Neuroprotection of Nicotiflorin in Permanent Focal Cerebral Ischemia and in Neuronal Cultures

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Nicotiflorin is a single component extracted from traditional Chinese medicine Flos Carthami. In this study, we investigated its neuroprotection in permanent focal cerebral ischemia model in rats, and in an in vitro model of ischemia. At doses of 2.5, 5, and 10 mg/kg, nicotiflorin administered immediately after the onset of ischemia markedly reduced brain infarct volume and neurological deficits. For primarily cultured neurons suffered 2 h hypoxia followed by 24 h reoxygenation, nicotiflorin significantly attenuated cell death and reduced LDH release. Morphological observation also directly confirmed its protective effect on neuron. These results provided strong pharmacological basis for its potential therapeutic role in cerebral ischemic illness.

Key words  nicotiflorin; neuroprotection; permanent focal brain ischemia; neuronal culture; hypoxia

Flavonoids, a group of polyphenolic compounds found ubiquitously in plants, have a variety of biological activities including anti-viral, anti-tumor, anti-inflammatory and vasodilatory actions.1) Because of these biological activities and their bioavailability in the brain in particular, they are supposed to have effects of neural protection.2) Nicotiflorin (kaempferol-3-β-rutinoside), a flavonoid glucoside originally derived from the flower petals of Flos Carthami, a medicinal plant traditionally used in prevention and treatment of cardiovascular diseases and thrombosis in Chinese medicine,3) is known to have potent analgesic, antihypertensive and antianaphylactic effects. In an analgesic model of writhing syndrome in mouse, nicotiflorin was to significantly reduce the number of writhings and stretchings induced by 1% acetic acid solution at a dose of 80 and 40 mg/kg.4) In normotensive pentothal anaesthetized rats, intravenous administration of nicotiflorin produced a dose dependent decrease in systolic, diastolic and mean arterial blood pressure. At a dose of 10 mg/kg the antihypertensive effect (41.60%±3.62) was observed with a duration of 2—3 min, during which the heart rate also decreased. These effects are not mediated through muscarinic, histaminergic and adeno ceptors.5) In antianaphylactic studies, it can significantly prevent fatal shock of hen egg-white lysozyme specific IgE-mediated anaphylaxis.6)

However, little information is available about the effects of nicotiflorin on the CNS. To determine whether it has neuroprotective effects in vivo, this study observed its effects on ischemic brain damage using a permanent model of focal ischemia in rats. Furthermore, in order to define the possible protective mechanism, we examined the effect of it on cell damage induced by an acute hypoxic insult in rat primarily cultured neurons.

MATERIALS AND METHODS

Preparation of Nicotiflorin Nicotiflorin was extracted from Flos Carthami, the purity of which was 99.5% by HPLC analysis and the structure of which was as follows:

Animals Animal experiments were approved by local committee review and were conducted according to policies on the use of animals of the Society for Neuroscience. Experiments were performed in male Sprague-Dawley (SD) rats weighing 250—350 g. Animals were housed under standard conditions with free access to rat chow and tap water before and after surgery. Rats were randomly divided into five groups, each consisting of ten animals.

Surgical Procedures Irreversible occlusion of the right middle cerebral artery was performed as described previously.7) Briefly, animals were anesthetized with chloral hydrate (350 mg/kg, i.p.) and, using an operating microscope, the right MCA was exposed by a subtemporal craniectomy. The artery and its lenticulostriate branches were then occluded by bipolar electrocoagulation. Afterwards, retracted soft tissues were replaced, wounds were sutured, anesthesia was discontinued, and the rats were put back into their cages. Body temperature was maintained at 37 °C by means of a rectal probe connected to a heating pad during surgery and until animals regained consciousness. Thereafter, rectal temperature was checked frequently (every 10—15 min) during the following 2 h and, if necessary, it was corrected to 37 °C using a heating pad which was placed under the cage. After this, animals were returned to their home cages and allowed free access to food and water.

Monitoring of Blood Gas Concentration and Cerebral Blood Flow In some of the experimental and control rats, a catheter was inserted into the femoral artery to measure arterial blood gases with a blood gas analyzer (Ciba-Corning 248, U.S.A.) before and after ischemia. Cerebral blood flow was monitored with a laser doppler perfusion monitor (Peri-flux 4001, Perimed, Sweden) in the cerebral cortex ipsilateral to the occluded MCA. Nicotiflorin treatment did not affect the cerebral blood flow, PaO2, PaCO2, and pH mean values in
D-glucose). The cortical tissues were dissociated to single cells by gentle 10—20 times trituration using a 1 ml pipet. The cell suspension was centrifuged at 310×g for 3 min, and the resulting pellets were resuspended in the whole culture medium and were seeded on 24-well tissue culture dishes (Costar) coated with 100 μg/ml poly-l-lysine (Sigma) at a density of 6×10^5 cells/ml in 300 μl/well. Cultures were incubated in 5% CO₂ and 95% air at 37°C and 100% humidity (Heraeus) for 24 h. Then the culture medium was replaced with serum-free Neurobasal Medium (Gibco), supplemented with 2% (vol/vol) B-27 (Gibco), the cultures were then fed every 4—5 d by half-changing the existing medium with fresh Neurobasal Medium and B-27. In order to decrease the number of non-neuronal cells, the antimitotic cytosine arabinoside (ara C, Sigma) was used at 10 μM on the third day after plating for 24 h. After one-week incubation, the neurons had matured and the dendritic network had been established. Cultures were maintained for 8—10 d prior to experimentation.10,11

**In Vitro Model of Ischemia** To model neuronal ischemia in vitro, cultures were exposed to combined hypoxia and glucose deprivation. The treatment was performed on 10-d-old cultures. Prior to exposure to hypoxia, the culture medium was replaced with a balanced salt solution (112 mM NaCl; 1.0 mM NaH₂PO₄; 5.2 mM KCl; 0.7 mM MgSO₄; 1.7 mM CaCl₂; 0.03 mM Phenol Red; pH 7.2) or balanced salt solution containing nicotiflorin (25, 50, 100 μg/ml) and continued cultured for 1 h. Then, the cultures were placed in an anaerobic system (Thermo Forma) containing 95% (v/v) N₂ and 5% (v/v) CO₂ and exposed to anoxia for 2 h at 37°C. The relative humidity was maintained as close to 100% by filling the bottom of the chamber with deionized sterile water. Cultures were then reincubated at 5% CO₂ and 95% air at 37°C and 100% humidity for 24 h. The normal control plates were kept in normoxic conditions for a corresponding time.

**Morphological Observations** Cell morphology was routinely assessed by means of a phase-contrast light microscopy (Olympus).

**Cell Viability Assay** Quantification of cell viability was made using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, this dye was added to each well (final concentration of 500 μg/ml, Sigma) and cells were incubated with MTT for 1 h at 37°C. When the assay was terminated, the dye was solubilized by acidified isopropanol (0.1 N HCl in isopropanol) and the absorbance intensity (540 nm) of each sample measured in a plate reader.12

**LDH Assay** Cell damage was also quantified by the measurement of lactate dehydrogenase (LDH) released from damaged cells into the extracellular fluid. LDH was measured after 24 h reoxygenation. Remaining LDH in the cells was lysed with 0.1 M phosphate buffer (0.1 M Na₂HPO₄ and 0.1 M KH₂PO₄, pH 7.4) containing 0.5% Triton X-100 and was measured. LDH release as an indicator of neuronal injury was represented by the percentage leakage of LDH into the culture medium, with respect to the total LDH activity measured.13

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**Pharmacological Treatment** Nicotiflorin was dispersed in an aqueous solution of Tween 80 (5% w/v) and administered intravenously via the tail vein at dosage of 2.5, 5 and 10 mg/kg immediately after occlusion. Rats in the control group received vehicle in the same volume and with the same time schedule as nicotiflorin-treated animals. Nimodipine (2 mg/kg) was selected as a positive control drug. The administering method was same as nicotoflorin.

**Quantification of Infarct Size** At 24 h after MCA occlusion, rats were sacrificed and their brains were removed. The cerebral cortex was coronally sectioned from +4 to −6 mm from the bregma (2 mm in thickness) using a microslicer, and five consecutive slices were stained with 2% 2,3,5-triphenyltetrazolium chloride at 37°C for 30 min. Photographs of the coronal sections were used to calculate the area of ischemic brain damage and the whole area of the cerebrum by using a computerized image analysis system (smartscape image software, China). The ischemic brain damage in each animal was expressed as a percentage of the sum of the damaged area compared with the sum of the whole area of the cerebrum.8)

**Neurological Deficits** A neurological examination was performed on each rat 24 h after the onset of ischemia using a modification of the procedure described by Suzuki et al. Briefly, neurological scores (NS) were derived using a 10-point sliding scale. Each animal was examined for reduced resistance to lateral push (score, 4), open field circling (score, 3), and shoulder adduction (score, 2) or contralateral forelimb flexion (score, 1) when held by the tail and suspended approximately 0.5 m above the floor. Rats extending both forelimbs toward the floor and showing any other signs of neurological impairment were scored 0. Using this procedure, maximal neurological severity was measured as NS of 10.

The determination of infarct volume and neurological scores were performed by one of the authors unaware of the treatment history of the rats.

**Neuronal Cell Culture** Cortical neurons were cultivated from fetuses aged within 24 h and cultured in neuron-defined medium. In brief, fetuses were decapitated and the cortex was dissected and collected under sterile conditions and kept in ice-cold Hank’s balanced salt solution (HBSS, 10 mM HEPES, pH 7.3), freed of meninges, olfactory bulbs, basal ganglia, and hippocampus, then incubated at 37°C for 15—30 min in Ca²⁺/Mg²⁺-free HBSS containing 0.25% trypsin (Gibco) and 0.2 mg/ml deoxyribonuclease (Sigma). The reaction was terminated by adding whole culture medium (DMEM (Gibco) supplemented with heat-inactivated 10% fetal calf serum and 10% horse serum (Gibco), with 33.3 mM d-glucose). The cortical tissues were dissociated to single animals.

**Table 1. Physiological Blood Parameters Following Nicotiflorin Treatment 15 min after the Onset of Ischemia**

<table>
<thead>
<tr>
<th></th>
<th>pH</th>
<th>PaCO₂</th>
<th>PaO₂</th>
<th>CBF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>7.39±0.01</td>
<td>42.13±0.48</td>
<td>114.67±5.62</td>
<td>31.50±3.11</td>
</tr>
<tr>
<td>Nicotiflorin</td>
<td>7.41±0.02</td>
<td>44.76±0.80</td>
<td>118.37±3.38</td>
<td>33.50±6.41</td>
</tr>
</tbody>
</table>

Values are mean±S.E. (n=3 for pH, PaO₂, and PaCO₂; n=4 for CBF). PaO₂ and PaCO₂ are expressed in mmHg. CBF values, evaluated 30 min after pMCAO, are expressed as percentage of baseline value. ANOVA followed by Newman–Keuls test revealed no significant intergroup difference for any variable.
cellular activities was considered to be the total LDH activity for each sample. These values were then converted to a percentage of the total LDH activity of the normoxia group (which was considered as 100%).

**Statistical Analysis** All values are expressed as mean±S.D. Significance of difference between groups for was tested using one-way analysis of variance (ANOVA) followed by post hoc Dunnett’s multiple comparison test. p values less than 0.05 were considered to be significant.

**RESULTS**

**Effect of Nicotiflorin on Ischemic Damage after Permanent Focal Cerebral Ischemia** Permanent occlusion of the right MCA resulted in ischemic brain damage within the MCA territory, i.e., the dorsolateral cortex and striatum. Nicotiflorin at all three doses of 2.5, 5 and 10 mg/kg administered intravenously immediately after MCA occlusion reduced the size of ischemic brain damage in a dose-dependent manner (Figs. 1, 2). p<0.01 (vehicle vs. nimodipine and nicotiflorin 5, 10 mg/kg). p<0.05 (vehicle vs. nicotiflorin 2.5 mg/kg).

**Effect of Nicotiflorin on Neurological Deficits after Permanent Focal Cerebral Ischemia** Twenty-four hours after the pMCAO, behavioral deficits were assessed by neurological scoring of the degree and duration of forelimb flexion and symmetry of movement and forepaw outstretching, as described in Materials and Methods. The mean score in vehicle-treated group was measured to be 7.1±0.7, whereas the scores in the nicotiflorin-treated groups were significantly decreased at all three doses (Fig. 3). p<0.01 (vehicle vs. nimodipine and nicotiflorin 2.5, 5 and 10 mg/kg).

**Effect of Nicotiflorin on the Morphology of Cultured Neurons Suffered Hypoxia** Figure 4 shows typical photographs of normal rat cultured cerebral cortical neurons (A) and those suffered hypoxia (B—E). Visual inspection using a phase-contrast microscopy indicated that the normal cells were viable (Fig. 4A). Many cells (though not all) suffered hypoxia showed obvious cell body damage, swelling, and cellular debris (Fig. 4B). In contrast, the cells treated with nicotiflorin (25—100 µg/ml) added into medium just before hypoxia were much better preserved and did not show damage like that of mere hypoxia (Figs. 4C—E). The protective effect of nicotiflorin on cultured neurons was also dose dependently.

**Neuroprotection by Nicotiflorin against Hypoxia-Induced Cell Death** Exposure of cortical cultures to hypoxia and glucose deprivation reduced neuronal viability, with maximal toxicity by 24 h. Treatment with nicotiflorin (25—100 µg/ml) added into medium just before hypoxia significantly attenuated neuronal death (Fig. 5). p<0.01 (hypoxia vs. normal and nicotiflorin 25, 50 and 100 µg/ml).

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**Fig. 1.** Histological Evidence for Nicotiflorin-Mediated Reduction in Infarct Size after Permanent Focal MCAO

The right MCA was occluded by electrocoagulation as described in Materials and Methods. Animals were treated with vehicle or 2.5, 5, 10 mg/kg nicotiflorin immediately after occlusion. Animals were killed 24 h after the onset of occlusion, and brain sections were treated with TTC (red). The brains shown are representative of 10 animals per condition.

**Fig. 2.** Neuroprotective Effects of Nicotiflorin on Brain Damage Induced by Electrocoagulation for Permanent Focal Ischemia in Rats

Nicotiflorin was administered intravenously at a total dose of 2.5, 5 or 10 mg/kg, immediately after occlusion. *p<0.05 and **p<0.01. n=10.

**Fig. 3.** Effects of Nicotiflorin on Neurological Deficits 24 h after the Onset of Ischemia

Rats were administered 2.5, 5, and 10 mg/kg nicotiflorin or vehicle immediately after occlusion. **p<0.01, compared with the vehicle-treated group. n=10.
Effect of Hypoxia on LDH Release

Cell death was also determined by measuring LDH release from the damaged cells, which is a marker of cell necrosis. As shown in Fig. 6, hypoxia induced LDH release increase significantly. Nicotiflorin (25—100 \( \mu \)g/ml) decreased the LDH release in a concentration-dependent manner. \( p < 0.01 \) (hypoxia vs. normal and nicotiflorin 25, 50 and 100 \( \mu \)g/ml).

DISCUSSION

*Flos Carthami*, dried flower petals of safflower (*Carthamus tinctorius*), is an important crude drug in traditional Chinese medicine for promoting blood circulation and removing obstruction in the channels. It has long been used in the prevention and treatment of cardiovascular diseases and thrombosis in China.\(^3\) Clinical experience from many hospitals in China has proved that *Flos Carthami* also has good efficacy in prevention and treatment of cerebrovascular diseases including cerebral thrombus, cerebral embolism, cerebral ischemia and lacuna embolism.\(^14^{--}16\) Our previous study and many other studies show that flavonoids are the main chemical constituents of *Flos Carthami*,\(^17^{--}20\) among which the content of nicotiflorin is the highest (>3%) in some varieties of *Flos Carthami*.\(^21\) We therefore presume that it is nicotiflorin that plays a key role in the effects of *Flos Carthami* against cerebral ischemia induced injuries.

In this study, we demonstrated a good neuroprotective effect of nicotiflorin in a permanent focal brain ischemia model in rats, finding that it reduced the MCAO induced infarct size. Consistent with these observations, we also found that nicotiflorin significantly improved the behavioral deficits caused by MCAO 24 h after induction of ischemia.

We also observed neuronal protection of nicotiflorin in an *in vitro* model of ischemia. Brain neurons are highly sensitive to changes in oxygen availability. Any transient incidences of hypoxia/ischaemia induce pathophysiological changes such as disturbances in energy metabolism\(^22\) and modifications in synaptic communication.\(^23\) The substantial membrane depolarisation that occurs after hypoxia leads to neuronal hyperexcitability, irreversible cellular dysfunction, and neurotoxicity.\(^24,25\) Vulnerability of neurons to oxygen deprivation depends on the magnitude of exposure\(^26,27\) or the CNS region affected.\(^28\) In our study, through morphology observation and cell viability determination, we verified nicotiflorin had a good direct protection on primarily cultured neurons suffered 2 h hypoxia followed by 24 h reoxygenation.

Dajas et al.\(^29\) had reported that in the *in vivo* experiments, when incubating PC12 cells with 200 \( \mu \)M hydrogen peroxide (H\(_2\)O\(_2\)) and kaempferol (main body of nicotiflorin, administered in lecithin preparations) for 30 min, it could not increase cell survival. In our experiment, however, it could increase the neuron survival damaged by hypoxia. We think this mainly due to the cell and damage model used in study. In our study, the primarily cultured neurons and hypoxia-reoxygenation model were more similar to the conditions of ischemia-reperfusion *in vivo*.

In recent years, other studies also revealed that extracts of some medical plants that contain diverse glycosides of kaempferol aglycone (such as *Ginkgo Biloba*) exhibited neuroprotective actions in many damage models.\(^30^{--}33\) In *Ginkgo biloba* leaves extracts, there are about 13 flavonol glycosides. Among them only 4 are with kaempferol aglycone, including nicotiflorin.\(^34\) In fact the neuroprotective effects of *Ginkgo biloba* leaves are provided not only by kaempferol glycosides, but also by other flavonoids glycosides. In our studies, the nicotiflorin was highly pure, so the protective effects came from nicotiflorin. Further investigations about the protective mechanism of kaempferol glycosides showed it involved their antioxidative activity, blockading caspase cascades, attenuating NMDA-induced neuronal...
toxicity, inhibiting monoamine oxidase (MAO) and excessive NO production, and so on. The neuroprotective actions were the result of a combination of many independent mechanisms.\textsuperscript{30} The studies also showed that kaempferol aglycone played an important role in such protective effects, meanwhile the glycosidation of aglycone and the substitute groups on the aglycone were important modulators to its biological activities.\textsuperscript{31} We considered the mechanism of neuroprotective effects of nicotiflorin was mainly attributed to the effects of kaempferol aglycone as above-mentioned.

In summary, we demonstrated and confirmed the neuroprotective effect of nicotiflorin using a permanent model of focal brain ischemia in rats. It was able to reduce the size of infarction induced induced by pMCAO and improve the behavioral deficits in rats. In an in vitro model of ischemia, it could provide a good direct protection on primarily cultured neurons suffer hypoxia. These findings may provide strong pharmacological basis for the use of nicotiflorin in the treatment of stroke.

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REFERENCES