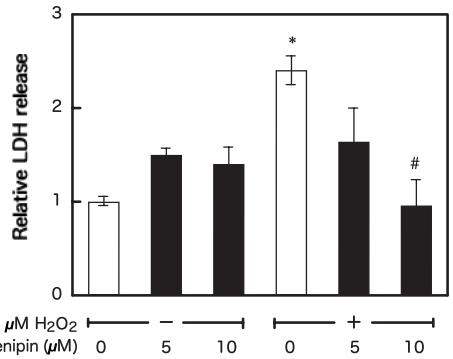

Fig. 6. Protective Effect of Genipin on Cytotoxicity Induced by H_2O_2

Neuro2a cells were treated with or without genipin (5–10 μM) and/or H_2O_2 (10 μM) for 24 hr. Cytotoxicity was evaluated by LDH release. Values represent the mean ± S.E. ($n = 6$). $^{*}p < 0.01$ vs. H_2O_2 (−)/Genipin (0) and $^{\#}p < 0.01$ vs. H_2O_2 (+)/Genipin (0).

by NO in PC12 cells.[23] Meanwhile, we have previously reported that genipin induces neuritogenesis through an NO-cGMP-PKG pathway in PC12h[5–7] and Neuro2a cells.[8] Therefore, genipin might exert the protective effect observed here *via* NO production, and this possibility should therefore be investigated. Catalposide, which like genipin is an iridoid-related compound, prevents H_2O_2-induced cytotoxicity *via* the expression of heme oxygenase-1 in Neuro2a cells.[24] This suggests that genipin may induce expression of some antioxidative stress factors for a protective effect. This possibility is of great interest and is under investigation.

In summary, the findings of the present study suggest that genipin attenuates cytotoxicity induced by 6-OHDA and H_2O_2 in Neuro2a cells. Therefore, genipin most likely acts as a new neurotrophic factor-like compound with both neuritogenic and neuroprotective effects. We anticipate that genipin has the potential to serve as a lead compound for the prevention and/or treatment of neurodegenerative diseases such as PD and AD. Further studies are now underway to clarify the mechanisms of these effects of genipin.

Acknowledgement This work is being supported by the "Academic Frontier" Project for Private Universities from the Ministry of Education, Culture, Sports, Science and Technology of Japan (2005–2009).

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