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Inhibitory Effects of Constituents of *Gastrodia elata Bl.* on Glutamate-Induced Apoptosis in IMR-32 Human Neuroblastoma Cells

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The inhibitory effects of the constituents of *Gastrodia elata Bl.* (GE) on glutamate-induced apoptosis in human neuronal cells were investigated using IMR32 human neuroblastoma cells. Glutamate (GLU) induced DNA fragmentation, a hallmark of apoptosis, in a dose-dependent manner. GLU also induced a slow and sustained increase in intracellular Ca^{2+} concentration. Treatment with EGTA, an extracellular Ca^{2+} chelator, in a nominal Ca^{2+}-free buffer solution abolished the GLU-induced intracellular Ca^{2+} increase, indicating that GLU stimulated Ca^{2+} influx pathway in the IMR32 cells. BAPTA, an intracellular Ca^{2+} chelator, significantly inhibited the GLU-induced apoptosis assessed by the flow cytometry measuring hypodiploid DNA content indicative of apoptosis, implying that intracellular Ca^{2+} rise may mediate the apoptotic action of GLU. Vanillin (VAN) and p-hydroxybenzaldehyde(p-HB), known constituents of GE, significantly inhibited both intracellular Ca^{2+} rise and apoptosis induced by GLU. These results suggest that the apoptosis-inhibitory actions of the constituents of GE may account, at least in part, for the basis of their antiepileptic activities. These results further suggest that intracellular Ca^{2+} signaling pathway may be a molecular target of the constituents of GE.

Key words : Human neuroblastoma cells, Vanillin, *p*-Hydroxybenzaldehyde, Epilepsy, Glutamate, Apoptosis, Intracellular Ca^{2+}

INTRODUCTION

Apoptosis is a highly organized cell death process characterized by ultrastructural modification (cytoskeletal disruption, cell shrinkage, and membrane blebbing), nuclear alteration (chromatin condensation and internucl eosomal DNA cleavage), and biochemical changes (activation of proteases) (Kidd, 1998). Induction of apoptosis appears to be a main pathological mechanism of many neurological diseases including epilepsy (Bengzon *et al.*, 1997). Excessive glutamate (GLU) release is regarded as a trigger for neuronal damage following epilepsy (Friedman, 1998). Moreover, exogenous administration of kainate, an agonist of one of GLU receptors, has been shown to induce both seizures and apoptosis in hippocampal neurons (Pollard *et al.*, 1994). *Gastrodia elata Bl.* (GE) is a traditional Chinese herbal drug, used for the treatment of convulsions and epilepsy (Liu and Mori, 1992; Huh *et al.*, 1998). Among the constituents of GE, vanillin (VAN) has been reported to suppress seizures induced by electrical stimulation to the basolateral amygdala (Wu *et al.*, 1989). Although VAN appears to act as an antioxidant in many cellular systems (Liu and Mori, 1993), the mechanism of antiepileptic action of GE is largely unknown, and remains to be determined. Thus, the purpose of this study was to elucidate the possible mechanism of antiepileptic action of GE. Specifically, we examined whether VAN and p-hydroxybenzaldehyde (p-HB) which are known as major constituents of GE (Liu and Mori, 1993; Yun-Choi *et al.*, 1998), inhibit GLU-induced apoptosis in IMR-32 human neuroblstoma cells. We also tested the involvement of intracellular Ca^{2+} signal in the mechanism of these actions, since intracellular Ca^{2+} is an important regulator of apoptosis in a variety of cell types (McConkey and Orrenius, 1997; Kim *et al.*, 1998),

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particularly in IMR-32 human neuroblastoma cells (Kim *et al.*, 1999).

MATERIALS AND METHODS

Materials

IMR-32 human neuroblastoma cell line was purchased from American Type Culture Collection (Rockville, MA). The powders, Eagle's minimum essential medium (MEM) and Earle's basal salt solution (EBSS), trypsin solution, sodium pyruvate, ethylene glycol-bis-(aminoethyl ether) N,N,N',N'-tetraacetic acid (EGTA), vanillin (VAN), *p*-hydroxybezaldehyde (p-HB), propidium iodide (PI), ribonuclease A, glutamate (GLU) and all salt powders were obtained from Sigma Chemical CO. (St. Louis, MO).1-(2,5-Carboxyoxazol-2-yl-6-aminobenzfuran-5-oxyl)-2-(2'- aminomethylphenoxy)-ethane-N,N,N'N' tetra-acetoxylmethyl ester (Fura-2) and bis-(o-amino-phenoxy)-ethane-N,N,N',N'-tetra-aceticacid acetoxy-methyl ester (BAPTA) were from Molecular Probes, Inc. (Eugene, OR). Fetal bovine serum (FBS) and antibiotics (penicillin and streptomycin mixture) were purchased from GIBCO (Grand Island, NY). VAN and p-HB were prepared as stock solutions in absolute ethanol, then diluted with aqueous medium to the final desired concentrations. The stock solution of drugs was sterilized by filtration through 0.2 m disc filters (Gelman Sciences: Ann Arbor, MI).

Cell culture

IMR-32 cells were grown at 37°C in a humidified incubator under 5% CO_2/ 95% air in a MEM supplemented with 10% FBS, 200 IU/ml penicillin, 200 μg/ml of streptomycin and 1 mM sodium pyruvate. Culture medium was replaced every other day. After attaining confluence the cells were sub-cultured following trypsinization.

DNA isolation and electrophoresis

IMR-32 cells were collected by centrifugation(200×g, 10min), washed twice in PBS (pH 7.4), and resuspended at a density of 4 x 10^6 cells/400 μl in hypotonic lysing buffer(5 mM Tris, 20 mM EDTA, pH 7.4) containing 0.5% Triton X-100 for 30 min at 4°C. The lysates were centrifuged at 13,000×g for 15 min at 4°C. Fragmented DNA was extracted from the supernatant with phenol-chloroform-isoamyl alcohol, precipitated by the addition of 2 volume of absolute ethanol and 0.1 volume of 3 M sodium acetate, and treated with RNAse A (500 U/ml) at 37°C for 3 hr. The pattern of DNA fragmentation was visualized by electrophoresis in 1.8% agarose gel, containing ethidium bromide and photographed under UV light. (Hockenbery *et al.*, 1990)

Flow cytometry assays

For flow cytometry analysis, IMR-32 cells were collected and washed twice with PBS buffer, pH 7.4. After fixing in 80% ethanol for 30 min, cells were washed twice, and resuspended in PBS buffer (pH 7.4) containing 0.1% Triton X-100, 5 mg/l PI and 50 mg/l ribonuclease A for DNA staining. Cells were then analyzed by a FACScan (BIO-RAD, Hercules, CA). At least 20,000 events were evaluated. All histograms were analyzed using WinBryte software (BIO-RAD, Hercules, CA) to determine percentage of nuclei with hypodiploid content indicative of apoptosis (Bombeli *et al.*, 1997).

Intracellular Ca^{2+} measurement

Aliquots of the IMR-32 cells, cultured for 3-5 days, were washed in EBSS. Then, 2 μM Fura-2 was added, and the cells were incubated for 60 min at room temperature (22-23°C). Unloaded Fura-2 was removed by centrifugation at 150 × g for 3 min. Cells were resuspended at a density of 2 ×10^6 cells/ml in Krebs-Ringer buffer (KRB) containing 125 mM NaCl, 5 mM KCl, 1.3mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 5 mM $NaHCO_3$, 25 mM HEPES and 6 mM glucose(pH 7.4). In the experiments for the identification of Ca^{2+} source, Ca^{2+}-free KRB containing EGTA (1mM) was used. Fura-2 loaded cells were transferred to a quartz cuvette and stirred continuously. Fluorescence emission (510 nm) was monitored with the excitation wavelength cycling between 340 and 380 nm at 37°C using a Hitachi F4500 fluorescence spectrophotometer. At the end of an experiment, fluorescence maximum and minimum values at each excitation wavelength were obtained by firstly permeabilizing the cells with 0.1% Triton X-100 (maximum) and then adding 10 mM EGTA (minimum). With the maximum and minimum values, the 340:380 nm fluorescence ratio was converted into free Ca^{2+} con-centration using Fura-2

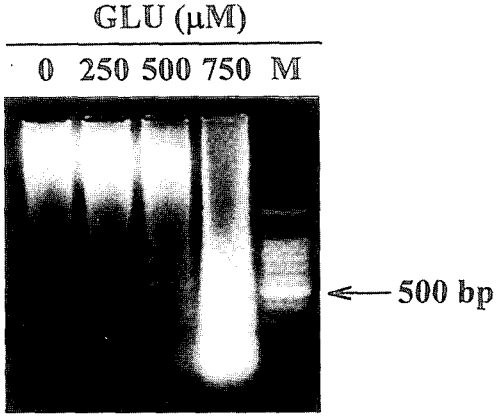

Fig. 1. GLU induces apoptosis in IMR-32 human neuroblastoma cells. Cells were treated for 48 hr with or without each concentration of GLU. DNA was isolated from the cells and analyzed by 1.8% agarose gel electrophoresis. Lane M represents DNA marker.

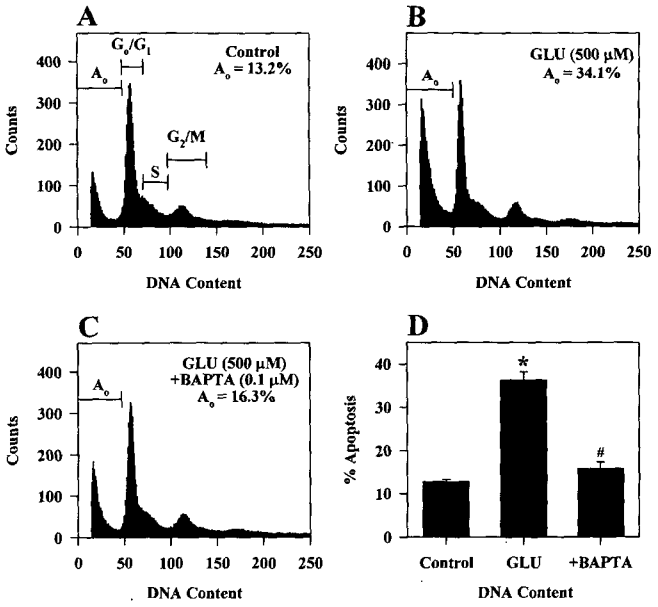

Fig. 2. Inhibitory effects of BAPTA, an intracellular Ca^{2+} chelator, on the GLU-induced apoptosis in IMR-32 human neuroblastoma cells. GLU (500 μM) was treated for 48 hr. BAPTA (0.1 μM) was added to the cells 2 hr before treatment with GLU. The number of apoptotic cells was measured by flow cytometry as described in text. The region to the left of the G_0/G_1 peak, designated A_0, was defined as cells undergoing apoptosis-associated DNA degradation (Bombeli *et al.*, 1997). Quantitative changes (D) were expressed as percent changes of the number of apoptotic cells compared to control condition in which the cells were grown in drug-free vehicle. *$p<0.05$ compared to control. #$p<0.05$ compared to GLU alone.

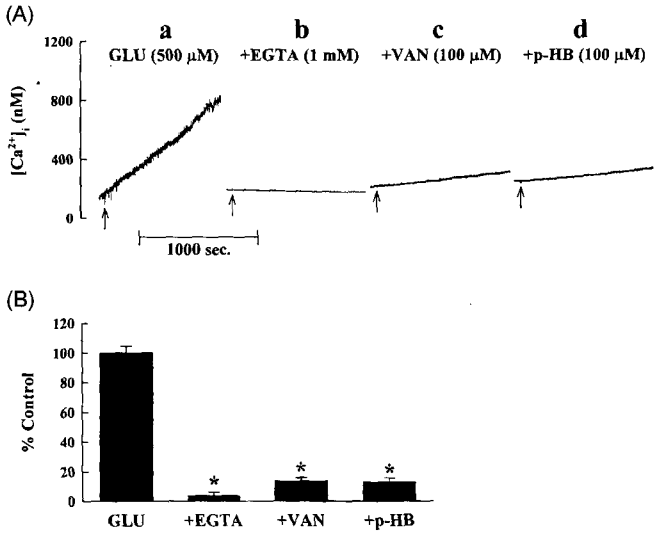

Fig. 3. Effects of the constituents of GE on GLU-induced Ca^{2+} influx in IMR-32 human neuroblastoma cells. Fura-2 loaded cells at a density of $2x10^6$ cells/ml were transferred to a quartz cuvet, and fluorescence emission was monitored at 37°C. Drugs were applied 10 min before fluorescence measurements. In the experiments for EGTA, a nominal Ca^{2+}-free medium was used. The data represent intracellular Ca^{2+} changes with time. Arrows show the time points for addition of 500 μM of GLU. Quantitative changes (B) were expressed as percent changes of increased intracellular Ca^{2+} concentration induced by the drug compared to GLU alone. Increased intracellular Ca^{2+} concentrations by GLU were calculated by difference at time 0 and 17 min after treatment with GLU. *$p<0.05$ compared to GLU alone.

Ca^{2+}-binding constant (224 nM) and the formula described by Grynkiewicz *et al.* (1985).

Data analysis

All experiments were performed four times. All data were displayed as % of control condition. Data were expressed as mean±standard error of the mean (SEM) and were analyzed using one way analysis of variance (ANOVA) and Student-Newman-Keul's test for individual comparisons. P values less than 0.05 are considered statistically significant.

RESULTS

Induction of apoptosis by GLU

The effect of GLU on apoptosis of the IMR-32 cells was examined by assessing DNA fragmentation, a hallmark of apoptosis (Wyllie *et al.*, 1984), using agarose gel electrophoresis. As represented in Fig. 1, GLU induced a dose-dependent DNA fragmentation in the IMR-32 cells. The apoptosis-inducing activity of GLU was observed at the concentration of 500μM. We used this concentration of GLU for the following intracellular Ca^{2+} and apoptosis experiments.

Intracellular Ca^{2+} mediates the GLU-induced apoptosis

Since intracellular Ca^{2+} signal has been implicated to mediate GLU-induced apoptosis in neuronal cells (Choi, 1990), we investigated the consistent role of intracellular Ca^{2+} signal in GLU-induced apoptosis in the IMR-32 cells. For this purpose we examined the effect of BAPTA, an intracellular Ca^{2+} chelator, on the GLU-induced apoptosis with flow cytometry by staining nuclei with PI and determining hypodiploid DNA content (Bombeli *et al.*, 1997). GLU (500 μM) induced 34.1% apoptosis designated as A_0 depicted in Fig. 2B. Pretreatment with BAPTA (0.1 μM) significantly suppressed the GLU-induced apoptosis as depicted in Fig. 2(C) and 2(D). To further define the role of intracellular Ca^{2+} signal in the observed GLU-induced apoptosis in IMR-32 cells, the direct effects of GLU on the intracellular Ca^{2+} concentration in the IMR32 cells were investigated. Intracellular Ca^{2+} levels were measured by the Fura-2 fluorescence method. As shown in Fig. 3 (A-a), GLU induced a slow and sustained increase in intracellular Ca^{2+} level at the concentration of 500 μM

at which GLU induced a significant DNA fragmentation. To determine the source of this increase, we measured intracellular Ca^{2+} concentrations using a nominal Ca^{2+}-free medium containing EGTA (1 mM), an extracellular Ca^{2+} chelator. This experimental protocol can effectively reduce extracellular free Ca^{2+} concentration, and thus, blunt available Ca^{2+} influx. Under these conditions cellular response to GLU was completely inhibited as illustrated in Fig. 3 (A-b) and 3(B). These results indicate that the GLU-induced intracellular Ca^{2+} rise was due to Ca^{2+} influx from the extracellular sites. To examine the role of Ca^{2+} influx in the GLU-induced apoptosis in the IMR-32 cells, we tested the effects of EGTA, and the results are shown in Fig. 4. Treatment with EGTA (1mM) significantly inhibited the GLU-induced apoptosis. These results suggest that Ca^{2+} influx may play a central role in the GLU-induced apoptosis.

Effects of constituents of GE on the GLU-induced intracellular Ca^{2+} rise and apoptosis

To examine the protective roles of constituents of GE in the insult of human neuronal cells, we first tested the effects of VAN and p-HB, known constituents of GE (Liu and Mori, 1993; Yun-Choi *et al.*, 1998), on the GLU-induced intracellular Ca^{2+} rise. VAN (100 μM) and p-HB (100 μM) significantly inhibited the GLU-induced intracellular Ca^{2+} rise as shown in Figs. 3 (A-c) and 3 (A-d), respectively. In addition, the GLU-induced apoptosis was also significantly inhibited by treatment with either VAN (100 μM) or p-HB (100 μM) as depicted in Fig. 4. These results indicate that the constituents of GE protected the IMR32 cells from the GLU-induced apoptosis through suppression of Ca^{2+} influx.

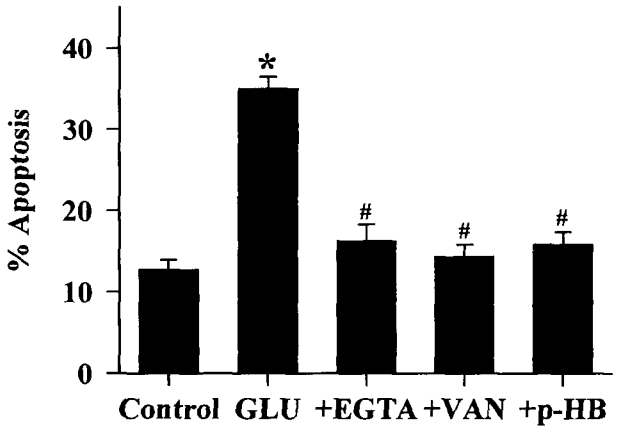

Fig. 4. Inhibitory effects of EGTA and the constituents of GE on the GLU-induced apoptosis in IMR-32 human neuroblastoma cells. Experimental methods are same as those in Fig. 2. Quantitative data are only presented. In these experiments EGTA (1 mM), VAN (100 μM) and p-HB (100 μM) were added to the cells 2 hr before treatment with GLU (500 μM). *$p<0.05$ compared to control. #$p<0.05$ compared to GLU alone.

DISCUSSION

The results of this study showed that treatment with GLU induced apoptotic cell death in the IMR-32 human neuroblastoma cells as assessed by two independent methods, DNA fragmentation assay (Fig. 1) and flow cytometry quantifying hypodiploid DNA contents (Fig. 2.) These results are consistent with those in the study using primary cultured neurons (Du *et al.*, 1997). Although the exact mechanism of GLU-induced apoptosis in neuronal cells is not completely known, intracellular Ca^{2+} increase (Leist and Nicotera, 1998) and oxygen free radical (OFR) formation (Tan *et al.*, 1998) appear to be essential mediators. In this study we showed that GLU increased intracellular Ca^{2+} concentration (Fig. 3) and that pretreatment with BAPTA, an intracellular Ca^{2+} chelator, almost completely inhibited the GLU-induced apoptosis (Fig. 2). These results indicate that intracellular Ca^{2+} signal may play an essential role in the induction of apoptosis by GLU in the IMR-32 human neuronal cell line. However, we can not exclude the contribution of OFRs to the observed GLU-induced apoptosis, since numerous studies have demonstrated that intracellular Ca^{2+} signal regulates the formation of OFRs in neurons (Carriedo et al., 1998), and vice versa (Facchinetti et al., 1998)

Apoptotic cell death has been implicated to act as a main pathological mechnism of various neurological diseases including epilepsy (Bengozon et al., 1997). Excessive amount of an excitotoxic neurotransmitter, GLU, is released during epileptic episode (Friedman, 1998), which appears to be associated with neuronal damage following epilepsy (Bittigau and IKonomidou, 1997). The induction of apoptosis by GLU in the IMR-32 cells found in this study suggest that this neuroblastoma cell line may be a suitable model celluar system for the studies on the mechanism of neuronal cell damage which occurs in many neuronal diseases, particularly in epilepsy(Meldrum, 1993).

GE is a traditional Chinese herbal drug which has been used for the treatment of neuronal diseases, particularly convulsions and epilepsy (Liu and Mori, 1992; Hsieh *et al.*, 1997). Previously, we have also reported that methanol extracts of GE have an inhibitory effect on convulsions induced by pentylenetetrazole (Huh *et al.*, 1998). However, the exact mechanism of the antiepileptic action of GE is largely unknown. The results of the present study showed that the constituents of GE, VAN and p-HB significantly inhibited the GLU-induced apoptosis in the IMR-32 cells (Fig. 4). Considering that the induction of apoptosis in neuronal cells is a major cause of various neuronal diseases including epilepsy described

earlier, these results suggest that the inhibitory effects of the constituents of GE on the GLU-induced apoptosis may be a major mechanism of antiepileptic activities of GE.

Since intracellular Ca^{2+} signal act as a mediator of apoptosis induced by GLU (Figs. 2 and 3), we evaluated whether this signaling system is a target of the constituents of GE. The results showed that pretreatment with VAN or p-HB significantly inhibited the GLU-induced intracellular Ca^{2+} increase Fig. 3 and apoptosis Fig. 4. These results strongly suggest that intracellular Ca^{2+} signal plays an essential role in the protective mechanism of GE against excitotoxic insults of neuronal cells. To our knowledge this is the first report which defines the role of intracellular Ca^{2+} signal in the mechanism of GE action. Although we did not identify (a) molecule(s) targeted by the constituents of GE, membrane GLU receptors and/or Ca^{2+} channels involved in the GLU-induced Ca^{2+} influx may be a candidate. This possibility may be supported by the facts that extracts of GE inhibit kainic acid binding to GLU receptors (Andersson *et al.*, 1995) and that VAN inhibits Ca^{2+} currents in Helix neurons (Erdelyi, 1992).

In addition to excitatory neurotransmitter GLU system, inhibitory neurotransmitter γ-aminobutyric acid (GABA) system appears to also be involved in pathogenesis of epilepsy (Troyer *et al.*, 1992; Lasley and Yan, 1994). Epilepsy is therapeutically managed by regulating the amount of GABA in brain (Petroff *et al.*, 1999), by activation of GABA receptor Cl^- channels (Sigel *et al.*, 1998), and by using inhibitors of GABA uptake (Morimoto *et al.*, 1997). These results raise the points that GABA system may be involved in unknown mechanism of the antiepileptic action of GE. This possibility is under study in our laboratory.

In conclusion, the constituents of GE inhibited the GLU-induced apoptotic cell death in a human neuronal cell line through regulation of intracellular Ca^{2+} signals. These results suggest that intracellular Ca^{2+} signaling pathways may be a central target of GE for its antiepileptic action.

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