Ginkgolides Protects the Cultured Rat Cortical Cells against Metabolic, Excitotoxic and Oxidative Insults

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INTRODUCTION

The neurotoxicity of L-glutamate (Glu) is associated with neurological disorders including hypoxic-ischaemic brain injury, Alzheimer’s disease, Huntington’s disease and Parkinson’s disease. Studies using cultured cortical neurons have demonstrated that exposure to Glu produced delayed degeneration of mature neurons.

Oxygen free radicals generated during aging, injury, and diseases have been postulated to be a major cause of death of neurons. H2O2 has recently been implicated as a cytotoxic agent in cortical neurons and other cells. Excessive release of the excitatory neurotransmitter Glu and the associated overactivation of Glu receptors are an important source of free radicals in neuron and, in turn, these free radicals may promote an increase in the extracellular concentration of Glu. Total ginkgolides (TG) is terpenoid constituent of Ginkgo biloba extract (EGb), which include ginkgolides A, B, C, M, J (Gin A, B, C, M, J) and bilobalide. We have previously obtained the results of TG’s beneficial effects on rat focal cerebral ischaemia model in vivo and also investigated the effects of TG on the enzymatic and nonenzymatic systems in the process of oxidative damage in the model of the reversible middle cerebral artery occlusion. Several studies have shown that the extract of Ginkgo biloba (EGb 761) could protect cultured neurons against damage induced by Glu, cyanide, staurosporine, serum deprivation, peroxynitrite, and hyreogen peroxide. Recently it has been reported that kaempferol, one of flavonoids in EGB, could protect rat cortical cultures against N-methyl-D-aspartate (NMDA)-induced neuronal toxicity in vitro. Zhu Li et al. reported that EGb and Gin B protected the neuronal viability against Glu-induced injury, and prevented the Glu-induced elevation in [Ca2+]. They, however, did not elucidate the function of TG. Comparing to the mono-ginkgolides, TG is easily prepared and effective Chinese traditional medicine. Until now, no report has been presented about the effects of TG on the models of the deprivation of oxygen and glucose in vitro and the oxidative stress damage on cortical neurons. In the present study, the effects of TG were assessed on cultured cortical neurons subjected to sugar-free and oxygen-free as well as Glu and...
H$_2$O$_2$ toxicity.

**MATERIALS AND METHODS**

**Cell Culture Agents and other Substances**

Eagle’s minimum essential medium (MEM) (containing 2 mM L-glutamine, 28 mM of glucose and 22 mM of sodium bicarbonate), Dulbecco’s modified MEM (DMEM) were obtained from Gibco (Grand Island, NY, U.S.A.); Fetal bovine serum was purchased from TBD Developing Centre of Biological Technology (Beijing, China); Poly-L-lysine, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphen-yl tetrazolium (MTT) was purchased from Sigma; Trypsin (bovine pancreas) was product from Sino-American Biotechnology Co. (Beijing, China) (Amresco); Horse serum was purchased from Zhengjiang Science and Technology Co. Ltd. (Tianjin, China); Cytosine arabinoside was obtained from Lianhua Pharmaceutical Co. Ltd. (Shanghai, China); L-Glutamic acid was product from Sino-American Biotechnology Co. (Serva 23000). Other reagents are analytical grade.

TG, purity > 95%, provided by professor Song You (Department of Biotechnology, Shenyang Pharmaceutical University). TG was initially dissolved in DMSO and then diluted with culture medium to the final concentration just before use. The DMSO concentration was limited to 0.1%.

**Primary Cortical Cell Cultures and the Evaluation of Cell Damage**

The study was carried out with permission from the ethical committee and in accordance with internationally accepted principles concerning the care and use of laboratory animals. Primary cultures were obtained from the cerebral cortex of fetal rats (17–19 days gestation). The procedures have been described previously.20,21 Briefly, cerebral cortices were isolated and digested in 0.25% trypsin. The resulting cell suspension was seeded into poly-L-lysine-coated 24-well tissue culture plates and cultivated in DMEM with 10% bovine serum and 10% horse serum in a humidified atmosphere of 95% air and 5% CO$_2$ at 37°C. After 2 days in culture, cytosine arabinoside (10 uM) was added to the medium for 24–36 hr to minimize glial cell proliferation. Only mature (10–12 days in vitro) cultures were used for experiments. Cell membrane damage was assessed by measuring the release of lactate dehydrogenase (LDH), a soluble cytoplasmic enzyme using the colorimetry.22 The supernatant medium of cell cultures was centrifuged at 2500 rpm for 10 min. The 20 ul supernatant was used to determine LDH activity at 340 nm by automatic biochemical analyzer. Changes in cell morphology were assessed by microscopic examination. Cell viability was quantified by the modified MTT reduction assay as described previously.23,24 The amount of crystals produced was determined by measuring its absorbances at 570 nm. By microscopic examination, the decrease in MTT reduction was confirmed to us parallel to cell death in all cases. Triplicate wells were used for each condition, and the averaged value was obtained. Each experiment was repeated in six separate cultures.

**Excitotoxic and Oxidative Insults**

Simultaneous application with Glu or H$_2$O$_2$: Cortical cell cultures were washed with DMEM and incubated simultaneously with TG (0.01, 0.1, 1, 10 and 100 ug/ml) and 1 mM Glu or 0.2 mM H$_2$O$_2$.16,25 for 12 hr, then the treatments were terminated by exchanging the culture medium with DMEM and the same supplements as described above. Neuronal viability was quantified 8 hr later by measuring dehydrogenase activity retained in living cells using the MTT assay. Neuronal integrity was assessed by spectrophotometric measurement of the efflux of LDH into the culture medium.

Pretreatment: Cortical cell cultures were washed with DMEM and pre-incubated with TG (0.01, 0.1, 1, 10 and 100 ug/ml) for 12 hr. The cultures were then exposed to 1 mM Glu or 0.2 mM H$_2$O$_2$ for 6 hr and treatments were terminated by exchanging the culture medium with DMEM and the same supplements. Neuronal viability was quantified and neuronal integrity was assessed 8 hr later as described above.

**Oxygen-Glucose Deprivation**

As shown previously,26,27 cultured neurons were damaged by exposure to oxygen-glucose deprivation. The oxygen-glucose deprivation treatment was performed on mature cultures. Cortical neurons were previously cultured in TG (0.01, 0.1, 1, 10 and 100 ug/ml) for 12 hr by dissolving TG in serum-free DMEM. The cultures were then exposed to MEM containing 20 nM KCN for 4 hr in order to achieve the oxygen-glucose deprivation and the deprivation was terminated by exchanging the culture medium with the same supplements as described above. Neuronal injury was assessed 8 hr later.

**Statistical Analysis**

Data were expressed as the mean ± S.D. The statistical significance of differences was determined by Dunnett’s multiple comparison test.
RESULTS

Glu and H₂O₂ Neurotoxicity

After 10 days in culture, neurons developed an extensive network, showing large and phase-bright cell bodies. When cultures were exposed to Glu and H₂O₂ for 12 hr, swelling and darkening of neurons were observed and debris occurred in some areas. Neurons in cultures dealt with H₂O₂ were injured worse than those in cultures exposed to Glu. Table 1 showed that the damage by H₂O₂ began at 0.1 mM whereas that by Glu began from 1.0 mM. This suggested that the damage by H₂O₂ was severer than that by Glu. The morphological change was confirmed by MTT assay. Glu (1 and 2 mM) and H₂O₂ (all the five concentrations) induced a marked decrease in A₅₇₀. Data are mean ± S.D. (n = 6). Asterisks indicate significant differences from the control group: *p < 0.05, **p < 0.01.

Table 1. Neuronal Viability (Confirmed by the Assay of MTT) Induced by Glu and H₂O₂ in Cortical Neurons of Rats

<table>
<thead>
<tr>
<th>Treatment (mM)</th>
<th>Absorbance at 570 nm</th>
<th>Treatment (mM)</th>
<th>Absorbance at 570 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.153 ± 0.008</td>
<td>Control</td>
<td>0.153 ± 0.008</td>
</tr>
<tr>
<td>Glu 0.1</td>
<td>0.138 ± 0.015</td>
<td>H₂O₂ 0.1</td>
<td>0.136 ± 0.011*</td>
</tr>
<tr>
<td>Glu 0.2</td>
<td>0.157 ± 0.017</td>
<td>H₂O₂ 0.2</td>
<td>0.128 ± 0.009**</td>
</tr>
<tr>
<td>Glu 0.5</td>
<td>0.151 ± 0.019</td>
<td>H₂O₂ 0.5</td>
<td>0.113 ± 0.007**</td>
</tr>
<tr>
<td>Glu 1.0</td>
<td>0.140 ± 0.007*</td>
<td>H₂O₂ 1.0</td>
<td>0.097 ± 0.006**</td>
</tr>
<tr>
<td>Glu 2.0</td>
<td>0.129 ± 0.011**</td>
<td>H₂O₂ 2.0</td>
<td>0.113 ± 0.005**</td>
</tr>
</tbody>
</table>

After the cultures were exposed to Glu (0.1–2.0 mM) and H₂O₂ (0.1–2.0 mM) for 12 hr, they were incubated with MTT for 4 hr, and then the amount of crystals produced was determined by measuring its absorbance at 570 nm. Glu (1 and 2 mM) and H₂O₂ (all the five concentrations) induced a marked decrease in A₅₇₀. Data are mean ± S.D. (n = 6). Asterisks indicate significant differences from the control group: *p < 0.05, **p < 0.01.

Table 2. Effects of Glu and H₂O₂ on LDH Release from Cultured Cortex Neurons

<table>
<thead>
<tr>
<th>Treatment (mM)</th>
<th>LDH (U/l)</th>
<th>Treatment (mM)</th>
<th>LDH (U/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>39.13 ± 1.96</td>
<td>Control</td>
<td>39.13 ± 1.96</td>
</tr>
<tr>
<td>Glu 0.1</td>
<td>38.47 ± 1.67</td>
<td>H₂O₂ 0.1</td>
<td>39.96 ± 3.24</td>
</tr>
<tr>
<td>Glu 0.2</td>
<td>38.98 ± 3.10</td>
<td>H₂O₂ 0.2</td>
<td>40.78 ± 5.89</td>
</tr>
<tr>
<td>Glu 0.5</td>
<td>39.83 ± 3.14</td>
<td>H₂O₂ 0.5</td>
<td>51.50 ± 1.93**</td>
</tr>
<tr>
<td>Glu 1.0</td>
<td>42.30 ± 2.91*</td>
<td>H₂O₂ 1.0</td>
<td>52.77 ± 3.81**</td>
</tr>
<tr>
<td>Glu 2.0</td>
<td>45.36 ± 0.77**</td>
<td>H₂O₂ 2.0</td>
<td>61.46 ± 5.60**</td>
</tr>
</tbody>
</table>

After the cultures were exposed to Glu (0.1–2.0 mM) and H₂O₂ (0.1–2.0 mM) respectively for 12 hr, the supernatant medium of cell cultures was gathered and then centrifuged at 2500 rpm for 10 min. The 20 ul supernatant was used to determine LDH activity at 340 nm by automatic biochemical analyzer according to the kit’s guide. With increasing of the concentrations of H₂O₂ and Glu, the activity of LDH in cultures was gradually increased. Data are mean ± S.D. (n = 6). Asterisks indicate significant differences from the control group: *p < 0.05, **p < 0.01.

MTT assay was more sensitive than the LDH assay. 0.1 mM H₂O₂ showed significant damage when MTT assay was used, but for the LDH assay, 0.5 mM H₂O₂ had measurable injury.

Protective Effects of TG Against Glu Neurotoxicity

When rat cortical neurons were simultaneously exposed to TG at 0.01, 0.1, 1, 10 and 100 ug/ml, respectively with Glu at 1 mM for 12 hr, Glu induced a marked decrease in A₅₇₀ and a significant increase of LDH in the medium, and TG could completely abolish the changes (Table 3).

Pretreatment of rat cortical cultures with TG for 12 hr also resulted in a dramatic reduction in Glu-induced neuronal damage. The protective effects of TG against Glu toxicity were concentration-dependent, that is, high concentration of TG demonstrated more protective effects than low concentrations of TG (Table 3).
Protective Effects of TG against H2O2 Toxicity

H2O2 at the concentration of 0.2 mM decreased the absorbance at 570 nm and increased the LDH release. The absorbance was found to be significantly increased in cultured neurons pretreated with 100 ug/ml TG 12 hr before the addition of H2O2 as well as in neurons treated with 10 and 100 ug/ml TG simultaneously with the H2O2 treatment compared with H2O2-treated group. TG is likely to be a potential agent in protecting the neurons suffered from oxidative stress induced by H2O2. The results were showed in Table 4.

Effects of TG on Cellular Viability and LDH Release of Cortical Neurons Induced by Ischaemia

After hypoxia and glucose-free (a model of ischaemia in vitro)26) for 4 hr, the absorbance at 570 nm of MTT was significantly decreased. The absorbance of the control group was 0.148 ± 0.009, but the absorbance value of the ischaemia group was decreased to 0.107 ± 0.001. As incubation with TG (concentrations from 0.01 ug/ml to 100 ug/ml) for 12 hr prior to ischaemia, the absorbance value of MTT gradually increased by a concentration-dependent manner. LDH release from the cultured medium was also examined and the results showed that ischaemia could increase the level of LDH from the neurons mediated via the leakage of cell membrane. When the pretreatment with TG (from 0.01–100 ug/ml) for 12 hr was followed by ischaemia for 4 hr, this leakage effect induced by ischaemia was significantly inhibited. TG at 10 ug/ml and 100 ug/ml abolished the ischaemia-induced leakage of LDH (Table 5).

DISCUSSION

Presently, the exact mechanism of ischaemia-induced neuronal cell death is not well understood. It is likely that during ischaemia, the lack of energy at the brain may cause to depolarize neurons resulting in significant increases in neurotransmitters such as Glu, aspartate, dopamine and serotonin.28,29) Glu, through an action on NMDA and α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) receptors, allows Ca2+ to enter the cell.30,31) Glu can...
also act on metabotropic receptors leading to the production of diacylglycerol and inositol tris-phosphate, which upon activation of enzymes leads to the release of Ca\textsuperscript{2+} from intracellular stores.\textsuperscript{32} The Ca\textsuperscript{2+} overload can lead to activation of proteases, nuclease, phospholipases, nitric oxide synthase and other degradative enzymes that lead to free radical production and cell death.\textsuperscript{28,29}

In this study, three models were used according to the theory described above, \textit{i.e.} the metabolic in-

### Table 4. Protective Effects of TG on H\textsubscript{2}O\textsubscript{2}-Induced Toxicity in Cultured Rat Cortical Neurons

<table>
<thead>
<tr>
<th>Treatment</th>
<th>MTT (Absorbance at 570 nm)</th>
<th>LDH (U/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (untreated)</td>
<td>0.129 ± 0.008**</td>
<td>35.12 ± 2.26*</td>
</tr>
<tr>
<td>TG (10 ug/ml)</td>
<td>0.132 ± 0.010**</td>
<td>36.01 ± 1.91*</td>
</tr>
<tr>
<td>H\textsubscript{2}O\textsubscript{2} (0.2 mM)\textsuperscript{a}</td>
<td>0.072 ± 0.003</td>
<td>38.90 ± 2.53</td>
</tr>
<tr>
<td>H\textsubscript{2}O\textsubscript{2} (0.2 mM)\textsuperscript{a} + TG (0.01 ug/ml)\textsuperscript{b}</td>
<td>0.070 ± 0.005</td>
<td>36.85 ± 1.70</td>
</tr>
<tr>
<td>H\textsubscript{2}O\textsubscript{2} (0.2 mM)\textsuperscript{a}</td>
<td>0.073 ± 0.005</td>
<td>37.67 ± 1.23</td>
</tr>
<tr>
<td>TG (1.0 ug/ml)\textsuperscript{b}</td>
<td>0.077 ± 0.006</td>
<td>36.06 ± 1.57*</td>
</tr>
<tr>
<td>TG (10 ug/ml)\textsuperscript{b}</td>
<td>0.078 ± 0.005*</td>
<td>35.78 ± 2.03*</td>
</tr>
<tr>
<td>TG (100 ug/ml)\textsuperscript{b}</td>
<td>0.080 ± 0.005**</td>
<td>34.68 ± 1.71**</td>
</tr>
</tbody>
</table>

### Table 5. Protective Effects of TG on Ischaemia-Induced Damage in Cultured Rat Cortical Neurons

<table>
<thead>
<tr>
<th>Treatment</th>
<th>MTT (Absorbance at 570 nm)</th>
<th>LDH (U/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (untreated)</td>
<td>0.143 ± 0.007**</td>
<td>35.87 ± 2.72**</td>
</tr>
<tr>
<td>TG (10 ug/ml)</td>
<td>0.146 ± 0.013**</td>
<td>35.92 ± 2.02**</td>
</tr>
<tr>
<td>H\textsubscript{2}O\textsubscript{2} (0.2 mM)\textsuperscript{a}</td>
<td>0.078 ± 0.003</td>
<td>47.19 ± 2.37</td>
</tr>
<tr>
<td>H\textsubscript{2}O\textsubscript{2} (0.2 mM)\textsuperscript{a}</td>
<td>0.076 ± 0.004</td>
<td>48.26 ± 2.15</td>
</tr>
<tr>
<td>TG (0.01 ug/ml)\textsuperscript{b}</td>
<td>0.078 ± 0.003</td>
<td>46.31 ± 2.45</td>
</tr>
<tr>
<td>TG (0.1 ug/ml)\textsuperscript{b}</td>
<td>0.079 ± 0.003</td>
<td>45.45 ± 1.64</td>
</tr>
<tr>
<td>TG (10 ug/ml)\textsuperscript{b}</td>
<td>0.080 ± 0.012</td>
<td>43.10 ± 2.72*</td>
</tr>
<tr>
<td>TG (100 ug/ml)\textsuperscript{b}</td>
<td>0.089 ± 0.006**</td>
<td>38.20 ± 1.54**</td>
</tr>
</tbody>
</table>

The damages were evaluated by the assay of MTT and LDH release from the cultured medium. TG was added simultaneously and 12 hr prior to addition of H\textsubscript{2}O\textsubscript{2}, and the cells were exposed to 0.2 mM H\textsubscript{2}O\textsubscript{2} for 6–12 hr. Data are mean ± S.D. (n = 6). Asterisks indicate significant differences from the group treated with H\textsubscript{2}O\textsubscript{2} alone: *p < 0.05, **p < 0.01. \textit{a) Cultures were treated with H\textsubscript{2}O\textsubscript{2} for 12 hr. \textit{b) Simultaneous application of H\textsubscript{2}O\textsubscript{2} (from 0.01 ug/ml to 100 ug/ml) with 0.2 mM H\textsubscript{2}O\textsubscript{2} for 12 hr. \textit{c) Cultures were treated with H\textsubscript{2}O\textsubscript{2} for 6 hr. \textit{d) Preincubation with TG (from 0.01 ug/ml to 100 ug/ml) for 12 hr followed by incubation with H\textsubscript{2}O\textsubscript{2} for 6 hr.}

The cultures were pretreated with TG (0.01–100 ug/ml) for 12 hr, and then were treated with hypoxia and glucose-free for 4 hr. The damages were evaluated by the assay of MTT and LDH release from the cultured medium. Data are mean ± S.D. (n = 6). Asterisks indicate significant differences from the ischaemic group: *p < 0.05, **p < 0.01. \textit{a) Cultures were treated with hypoxia and glucose-free for 4 hr. \textit{b) Preincubation with TG (from 0.01 ug/ml to 100 ug/ml) for 12 hr followed by incubation with hypoxia and glucose-free for 4 hr.}
sult induced by glucose deprivation and hypoxia, the excitotoxic insult induced by Glu and the oxidative stress damage induced by H$_2$O$_2$. Results showed that TG could protect neurons from all the three insults in a concentration-dependent manner.

In the metabolic insult model, protection of TG was observed at the concentration ranging from 1.0 to 100 ug/ml (as to LDH, 10 and 100 ug/ml) when incubation was performed for 12 hr prior to damage. The protection of neurons by TG appeared not to be involved in an increase in energy availability to the neurons since at the end it did not prevent the depletion of cellular ATP caused by glucose withdrawal although TG protected cultured cortical neurons against glucose deprivation-induced injury.

During glucose deprivation, mitochondrial metabolism would be a preferred route of energy production. It has been shown that superoxide and hydroperoxide were produced as by-products of mitochondrial respiration. Therefore, it is reasonable to hypothesize that glucose deprivation would create a metabolic state where superoxide and hydroperoxide concentrations increased. In addition to glucose deprivation, neurons are also deprived of oxygen in stroke. Earlier work indicated that activation of NMDA receptors and calcium accumulation played a role in hypoxic neuronal injury. Since our previous studies showed that TG could interfere the radical system, we have investigated whether glucose deprivation and hypoxia-induced cytotoxicity might be antagonized by TG. Results showed that TG protected neurons from the toxicity in a concentration-dependent manner. Pae et al. reported that antioxidant N-acetyl cysteine showed protection of the cell from glucose deprivation-induced cytotoxicity and proposed that oxidative stress might be involved in glucose deprivation-induced cytotoxicity.

Moreover, neuroprotection was observed at concentrations from 0.01 to 100 ug/ml when TG was added to the culture medium 12 hr before or simultaneously with the induction of damage by glutamic acid. As discussed above, the excitotoxic injury played a major role in the pathophysiology of cerebral ischaemia and neurodegenerative disorders. Cortical neurons possess Glu receptors and so that they are vulnerable to excitatory amino acid stimulation. However, the mechanism by which TG protected cultured cortical neurons against Glu-induced neurotoxicity has not been fully understood at present. Competitive or non-competitive blockers of the NMDA subclass of Glu receptor have been shown to protect cultured cortical neurons from Glu cytotoxicity, suggesting that the NMDA receptor was the predominant mediator of Glu-induced toxicity in these neurons. Moreover, Du et al. deduced that tumor necrosis factor (TNF)-$\alpha$ and NO might be involved in excitatory amino acid Glu insult. They demonstrated that Gin A and Gin B decreased TNF-$\alpha$ and NO production in a dose-dependent manner. Zhang et al. speculated that TG had effects on calcium and postulated that TG stabilized the calcium homeostasis of central neurons, thereby protected them against environmental insults. In addition, free radicals are generated in response to Glu receptor activation and appear to play a role in excitotoxic injury. Studies indicated that EGb 761 or its some ingredients enhanced free radical defense systems in cells. For example, pretreatment of cells with EGb delayed a time-dependent increase in the number of dead neurons during exposure to H$_2$O$_2$ and reduced hydroxyl radical induced cell apoptosis and DNA fragmentation. Bilobalide enhanced the percentage of viable neurons in primary cultures from chick embryo hemispheres when damaged with cyanide. Gin A and Gin B inhibited NO production in lipopolysaccharide stimulating microglia, in rat C$_6$ astrocytoma cells and in human BT 325 astrocytoma cells. For a complex product like TG, which contains several active chemical components, multiple effects at various regulation sites are plausible. Further studies are required to define the mechanism of action.

In Tables 2 and 4, 0.2 mM H$_2$O$_2$ yielded different results on LDH release. The reason for the disparity may be as following. The cell injury by 0.2 mM H$_2$O$_2$ was obvious by MTT but not by LDH. It suggested that the mitochondrial dysfunction after cell injury occurs first and then LDH released appeared after cell death. 0.2 mM H$_2$O$_2$, the low dose for cell injury, was chosen to study the protection of TG, for the fear that the protective effect of TG would not be obvious when a high dose of H$_2$O$_2$ was used.

In conclusion, we have found that TG protected rat cortical neurons from Glu-induced toxicity, from oxidative stress damage, and from the deprivation damage of oxygen and glucose. This observation suggests that TG may potentially be useful for the treatment of neurodegenerative changes due to cerebral ischaemia and others.

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REFERENCES


