JPP
Journal of
Pharmacy and Pharmacology

JPP 2007, 59: 521–528
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Received September 14, 2006
Accepted December 4, 2006
DOI 10.1211/jpp.59.4.0006
ISSN 0022-3573

Protective action of 9-hydroxypinoresinol against oxidative damage in brain of mice challenged with kainic acid

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Abstract

The neuroprotective effect of 9-hydroxypinoresinol was examined in mice challenged with kainic acid (KA), a potent central nervous system excitotoxin. For this purpose, mice were administered intraperitoneally with 9-hydroxypinoresinol before KA injection. A remarkable neuroprotective effect was observed with a single dose of 9-hydroxypinoresinol (30 mg kg^{-1}) 24 h before KA challenge. Furthermore, 9-hydroxypinoresinol (20 mg kg^{-1}) administered for 3 days before KA challenge reduced the mortality (60%) induced by KA to zero, and alleviated behavioural signs of KA neurotoxicity. Additionally, pretreatment with 9-hydroxypinoresinol (20 mg kg^{-1}) prevented the decrease in the levels of total glutathione (GSH) and thiobarbituric acid reactive substances ($P < 0.05$). GSH peroxidase activity in brain tissue was restored to control levels, although GSH reductase activity and GSH *S*-transferase activity were not affected. Such a protective action was also observed even with a lower dose (10 mg kg^{-1}) of 9-hydroxypinoresinol administered for 3 days, albeit to a lesser extent. From the results, it is proposed that 9-hydroxypinoresinol exerts a potent neuroprotective effect mainly by preventing oxidative stress in brain tissue of mice challenged with KA.

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Funding: This study was supported by a Leading Women Scientists' Research Foundation Grant, Republic of Korea (grant no: 2005-6521).

Introduction

Kainic acid (KA) is a potent central nervous system excitotoxin producing epileptiform activity and, ultimately, irreversible neuropathological changes (Sperk 1994). KA is known to induce brain lesions by directly activating a subtype of ionotropic glutamate receptors and further provoking the release of neurotoxic amounts of glutamate (Olney et al 1974; Coyle & Puttfarcken 1993). Therefore, KA has been used as a model agent for the study of neurotoxicity of excitatory amino acids such as glutamate. KA-induced neuronal death is known to result from the generation of reactive oxygen species (ROS) in brain tissue and subsequent membrane destruction (Floreani et al 1997; Saija et al 1997). Brain tissue is particularly vulnerable to oxidative stress as it consumes a large amount of oxygen while having a relative paucity of protective systems (Olney et al 1974; Meister & Anderson 1983). Antioxidants such as glutathione (GSH) participate in maintaining cellular redox balance in brain tissue and in protecting against ROS-mediated oxidative damage (Meister & Anderson 1983; Alams et al 1991; Sian et al 1994). Although the intracellular level of reduced GSH is maintained mainly by GSH reductase (GR) (Meister & Anderson 1983), antioxidant enzymes such as superoxide dismutase, catalase and GSH peroxidase (GP) also contribute to the preservation of the intracellular GSH level by removing ROS (Meister 1995). Furthermore, earlier studies have shown that the administration of antioxidants such as GSH ester or melatonin prevented KA-induced reduction of the intracellular GSH level in brain tissue (Floreani et al 1997; Gupta et al 2002). Plant polyphenols were also found to prevent the neurotoxicity of excitotoxins in cell culture systems (Gilgun-Shrki et al 2001; Hur et al 2001; Jang et al 2002; Min et al 2004). However, there have been only a limited number of reports on the neuroprotective effects of polyphenolic antioxidants in in-vivo systems. Even *trans*-resveratrol, which alleviated lipid peroxidation in the brain tissue of rats administered with KA, failed to prevent the reduction of GSH level in brain (Gupta et al 2002). Our group has observed that oral administration of petaslignolide A, corresponding to 9-hydroxypinoresinol glycoside, showed a remarkable neuroprotective effect via antioxidant action in brain tissue,

in contrast to the negligible neuroprotective effect by pinoresinol glycoside (Cui 2005; Cui et al 2005). Recent studies suggest that some orally administered furfuran or butyrofuran lignan compounds may have antioxidant effects in-vivo after metabolic deglycosylation in the gut (Niemeyer et al 2003; Jansen et al 2005; Milder et al 2005). Thus, it is very likely that the glycosidic form of 9-hydroxypinoresinol is cleaved to 9-hydroxypinoresinol in the intestine before transport across the blood–brain barrier (Jansen et al 2005; Milder et al 2005). So, 9-hydroxypinoresinol, which has a greater antioxidant effect than its precursor, is thought to be actively implicated in the neuroprotective effect seen in-vivo. However, there has been no experiment to test this assumption.

A recent report by Steffan et al (2005) indicated that the antioxidant effect of 9-hydroxypinoresinol (isolated from plants used in traditional Indonesian medicine) was not impressive in an in-vitro assay, and activity was even less than that of flavonoid compounds such as kaempferol or luteolin. However, quercetin, a potent flavonoid antioxidant, also failed to show a neuroprotective action in-vivo, probably due to its prooxidant action (Dajas et al 2003). Thus, the neuroprotective effect of polyphenols cannot be simply explained by their in-vitro antioxidant effects alone.

The aim of the present study was to assess the neuroprotective effect of 9-hydroxlpinoresinol against oxidative damage in the brain of mice treated with KA, and to determine the mechanism by which 9-hydroxlpinoresinol exerts its neuroprotection.

Materials and Methods

Materials

Naringinase (from *Penicillium decumbens*), GR (Type III from baker's yeast), GSH, oxidized GSH (GSSG), 5,5′-dithio-2-nitrobenzoic acid (DTNB), NADP, NADPH, NADH, FAD, thiobarbituric acid, dichlorophenolindophenol, 1-choloro-2,4-dinitrobenzene and bovine serum albumin were products of Sigma Chemical Co. (St Louis, MO, USA).

Preparation of 9-hydroxypinoresinol

Petaslignolide A (9-hydroxypinoresinol β-D-glucopyranoside; Figure 1) was isolated from the methanol extract of *Petasites japonicus* leaves by solvent fractionation and chromatography, and then 9-hydroxypinoresinol (see Figure 1) was prepared from enzymatic deglycosylation of petaslignolide A with naringinase as described previously (Cui et al 2005; Min et al 2005). The purity of 9-hydroxypinoresinol was estimated to be >90%. The negative ion atmospheric pressure ionization mass spectrometry analysis indicated that the hydrolysis product had a quasi-molecular ion at m/z 373 $[M-H]^-$, in compliance with the molecular formula, $C_{20}H_{20}O_6$. Its structure was determined to be 7′α-(4′-hydroxy-3′-methoxyphenyl)-7α-(4-hydroxy-3-methoxyphenyl)-9-hydroxy-3,7-dioxabicyclo[3.3.0]-octane, namely 9-hydroxypinoresinol, by analysis of the nuclear magnetic resonance spectrum, in comparison with the nuclear magnetic resonance spectrum of its precursor, petaslignolide A (Min et al 2005).

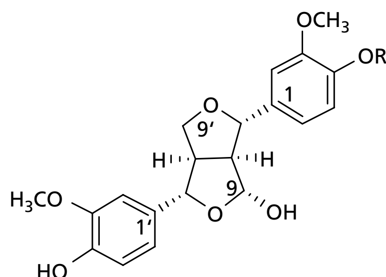

Figure 1 Structure of 9-hydroxypinoresinol (R = H) and 9-hydroxypinoresinol β-D-glucopyranoside (R = glucose).

Measurement of the antioxidant effect of 9-hydroxypinoresinol

Low density lipoprotein (LDL; 0.1 mg protein mL^{-1}), prepared from sequential ultracentrifugation (Schumaker & Puppione 1986), was incubated with 5 μM Cu^{2+} in the presence or absence of each antioxidant in 200 μL of 10 mM phosphate-buffered saline (PBS) at 37°C for 3 h, and the production of thiobarbituric acid reactive substances (TBARS) was measured as previously described (Esterbauer & Cheeseman 1990). DPPH radical scavenging activity was carried out by adding each sample (10 μL in dimethylsulfoxide) to 190 μL of 150 μM DPPH in methanol, and monitoring the decrease in absorbance at 517 nm as described previously (Cotelle et al 1996).

Animal experiments

Male ICR mice, 20–22 g, 6–8 weeks old, were housed in polycarbonate cages (10 per cage) and fed unrestricted amounts of filtered water and pelleted commercial diet (Samyang Co., Korea). The temperature and relative humidity were 23 ± 3°C and 55 ± 10%, respectively, and a 12-h light/dark cycle was maintained.

The experiments were conducted according to the Guiding Principles in the Use of Animals in Toxicology adopted by the Society of Toxicology (USA) in July 1989 and revised in March 1999. The protocol was approved by the Institutional Animal Care and Use Committee of Pharmacy Animal Research Group (No. 6-0-1), Chungnam National University, Korea.

Mice were assigned randomly to treatment groups (10 mice per group) and were weighed individually every day. Mice were administered intraperitoneally with 9-hydroxypinoresinol (10 or 20 mg kg^{-1}; 5 mL kg^{-1}) suspended in saline containing 5% Tween 80 for 3 consecutive days before intraperitoneal injection of KA (50 mg kg^{-1}; 3 mL kg^{-1}) dissolved in 10 mM PBS, pH 7.4 (Sok et al 2003; Cui et al 2005). Control animals were injected with saline containing 5% Tween 80. Separately, mice were administered intraperitoneally with 9-hydroxypinoresinol (30 mg kg^{-1}; 5 mL kg^{-1}) 2 or 24 h before KA challenge. Following KA administration, the onset time (min) of neurobehavioural activities such as tail arch, tremors and seizures was measured. Death of animals was monitored for 1 h (Tan et al 1998), and the time (min) to death after KA injection was recorded. The mortality was expressed as the percentage of dead mice among the total number of mice (10 mice). At 1 day after KA administration, the brain tissues of mice were

removed after intracardial perfusion under light anaesthesia with ether, with cold saline to avoid blood contamination.

Measurement of lipid peroxidation in brain tissue

Brain tissue, rinsed with 0.15 M KCl solution containing 2 mM EDTA, was homogenized in 9 volumes of 10 mM phosphate buffer (pH 7.4) using a tissue homogenizer with a teflon pestle. To the brain homogenate (1.0 mL) was added 1.0 mL of SDS (8.1%), 2 mL of acetic acid (20%) and 1 mL of thiobarbituric acid (0.75%). The mixture was boiled for 30 min, and the absorbance of the supernatant after centrifugation (18 000 *g*, 10 min) was measured at 532 nm as previously described (Bidlack & Tappel 1973).

Determination of the total GSH in brain tissue

At 1 day after KA administration, the brain tissues of mice were removed after intracardial perfusion and kept at –60°C until used. Frozen brain tissue (about 0.27–0.30 g wet weight) was pulverized in a cooled ceramic percussion mortar with 6% metaphosphoric acid, and the mixture was centrifuged (27 000 *g*, 20 min) at 4°C. Total GSH was determined enzymatically according to Roberts & Francetic (1993) with a slight modification. To the supernatant (0.02 mL) was added 100 mM phosphate buffer, pH 7.4 (0.39 mL) containing 5 mM EDTA, 10 mM DTNB (0.025 mL) and 5 mM NADPH (0.08 mL). After 3 min equilibration at 25°C, the reaction was started by adding GR (1 unit), and the formation of 2-nitro-5-thiobenzoate was monitored at 412 nm. The amount of total GSH was determined from a standard curve.

Assay of GP and GR

Brain tissue was homogenized in 9 volumes of 20 mM phosphate buffer containing 0.1 M KCl, 1 mM EDTA and 0.5% Triton X-100. The homogenate was centrifuged (15 000 *g*, 15 min) and the supernatant was recentrifuged (105 000 *g*, 30 min). The last supernatant was retained for enzyme assay. The assay of GP activity was carried out as described by Raymond et al (1995). A mixture containing 0.1 M phosphate buffer (pH 7.0), 3 mM EDTA, 1 mM GSH, 0.1 mM NADPH, 2 units of GR and 0.05 mL of supernatant was incubated for 3 min, and then 0.01 mL of 10 mM cumene hydroperoxide was added to the reaction mixture. GR activity was measured by monitoring the change of absorbance at 340 nm in a mixture containing 0.1 M phosphate buffer (pH 7.0), 0.5 mM EDTA, 1 mM GSSG, 0.1 mM NADPH and 0.05 mL of the supernatant (Raymond et al 1995).

Assay of GSH *S*-transferase (GST) and quinone reductase activity

The supernatant (20 μL) of brain homogenate was included in 480 μL of 0.1 M potassium phosphate buffer, pH 6.5 containing 1 mM reduced GSH (0.5 mL) and 1 mM CDMB (0.5 mL), and the absorbance at 340 nm was monitored for the determination of GST activity (Habig et al 1974). Separately, the supernatant (20 μL) was included in 25 mM Tris buffer (pH 7.4) containing 0.01% Tween 20, 1 mM dichlorophenolindophenol, 0.25 mM FAD and 10 mM NADH, and the absorbance at 660 nm was monitored in the presence or absence of 20 mM dicumarol for the determination of quinone reductase activity (Ernster 1967). Quinone reductase activity was expressed as dicumarol-suppressed activity.

Effect of KA and 9-hydroxypinoresinol on bodyweight and brain weight of mice

Mice were administered with 9-hydroxypinoresinol (20 mg kg^{-1}, i.p.) for 3 days before KA (50 mg kg^{-1}) injection. Change in bodyweight was determined after intraperitoneal administration of 9-hydroxypinoresinol (20 mg kg^{-1}, 5 mL kg^{-1}) suspended in saline containing 5% Tween 80, or saline containing 5% Tween 80 for 3 days. At 1 day after KA administration, brains were removed and used for the determination of brain weight.

Protein determination

Protein was determined according to the method of Bradford (1976) using bovine serum albumin as a standard.

Statistical analysis

All statistical analyses were performed using an SAS program (Statistics version 6.12, 1997; SAS Institute Inc., Cary, NC, USA). Statistical assessments were performed using the Student's *t*-test or analysis of variance, followed by post-hoc Duncan's multiple-range test for biochemical and neurobehavioural changes (Steel & Torrie 1960). All data are presented as mean ± s.e. A value of $P < 0.05$ was considered statistically significant.

Results

Since the neuroprotective effect of petaslignolide A, corresponding to 9-hydroxypinoresinol glycoside, required it to be administered prior to KA challenge (Cui et al 2005), it was supposed that a metabolite of petaslignolide A would most likely be the active species responsible for the neuroprotective effect in-vivo. Previous studies have shown that the primary metabolism of lignan glycoside in-vivo is enzymatic deglycosylation by microfloral glycosidase in the intestine (Jansen et al 2005; Milder et al 2005). Thus, a deglycosylation product of petaslignolide A was supposed to be responsible for its neuroprotective action. In this respect, 9-hydroxypinoresinol, prepared from enzymatic hydrolysis of petaslignolide A, was examined for antioxidant action in-vitro as well as neuroprotective action in-vivo.

Antioxidant action of 9-hydroxypinoresinol in-vitro

9-Hydroxypinoresinol was tested for antioxidant action in preventing Cu^{2+}-induced oxidation of LDL. As shown in Table 1, 9-hydroxypinoresinol (IC50 44.0 μg mL^{-1}) was fourfold more potent than petaslignolide A (IC50 181.1 μg mL^{-1}) in exerting an antioxidant action against LDL oxidation.

When 9-hydroxypinoresinol was tested for DPPH radical scavenging activity, 9-hydroxypinoresinol (IC50 25.5 μg mL^{-1}) was 4.5-fold more effective than petaslignolide A (IC50 113.0 μg mL^{-1}). Thus, the enzymatic deglycosylation of petaslignolide A led to an increase in both antioxidant activities. In comparison, the antioxidant action of 9-hydroxypinoresinol was comparable with that of α-tocopherol (IC50 17.2 μg mL^{-1}) in DPPH radical scavenging activity. From these results, 9-hydroxypinoresinol was thought to efficiently remove lipid radicals or oxidant radicals.

Neuroprotective action of 9-hydroxypinoresinol in-vivo

Given the above, it was supposed that 9-hydroxypinoresinol could prevent oxidative stress in brain tissue of mice challenged with KA and thereby alleviate the neurotoxicity of KA. To test this notion, 9-hydroxypinoresinol (30 mg kg^{-1}) was administered intraperitoneally to mice 2 or 24 h before KA challenge (50 mg kg^{-1}, 3 mL kg^{-1}), and its protective effect was examined by assessing behavioural signs such as sustained seizure, tremor or mortality. When 9-hydroxypinoresinol was administered 2 and 24 h before KA challenge, the onset time of seizure was 15.0 min and 22.0 min, respectively, compared with 14.3 min for control mice treated with KA alone (Table 2). In addition, pretreatment with 9-hydroxypinoresinol 2 and 24 h before KA challenge delayed the time of death to 46.7 min (30% mortality) and 80.0 min (10% mortality), respectively, compared with 31.4 min (50% mortality) for control mice. Thus, it is suggested that the time interval between 9-hydroxypinoresinol administration and KA challenge may be critical for effective protection against KA neurotoxicity. In the subsequent experiment to determine the effect of multiple administrations (Table 3), 9-hydroxypinoresinol (20 mg kg^{-1}) was intraperitoneally administered successively for 3 days prior to KA challenge, so that the neuroprotective effect of 9-hydroxypinoresinol could be increased. As shown in Table 3, the multiple administration of 9-hydroxypinoresinol (20 mg kg^{-1}) for 3 days was more effective than the single administration (Table 2) in reducing mortality: there was no mortality in the 9-hydroxypinoresinol/KA-treated group compared with 60% mortality in the vehicle/KA-treated group. In addition, pretreatment with 9-hydroxypinoresinol ameliorated behavioural signs caused by KA. These results suggest that an accumulation of 9-hydroxypinoresinol or its metabolite may be important for the protection against KA-induced oxidative damage in brain tissue. A significant reduction in mortality was also expressed by a low dose of 9-hydroxypinoresinol (10 mg kg^{-1}) administered for 3 days

Table 1 Antioxidant action of compounds expressed as the IC50

Compound	Protection against Cu^{2+}-induced LDL oxidation (μgmL^{-1})	DPPH radical scavenging activity (μgmL^{-1})
Petaslignolide A	181.1 ± 3.44^{a}	$113.04 \pm 4.21^{a*}$
9-Hydroxypinoresinol	43.98 ± 0.14^{b}	25.49 ± 0.72^{b}
α-Tocopherol	11.64 ± 0.33^{c}	17.16 ± 0.82^{c}

IC50 values were determined by regression analyses and expressed as mean ± s.d. of three replicates. Any two values in the same row with different letters are significantly different ($P < 0.05$). *DPPH radical scavenging activity of petaslignolide A was reported previously (Min et al 2005).

Table 2 Change of behaviour and mortality in mice administered with 9-hydroxypinoresinol before kainic acid (KA) injection

Group	Tail arch (min)	Tremors (min)	Seizures (min)	Death (min)	Mortality (%)
KA (50 mg kg^{-1})	7.6 ± 0.1^{a}	9.7 ± 0.9^{a}	14.3 ± 1.1^{a}	31.4 ± 3.4^{a}	50
KA + 9-hydroxypinoresinol (2 h)	7.7 ± 0.9^{a}	11.3 ± 1.2^{b}	15.0 ± 1.6^{a}	46.7 ± 9.6^{b}	30
KA + 9-hydroxypinoresinol (24 h)	12.7 ± 1.7^{b}	16.7 ± 1.5^{c}	22.0 ± 2.2^{b}	80.0 ± 0.0^{c}	10

Mice were administered once with 9-hydorxypinoresinol (30 mg kg^{-1}, i.p.) 2 or 24 h before KA injection (50 mg kg^{-1}, 3 mL kg^{-1}) and then behavioural signs were monitored for 60 min to determine the onset time of behavioural signs and the % mortality. Values are means ± s.d. (10 mice per group). Any two values in the same row with different letters are significantly different ($P < 0.05$).

Table 3 Change of behaviour and mortality in mice administered with 9-hydroxypinoresinol for 3 days before kainic acid (KA) injection

Group	Tail arch (min)	Tremors (min)	Seizures (min)	Death (min)	Mortality (%)
KA (50 mg kg^{-1})	5.9 ± 1.4**	8.7 ± 2.0**	15.5 ± 3.2**	22.2 ± 6.7	60
KA + 9-hydroxypinoresinol (20 mg kg^{-1})	11.5 ± 1.6	15.9 ± 2.4	28.4 ± 3.1	No death	0
KA (50 mg kg^{-1})	6.3 ± 2.0**	9.8 ± 2.0**	14.5 ± 2.3**	21.3 ± 4.6 (NS)	40
KA + 9-hydroxypinoresinol (10 mg kg^{-1})	9.4 ± 2.7	13.1 ± 2.9	18.0 ± 2.8	21.0 ± 0.0	10

Mice were intraperitoneally administered with KA and then behavioural signs were monitored for 60 min to determine the onset time of behavioural signs and the % mortality. In separate experiments mice were administered with 9-hydorxypinoresinol (20 or 10 mg kg^{-1}, i.p.) for 3 days before KA (50 mg kg^{-1}, 3 mL kg^{-1}) injection. Values are means ± s.d. (10 mice per group). **Significantly different compared with the KA + 9-hydroxypinoresinol group at $P < 0.01$ (Student's *t*-test). NS, not significant at $P < 0.01$.

before KA challenge (Table 3). To determine if the neuroprotective action of 9-hydroxypinoresinol pretreatment was related to its protective effects against oxidative stress in the brain tissue of mice intoxicated with KA, we examined the effect of 9-hydroxypinoresinol on the level of MDA, a biochemical marker of lipid peroxidation, in the brain of mice administered with KA. As presented in Table 4, the TBARS value was increased to 136% of the control value ($P < 0.05$) in the homogenate of whole brain of mice treated with KA. Pretreatment with 9-hydroxypinoresinol (20 $mg\,kg^{-1}$) for 3 days reduced the TBARS value, which was enhanced by KA challenge, to the level of the control group ($P < 0.05$). In a related experiment, we examined the effect of 9-hydroxypinoresinol on the level of total GSH, another biomarker of oxidative damage, in the brain of mice administered with KA. As shown in Table 4, the administration of 9-hydroxypinoresinol (20 $mg\,kg^{-1}$) increased the level of total GSH (80%) in the brain cytosol of the KA-treated group to control levels, further supporting the notion that the neuroprotective effect of 9-hydroxypinoresinol might be related to its antioxidant action in preventing oxidative damage in the brain. A beneficial effect of 9-hydroxypinoresinol on the levels of TBARS and total GSH was also observed in a separate experiment where a lower dose of 9-hydroxypinoresinol (10 $mg\,kg^{-1}$) was administered for 3 days (data not shown). In further studies, the change in activity of GSH-related antioxidant enzymes such as GP and GR was examined. Table 5 indicates that the loss of the GP activity caused by KA was recovered by pretreatment with 9-hydroxypinoresinol (20 $mg\,kg^{-1}$). In contrast, there was no significant difference in GR activity between the vehicle-treated group, KA-treated group and 9-hydroxypinoresinol/KA-treated group (Table 5). In an experiment to determine if the neuroprotective action of 9-hydroxypinoresinol may be related to the induction of phase II detoxification enzymes (Kraft et al 2004), the change of GST or quinone reductase activities in brain tissue was examined. However, there was no significant alteration in the activity of the two enzymes among the vehicle-treated group, KA-treated group and 9-hydroxypinoresinol/KA-treated group (Table 6), indicating that the neuroprotective action of 9-hydroxypinoresinol may not be related to the induction of phase II enzymes.

Table 4 Effect of 9-hydroxypinoresinol on thiobarbituric acid reactive substances (TBARS) and total glutathione (GSH) level in the brain tissues of mice administered with kainic acid (KA)

Group	TBARS (nmol (mg protein)$^{-1}$)	Total GSH (nmol (mg protein)$^{-1}$)
Control	0.44 ± 0.01^a	10.8 ± 0.5^a
KA (50 mg kg^{-1})	0.57 ± 0.02^b	8.6 ± 0.4^b
KA + 9-hydroxypinoresinol (20 mg kg^{-1})	0.43 ± 0.02^a	10.6 ± 0.5^a

Mice were administered with 9-hydroxypinoresinol (20 mg kg^{-1}, i.p.) for 3 days before KA (50 mg kg^{-1}, 3 mL kg^{-1}) injection. The brain homogenate was used for the determination of the TBARS value. Brain tissues were homogenized with 6% metaphosphoric acid and, after centrifugation, the supernatant was used for the determination of total GSH. Any two values in the same row with different letters are significantly different ($P < 0.05$). Values are means ± s.d. of triplicate determinations (10 mice per group, except four mice for the KA group).

Table 5 Effect of 9-hydroxypinoresinol on glutathione peroxidase (GP) and glutathione reductase (GR) activity in the brain tissue of mice administered with kainic acid (KA)

Group	GP (nmol min (mg protein)$^{-1}$)	GR (nmol min mg protein)$^{-1}$)
Control	8.6 ± 0.5^a	17.5 ± 1.9^a
KA (50 mg kg^{-1})	7.5 ± 0.6^b	17.3 ± 2.8^a
KA + 9-hydroxypinoresinol (20 mg kg^{-1})	8.7 ± 0.6^a	17.1 ± 1.9^a

Brain tissue was homogenized in phosphate-buffered saline for the determination of GP activity or GR activity. Values are mean ± s.d. of triplicate determinations (10 mice per group, except four mice for the KA group). Any two values in the same row with different letters are significantly different ($P < 0.05$).

Table 6 Effect of 9-hydroxypinoresinol on glutathione *S*-transferase (GST) and quinone reductase activity in the brain tissue of mice administered with kainic acid (KA)

Group	GST (nmol min (mg protein)$^{-1}$)	Quinone reductase (nmol min (mg protein)$^{-1}$)
Control	90.6 ± 22.0	81.0 ± 21.6
KA (50 mg kg^{-1})	95.7 ± 15.8	79.4 ± 16.2
KA + 9-hydroxypinoresinol (20 mg kg^{-1})	86.1 ± 22.5	80.1 ± 15.3

Brain tissue was homogenized in phosphate-buffered saline for the determination of GST or quinone reductase activity. Values are mean ± s.d. of triplicate determinations (10 mice per group, except four mice for the KA group). Any two values in the same row with different letters are significantly different ($P < 0.05$).

Effect of 4-hydroxypinoresinol on bodyweight and brain weight

9-Hydroxypinoresinol (20 mg kg^{-1}, 3 days) was administered intraperitoneally to mice before exposure to KA. The administration of 9-hydroxypinoresinol caused no significant changes in bodyweight or brain weight (Table 7), suggesting that 9-hydroxypinoresinol at the doses used had no toxic effects. Further, in a separate experiment, 9-hydroxypinoresinol alone had no effect on liver weight (data not shown).

Discussion

Since the ability of endogenous antioxidants to combat oxidative stress in brain tissue is limited, the administration of antioxidants has been suggested as a pharmacological strategy to protect brain tissue from oxidative damage (Saija et al 1997; Gupta et al 2002). For this purpose, the use of plant phenolic compounds, which demonstrate potent antioxidant action,

Table 7 Change in bodyweight and brain weight of mice administered with kainic acid (KA) and 9-hydroxypinoresinol

	Initial bodyweight (g)	Final bodyweight (g)	Change in bodyweight (g)	Brain weight (g)
Control	21.5 ± 0.6	24.6 ± 0.9	3.1 ± 0.6	0.322 ± 0.012
KA (50 mg kg^{-1})	21.3 ± 0.9	24.9 ± 0.9	3.6 ± 0.8	0.316 ± 0.012
KA + 9-hydroxypinoresinol (20 mg kg^{-1})	21.7 ± 0.7	24.5 ± 0.8	2.8 ± 0.6	0.313 ± 0.010

Mice were administered with 9-hydorxypinoresinol (20 mg kg^{-1}, i.p.) for 3 days before KA (50 mg kg^{-1}) injection. Change in bodyweight was determined after administration of 9-hydroxypinoresinol. At 1 day after KA administration, brains were removed and used for the determination of brain weight. Values are means ± s.d. (10 mice per group, except four mice for the KA group for determination of brain weight). Any two values in the same row with different letters are significantly different ($P < 0.05$).

could be a useful approach. However, although there have been numerous studies on the antioxidant action of polyphenolic compounds in-vitro (Jang et al 2002; Min et al 2004; Lin et al 2005; Milder et al 2005; Saleem et al 2005), there are few reports on the neuroprotective action of plant polyphenols in-vivo (Gupta et al 2002; Lin et al 2005; Zbarsky et al 2005). Naringenin expressed a only a small neuroprotective effect, while quercetin showed a negligible effect (Zbarsky et al 2005). Other polyphenols have shown interesting neuroprotective actions in-vivo. *trans*-Resveratrol successfully prevented lipid peroxidation in brain tissue of rat, although it failed to maintain the GSH level in the brain (Gupta et al 2002). Curcumin, another lignan antioxidant, expressed a partial neuroprotective effect against brain damage in a model of Parkinson's disease (Zbarsky et al 2005). Petaslignolide A, a new lignan glycoside from *Petasites japonicus*, demonstrated a remarkable neuroprotective action against KA toxicity in mice (Cui et al 2005).

Since 9-hydroxypinoresinol was more antioxidative than petaslignolide A (corresponding to 9-hydroxypinoresinol glycoside) in-vitro, it is conceivable that 9-hydroxypinoresinol glycoside may be converted to 9-hydroxypinoresinol before exerting its neuroprotective action in-vivo. In support of this, the intraperitoneal administration of 9-hydroxypinoresinol at a dose of 20 mg kg^{-1} for 3 days was no less effective than the oral dose of 9-hydroxypinoresinol glycoside (40 mg kg^{-1}, 4 days) (see Cui et al 2005). The difference in the effective dose of 9-hydroxypinoresinol and petaslignolide A may be caused by the different administration routes. However, the possibility is not excluded that the lower dose of 9-hydroxypinoresinol in comparison with petaslignolide A may be due to its greater antioxidative and non-polar properties, which is favourable for penetration through the brain membrane (Youdim et al 2004). Nonetheless, 9-hydroxypinoresinol glycoside may be directly involved in the neuroprotective action and this needs further study.

9-Hydroxypinoresinol seems to be more efficient than other lignan phenols such as resveratrol, curcumin and naringenin with respect to the effective dose (Gupta et al 2002: Zbarsky et al 2005). Although the mechanism for the neuroprotective action of 9-hydroxypinoresinol is not fully elucidated here, the primary action of 9-hydroxypinoresinol may be related to the removal of reactive oxidants responsible for KA-induced oxidative stress as evidenced by the GSH-sparing activity as well as the protective effect on lipid peroxidation in brain tissue. Further support may be gained from the substantial protection by 9-hydroxypinoresinol against KA-induced loss of GP activity, which was reported to be susceptible to oxidants (Tabatabaie & Floyd 1994). It is also possible that the neuroprotective action of 9-hydroxypinoresinol may implicate the induction of antioxidant enzymes. However, 9-hydroxypinoresinol failed to affect the activity of other antioxidant enzymes such as GR, GST and quinone reductase. Therefore, it is more likely that the primary neuroprotective action of 9-hydroxypinoresinol may be attributed to the direct removal of reactive oxidants responsible for the oxidative stress in brain tissue, rather than the induction of antioxidant enzymes/phase II enzymes. It is also conceivable that the neuroprotective action of the 9-hydroxypinoresinol may be due to other activities such as the restoration of the imbalance of glutamate or GABA content (Olney et al 1974; Coyle & Puttfarcken 1993; Sperk 1994), or the ability to antagonize the binding of KA to receptors as had been observed with butyrofuran lignan (Jang et al 2002). It is not excluded that the action of 9-hydroxypinoresinol may also be partly related to the suppression of pro-inflammatory processes, as suggested by the anti-inflammatory action of some lignan antioxidants (Hwang et al 2003; Tse et al 2005).

The neuroprotective action of 9-hydroxypinoresinol required prior administration at least 24 h before KA injection. Additionally, the neuroprotection was more marked after multiple administrations (20 mg kg^{-1} for 3 days) compared with a single injection (30 mg kg^{-1} for 1 day). These results suggest that 9-hydroxypinoresinol has to undergo metabolism to exert its neuroprotective action. It is possible that 9-hydroxypinoresinol may be oxidatively metabolized by the cytochrome P450 system to demethylated or hydroxylated products, as had been proposed for the metabolism of lignans such as secoisolariciresinol or matairesinol (Niemeyer et al 2003). Alternatively, the accumulation of 9-hydroxypinoresinol or its metabolite in brain tissue may be important for the neuroprotective action, since the effect of 9-hydroxypinoresinol required prior or multiple administrations. Further study of the structure–activity relationships of 9-hydroxypinoresinol metabolites would disclose the significance of metabolic pathways in the neuroprotection in-vivo.

Conclusion

A key finding of the present study was that 9-hydroxypinoresinol at relatively low doses (10–20 mg kg^{-1}) has a remarkable protective action against KA neurotoxicity. It is likely that the primary mechanism for the antioxidant action of 9-hydroxypinoresinol in brain tissue is mainly attributed to the removal of reactive oxidants responsible for oxidative stress in brain tissue, rather than the induction of antioxidant enzymes/phase II metabolic enzymes. Further, it seems that 9-hydroxypinoresinol or its metabolite may accumulate in-vivo after multiple administrations before exerting the neuroprotective action. The results suggest that 9-hydroxypinoresinol may have potential as a neuroprotective agent against oxidative stress-related neurodegenerative disorders.

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