Cerebral Protective and Cognition-Improving Effects of Sinapic Acid in Rodents

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Received October 20, 2006; accepted December 12, 2006

We previously demonstrated that tenuifoliside B and 3,6’-disinapoylsucrose in Polygalae Radix, the root of Polygala tenuifolia Willdenow, inhibited potassium cyanide (KCN)-induced hypoxia and scopolamine-induced memory impairment in mice. Because both ingredients have a common sinapoyl moiety in their structure, we inferred that the sinapoyl moiety could inhibit hypoxia and memory impairment. In the present study to clarify the hypothesis, sinapic acid inhibited KCN-induced hypoxia and scopolamine-induced memory impairment as well as tenuifoliside B and 3,6’-disinapoylsucrose did. In addition, sinapic acid inhibited decompression- or bilateral carotid artery ligation-induced hypoxia (or mortality) and CO2-induced impairment in mice, and basal forebrain lesion-induced cerebral cholinergic dysfunction (decreases in acetylcholine concentration and choline acetyltransferase activity) in rats. These results, taken together, suggest the possibilities that sinapic acid is not only a very important moiety in the pharmacological activities of tenuifoliside B and 3,6’-disinapoylsucrose but also a candidate for a cerebral protective and cognition-improving medicine.

Key words Polygalae Radix; sinapic acid; memory impairment; anoxia; acetylcholine; choline acetyltransferase

Senile dementia of Alzheimer type and multi-infarct dementia are considered to be major problems of contemporary societies. Recently, Kami-kihi-to, Ninjin-yoei-to and Kami-untan-to, which are traditional Japanese herbal medicines (Kampo medicines) have been reported to improve memory disturbance and the related behaviors.1–4) Because these Kampo Medicines contain Polygalae Radix (Onji in Japanese), the root of Polygala tenuifolia Willdenow as a common herb, it is suggested that the constituent herb may possess the activity to improve memory disturbance. Polygalae Radix itself has been utilized empirically in Chinese medicine, works to relieve amnesia, neurasthenia, palpitations, nocturnal emission, and insomnia.5) Recently, evidence of the memory-enhancing effects of Polygalae Radix has been accumulated by several in vivo and in vitro studies; it was reported that a water extract of Polygalae Radix improved scopamine-induced memory impairment in mice,6) and that DX-9368 belongs to the group of Polygalae Radix agents ameliorated ethanol- and scopamine-induced memory impairment in mice.7) Yabe et al.8) reported that the water extract up-regulated choline acetyltransferase (ChAT) activity and increased nerve growth factor (NGF) secretion in vitro. However, the pharmacological effects of the ingredients in Polygalae Radix have been little investigated. In these background, more recently, we demonstrated that tenuifoliside B [6’-(p-hydroxybenzoyl)-3-sinapoylsucrose] and 3,6’-disinapoylsucrose among the components isolated from Polygalae Radix inhibited potassium cyanide (KCN)-induced hypoxia and scopolamine-induced memory impairment.9) From these results, we suggested that the two ingredients were instrumental in the cerebral protective and cognition-improving effects of Polygalae Radix. More interestingly, both ingredients have a common sinapoyl moiety in their chemical structures. These suggest that sinapic acid might be not only a main active structure in the pharmacological effects of tenuifoliside B and 3,6’-disinapoylsucrose but also a candidate for cerebral protective and cognition-improving medicine.

In the present study, in order to ascertain the hypothesis, we examined whether sinapic acid possesses cerebral protective and cognition-improving effects in various animal models of hypoxia and amnesia.

MATERIALS AND METHODS

Drug and Reagents Sodium sinapate (Fig. 1), which is the water-soluble form of sinapic acid, was used in the following experiments. Sodium sinapate (4.92 g) was obtained by lyophilizing a solution of sinapic acid (4.48 g) purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan) in 200 ml of 0.1 M sodium hydroxide. Ethylhomocholine iodide (EHC), an internal standard for determination of acetylcholine (ACh), was synthesized from 3-dimethylamino-1-propanol and iodoethane purchased from Sigma-Aldrich Fine Chemicals Japan Co., Ltd. (Tokyo, Japan). Other reagents used for analysis were the highest purity commercially available.

Animals Male ICR mice (7 weeks old) to evaluate KCN-induced coma and hypobaric hypoxia-induced survival and male ddY mice (8 weeks old) to evaluate CO2- or scopolamine-induced amnesia were purchased from Japan SLC Co., Ltd. (Hamamatsu, Japan). Male Wistar rats (8 weeks old) to evaluate cerebral acetylcholine (ACh) level and choline acetyltransferase (ChAT) activity were purchased from Charles River Japan (Tsukuba, Japan). They were housed in a facility at a temperature of 23±2 °C and relative humidity of 55±10%, and controlled lighting with lights on

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Fig. 1. Chemical Structure of Sodium Sinapate

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from 07:00 to 19:00 daily. The animals were allowed free access to water and standard laboratory chow (MF, Oriental Yeast Co., Ltd., Japan). They were fasted for 18 h prior to the beginning of the experiments.

All experimental procedures were performed according to the ‘Guidelines for the Care and Use of Laboratory Animals’ approved by the Laboratory Animal Committee of Tsumura & Co.

**Effect of Sinapic Acid on KCN-Induced Hypoxia**

Sinapic acid at 3 mg/kg (n = 15), 10 mg/kg (n = 12), or 100 mg/kg (n = 8) or 10 ml/kg of vehicle (n = 17) was orally (p.o.) administered to mice. Sixty minutes later, all animals were intravenously (i.v.) injected with KCN (2 mg/kg) dissolved in saline (10 ml). The duration of the coma induced by KCN was recorded. The coma status was judged by disappearance of a righting reflex after injection of KCN.

**Effect of Sinapic Acid on Hypobaric Hypoxia**

Sinapic acid (10 and 100 mg/kg, p.o., n = 16/each group) or the vehicle (10 ml/kg saline, p.o., n = 16) was administered to mice. Sixty minutes later, each animal was placed in a decompression chamber, and then the inside pressure was lowered to 160 mmHg with a vacuum pump. The effect of sinapic acid on hypobaric hypoxia was evaluated by measuring the mortality; the survival time from the start of decompression until the respiratory movements stopped was recorded.

**Effect of Sinapic Acid on Carotid Artery Ligation-Induced Hypoxia**

The effect of sinapic acid on carotid artery ligation-induced hypoxia was evaluated by measuring the mortality after ligation. In brief, 10 mg/kg (n = 24) and 100 mg/kg (n = 28) of sinapic acid or the vehicle (10 ml/kg saline, n = 25) as control was administered p.o. to mice. Sixty minutes later, bilateral carotid arteries in all animals were ligated under an unanesthetized condition. The ligation-induced mortality was recorded at 30 min intervals for 240 min after ligation.

**Effect of Sinapic Acid on CO2-Induced Amnesia**

Forty-eight mice were divided into control+vehicle (10 ml/kg saline, n = 12) and CO2-treated groups (n = 36). The CO2-treated group was further divided into three groups: CO2+vehicle (n = 13), CO2+10 mg/kg sinapic acid (n = 14), and CO2+100 mg/kg sinapic acid (n = 9). Sinapic acid (10 or 100 mg/kg, i.p.) or saline (10 ml/kg) was administered 60 min before the acquisition trial of the passive-avoidance test.

The effect of sinapic acid on CO2-induced amnesia was evaluated using a step-through passive-avoidance task described in the previous section. In brief, the acquisition trial was performed for all mice in each group. Scopolamine (0.3 mg/kg, i.p.) or vehicle saline (10 ml/kg, i.p.) was administered 15 min before the acquisition trial. Twenty-four hours later, the retention trial was performed. Sinapic acid (3, 10, or 100 mg/kg, p.o.) or vehicle saline (10 ml/kg, p.o.) was administered 60 min before the retention trial. The cut-off time was 300 s.

**Effect of Sinapic Acid on Basal Forebrain Lesion-Induced Decreases in Cerebral ACh Level and ChAT Activity**

Eighty rats were divided into sham (10 ml/kg saline, n = 20) and ibotenic acid-induced forebrain lesion groups (n = 60). The forebrain lesion group was further divided into three groups: ibotenic acid+vehicle (n = 20) and ibotenic acid+sinapic acid (3 or 10 mg/kg, n = 20/each group).

All rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.). Ibotenic acid (10 μg/2.0 μl) in forebrain lesion group or the vehicle (2.0 μl of saline) in sham group was stereotaxically injected into the bilateral nucleus basalis of Mynert (coordinates: posterior 1.8 mm and bilateral 3.2 mm from the bregma; ventral 6.5 mm from the dura). Sinapic acid (3, 10 and 30 mg/kg, n = 20 in each group) or water (10 ml/kg, n = 20) as control was orally administered for 7 d after the injection of ibotenic acid. Sham-operated rats were administered water (10 ml/kg, p.o., n = 20) for 7 d. On day 8, half of the animals (n = 10) in each group were killed by microwave irradiation at 8.5 kW for 1.10 s (NJE model 2603, New Japan Radio, Saitama, Japan) for determination of ACh in the brain. The other half (n = 10) was decapitated for determination of ChAT activity in the brain.12)

For determination of the ACh level in the brain,12) the microwave-irradiated brain was quickly removed, and the frontal and parietal cortices were dissected. The tissue was homogenized for 60 s in 1 ml of 0.05 M HClO₄ containing 10 nmol EHC as an internal standard with an ultrasonic cell disruptor. The homogenate was centrifuged at 20000g at 4°C for 15 min. The supernatant was purified by passage through a 0.45 μm Milipore filter. An aliquot of the filtrate was injected into a liquid chromatograph equipped with an electrochemical detector (LC-ECD).

For determination of ChAT activity in the brain,13) the brain was quickly removed from the decapitated rat, and the parietal cortex was dissected on a cold glass plate. The tissue was homogenized in 10 ml of cold 0.1 M potassium phosphate buffer, pH 7.4, using an ultrasonic cell disruptor. The homogenate was used for assay of ChAT by the LC-ECD,13) and
for protein assay using a Bio-Rad protein kit (Bio-Rad Laboratory, Richmond, CA, U.S.A.).

**Statistical Analysis** The results are expressed as the mean±S.E.M. One-way ANOVA followed by Dunnett’s test was used to evaluate the significance between groups in the experiments of KCN-induced amnesia, hypobaric hypoxia, and carotid artery ligation-induced hypoxia, and basal forebrain lesion-induced decreases in cerebral ACh level and ChAT activity experiments. The Mann–Whitney test was used in the experiments of CO2-induced and scopolamine-induced amnesia. The significance level was accepted at p<0.05.

**RESULTS**

**Effect of Sinapic Acid on KCN-Induced Hypoxia** The effect of sinapic acid on KCN-induced hypoxia was evaluated by measuring the duration of a coma that was judged by disappearance of the righting reflex. As shown in Table 1, i.v. injection of KCN (2 mg/kg) lost the righting reflex for 281.0±33.9 s. Pretreatment with sinapic acid (3—100 mg/kg) significantly inhibited the KCN-induced coma time in a dose-dependent manner.

**Effect of Sinapic Acid on Hypobaric Hypoxia** The effect of sinapic acid on hypobaric hypoxia was evaluated by measuring the time until the animal died in a decompression chamber. The results are shown in Table 2. The survival time of the vehicle-treated control was 66.5±2.0 s in a decompression chamber. Pretreatment with sinapic acid (10, 100 mg/kg) significantly prolonged the survival time.

**Effect of Sinapic Acid on Carotid Artery Ligation-Induced Hypoxia** The effect of sinapic acid on carotid artery ligation-induced hypoxia was evaluated by measuring mortality for 240 min after the ligation in mice. As shown in Table 3, the mortality in vehicle-treated controls gradually increased in a time-dependent manner, and was 92% at 240 min after the ligation. Pretreatment with sinapic acid (10, 100 mg/kg) significantly inhibited the ligation-induced mortality in a dose-dependent manner. Significant inhibition was observed at 60 min and 120—240 min after the ligation in the 100 mg/kg sinapic acid-treated group.

**Effect of Sinapic Acid on CO2-Induced Amnesia** Table 4 shows the percentage of mice that learned avoidance and the mean latency time in the retention trial. Seventy-five per cent (9/12 mice) in the non-CO2 group stayed in the illuminated compartment over 600 s, and the mean latency time was 543.3±35.7 s. On the other hand, both parameters were significantly shortened in CO2-loaded mice. The shortening of both parameters was inhibited by pre-treatment with sinapic acid (10, 100 mg/kg) in a dose-dependent manner.

**Effect of Sinapic Acid on Scopolamine-Induced Amnesia** Table 5 shows the percentage of mice that learned avoidance and the mean latency time in the retention trial. The percentage (10%) and mean latency time (153.2±18.7 s) of the scopolamine-treated mice were significantly decreased (p<0.001) compared to those (80% and 268.9±15.8 s) of non-scopolamine controls. The decreases of both parameters

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**Table 1. Effects of Sinapic Acid on KCN-Induced Hypoxia**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (mg/kg, p.o.)</th>
<th>n</th>
<th>Coma time (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>—</td>
<td>17</td>
<td>281.0±33.9</td>
</tr>
<tr>
<td>Sinapic acid</td>
<td>3</td>
<td>15</td>
<td>162.8±50.2**</td>
</tr>
<tr>
<td>Sinapic acid</td>
<td>10</td>
<td>12</td>
<td>149.5±30.8*</td>
</tr>
<tr>
<td>Sinapic acid</td>
<td>100</td>
<td>8</td>
<td>116.3±21.0***</td>
</tr>
</tbody>
</table>

Each value represents the mean±S.E. *p<0.05, **p<0.01 compared with the control value. The degree of hypoxia was evaluated by measuring the duration of coma induced by KCN.

**Table 2. Effects of Sinapic Acid on Hypobaric Hypoxia**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (mg/kg, p.o.)</th>
<th>n</th>
<th>Survival time (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (saline)</td>
<td>—</td>
<td>16</td>
<td>66.5±2.0</td>
</tr>
<tr>
<td>Sinapic acid</td>
<td>10</td>
<td>16</td>
<td>76.8±2.3**</td>
</tr>
<tr>
<td>Sinapic acid</td>
<td>100</td>
<td>16</td>
<td>79.0±2.4***</td>
</tr>
</tbody>
</table>

Each value represents the mean±S.E. *p<0.01, **p<0.001 compared with the control value. Drugs were administered 60 min before the induction of hypoxia (30 s at 160 mmHg). The degree of hypoxia was measured by survival time until mice died in the decompression chamber.

**Table 3. Effect of Sinapic Acid on Bilateral Carotid Artery-Ligated Mice**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (mg/kg)</th>
<th>n</th>
<th>Mortality (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Control (saline)</td>
<td>—</td>
<td>25</td>
<td>0</td>
</tr>
<tr>
<td>Sinapic acid</td>
<td>10</td>
<td>24</td>
<td>0</td>
</tr>
<tr>
<td>Sinapic acid</td>
<td>100</td>
<td>28</td>
<td>0</td>
</tr>
</tbody>
</table>

*p<0.05, **p<0.01 compared with the control value. Drugs or saline (10 ml/kg) were administered p.o. 60 min before the ligation of bilateral carotid arteries.

**Table 4. Effect of Sinapic Acid on CO2-Induced Impairment of Passive Avoidance Performance**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (mg/kg)</th>
<th>n</th>
<th>CO2</th>
<th>Number of animals retaining memory (%)</th>
<th>Latency (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (saline)</td>
<td>—</td>
<td>12</td>
<td>—</td>
<td>9/12 (75.0%)</td>
<td>543.3±35.7</td>
</tr>
<tr>
<td>Control (saline)</td>
<td>—</td>
<td>13</td>
<td>+</td>
<td>1/13 (7.6%)</td>
<td>279.3±41.6*</td>
</tr>
<tr>
<td>Sinapic acid</td>
<td>10</td>
<td>14</td>
<td>+</td>
<td>3/14 (21.4%)</td>
<td>342.6±46.4</td>
</tr>
<tr>
<td>Sinapic acid</td>
<td>100</td>
<td>9</td>
<td>+</td>
<td>5/9 (55.6%)</td>
<td>443.9±63.4*</td>
</tr>
</tbody>
</table>

Each value represents the mean±S.E. *p<0.05 vs. non-CO2-treated control, *p<0.05 compared with the CO2-treated control (Mann–Whitney test). Drugs or saline (10 ml/kg) were administered i.p. 60 min before the acquisition trial of the passive-avoidance test.
by scopolamine were inhibited by pre-treatment with sinapic acid (3—100 mg/kg) in a dose-dependent manner.

**Effects of Sinapic Acid on Basal Forebrain Lesion-Induced Decreases in Cerebral ACh Level and ChAT Activity** The effects of sinapic acid (3, 10 mg/kg) on ACh levels in the parietal cortex and frontal cortex of ibotenic acid-treated rats are shown in Figs. 2 and 3, respectively. Ibotenic acid decreased ACh levels in the parietal and frontal cortices. The decreases in ACh levels in both regions were inhibited by pretreatment with sinapic acid in a dose-dependent manner. Significant inhibition was observed at a dose of 10 mg/kg in both regions.

The effect of sinapic acid (3, 10 mg/kg) on ChAT activity in the parietal cortex of ibotenic acid-treated rats is shown in Fig. 4. Ibotenic acid induced a significant decrease in ChAT activity in the parietal cortex. The decrease in ChAT activity was inhibited by pretreatment with sinapic acid in a dose-dependent manner. Significant inhibition was observed at a dose of 10 mg/kg.

**DISCUSSION**

In the present study, we examined the cerebral protective effects of sinapic acid in several hypoxia models, *i.e.*, by measuring the duration of KCN-induced coma and the mortality induced by vacuum pressure or carotid artery ligation. Coma induced by KCN is considered to be due to cerebral cytotoxic hypoxia caused by the inhibition of mitochondrial cytochrome oxidase, which is a cellular respiration enzyme. Decompression and carotid-artery ligation induce a decrease in oxygen supply to the brain (hypoxia), the continuance of which leads to death. Sinapic acid inhibited KCN-induced coma and decompression- or carotid-artery ligation-induced mortality. These results strongly suggest that sinapic acid inhibits cerebral hypoxia. It is known that...
hypoxia and ischemia induce cerebral neuronal death.\textsuperscript{16,17} Taken together, sinapic acid is thought to protect against cerebral neuronal damage and death.

It is well known that memory and learning are impaired by hypoxia in animals and humans.\textsuperscript{18,19} For instance, transient cerebral ischemia and hypoxia lead to delayed neuronal damage in the cortex and hippocampus, which are very important in mnemonic function.\textsuperscript{20,21} These findings suggest the possibility that sinapic acid also inhibits hypoxia-induced memory impairment. In order to clarify the hypothesis, the effect of sinapic acid on hypoxia-induced memory impairment was examined in \textit{CO}_2-induced memory impairment, which is known as one of the hypoxia-induced amnesia models. The step-through latency in the retention test was significantly decreased by inhaling \textit{CO}_2\textsuperscript{22,23} and the decreased latency time implied an impairment of memory. Sinapic acid significantly inhibited the \textit{CO}_2-induced memory impairment, suggesting that the anti-amnesic effect of sinapic acid may be achieved by preventing the neuronal death or damage resulting from hypoxia.

Hypoxia induces not only neuronal death but also presynaptic cholinergic dysfunction, which is closely related to memory disturbance; KCN reduces potassium-stimulated ACh release from isolated nerve endings, \textit{i.e.}, synaptosomes.\textsuperscript{24,25} The presynaptic terminals of the cholinergic neuron are vulnerable to ischemic insult, and cholinergic dysfunction precedes postsynaptic CA1 pyramidal cell death in the hippocampus.\textsuperscript{16} Consequently, in order to confirm whether sinapic acid directly modulates the cholinergic function, its effect on scopolamine-induced amnesia was examined. Scopolamine causes profound amnesia or impairment of memory by inhibiting muscarinic ACh receptors,\textsuperscript{26} which is not a hypoxic amnesia. Sinapic acid inhibited the scopolamine-induced decrease in the step-through latency in the retention test suggesting amnesia (Table 5). In addition, the effects of sinapic acid on cerebral cholinergic neurochemical parameters, \textit{i.e.}, ACh level as a cholinergic neurotransmitter and ChAT activity as the enzyme synthesis, were examined in the basal forebrain-lesioned rats by injection of ibotenic acid, a glutamate analog, that selectively destroys the neuronal cell body.\textsuperscript{27} The cholinergic neurons of the basal forebrain provide a major source of cholinergic innervation of the cortex, which plays an important role in learning and memory processes.\textsuperscript{28,29} Therefore, the destruction of the basal forebrain with ibotenic acid is often used as a powerful tool to decrease cholinergic function in the cerebral cortex. A microinjection of ibotenic acid into the basal forebrain decreased ACh concentrations in the parietal and frontal cortices and ChAT activities in the parietal cortex. Sinapic acid inhibited the decreases in ACh concentrations and ChAT activities (Figs. 2, 3, 4). Yabe \textit{et al}.\textsuperscript{3} also reported that sinapic acid inhibited the decrease in ChAT activity in the frontal cortex of basal forebrain lesioned rats. These results suggest that sinapic acid is improved memory disturbance by activating cholinergic function (ACh and ChAT). However, the decrease in oxygen supply to the brain (hypoxia or ischemia) is reported to be changed various cerebral neuronal systems including catecholaminergic, serotonergic and glutamatergic neurons, and neurotrophins such as NGF.\textsuperscript{8,30—34} Therefore, further investigations to assess the mechanism underlying curative effects by sinapic acid will be necessary in near future.

We previously demonstrated that the methanol extract (50—500 mg/kg) of \textit{Polygalae Radix} inhibited KCN-induced hypoxia and scopolamine-induced memory impairment in mice.\textsuperscript{35} The contents of tenuifoliside B and 3,6'-disinapoylsucrase in the methanol extract of \textit{Polygalae Radix} (50—500 mg/kg) are 1.1% (0.55—5.5 mg/kg) and 7.3% (3.65—36.5 mg/kg), respectively. In the present study, sinapic acid (10—100 mg/kg), a common sinapoyls moiety in tenuifoliside B and 3,6'-disinapoylsucrose, inhibited KCN-induced hypoxia and scopolamine-induced memory impairment as well as tenuifoliside B (3—100 mg/kg) and 3,6'-disinapoylsucrose (10—100 mg/kg) did. In addition, sinapic acid inhibited hypobaric hypoxia-induced or bilateral carotid artery ligation-induced mortality, \textit{CO}_2-induced memory impairment in mice, and forebrain lesion-induced cerebral cholinergic dysfunction (decreases in ACh concentration and ChAT activity) in rats. These results, taken together, suggest the possibilities that sinapic acid is not only a very important moiety in the pharmacological activities of tenuifoliside B and 3,6'-disinapoylsucrose but also a candidate for a cerebral protective and cognition-improving medicine. However, \textit{p}-hydroxybenzoic acid is also inferred to be a common moiety in the structures of tenuifoliside B and 3,6'-disinapoylsucrose. It is still unclear whether or not sinapic acid is derived from tenuifoliside B and 3,6'-disinapoylsucrose administered to animals. Therefore, further investigations are necessary in order to more clarify the possibilities suggested in the present study.

Acknowledgements The authors thank Dr. S. Iizuka for excellent technical assistance.

REFFERENCES

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