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Neuroprotective Effects of Resveratrol on Cerebral Ischemia-Induced Neuron Loss Mediated by Free Radical Scavenging and Cerebral Blood Flow Elevation

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Resveratrol is a natural phytoestrogen and possesses many biological functions such as anti-inflammatory activity and protection against atherosclerosis and myocardial infraction. The present study was carried out to elucidate the neuroprotective effect and possible mechanism of resveratrol on cerebral ischemia-induced hippocampus neuron loss. Sixty adult male rats underwent general anesthesia (urethane, 1.4 g/kg, i.p.) and were divided into three groups: sham operation, ischemia treatment, and ischemia combined with resveratrol administration (20 mg/kg, i.v.). The carotid artery was bilaterally ligated to induce cerebral ischemia. Microdialysis and high-performance liquid chromatography were used to analyze dihydroxybenzoic acid (DHBA) that reflected the hippocampal hydroxyl radical level. Hippocampal nitric oxide was assayed among different groups. During cerebral ischemia, the hydroxyl radical levels were elevated in rats and animals displayed severe neuronal loss. A single dose of resveratrol significantly increased the nitric oxide level and decreased the hydroxyl radical level. The reduction of cerebral blood flow and neuronal loss were also attenuated by resveratrol treatment. The results demonstrated that a single infusion of resveratrol could elicit neuroprotective effects on cerebral ischemia-induced neuron damage through free radical scavenging and cerebral blood elevation due to NO release.

KEYWORDS: Phytoestrogen; ischemia; free radicals; nitric oxide; neuronal damage

INTRODUCTION

Resveratrol (3,5,4′-trihydroxystilbene) is a polyphenol belonging to the phytoalexin family and is synthesized by the enzyme resveratrol synthase from coumaroyl CoA and malonyl CoA in response to stress, injury, infection, or UV irradiation. It has been found in the seeds of various plant species including grapes and peanuts and constitutes one of the components of red wine (*1*, *2*). Resveratrol possesses many biological functions such as anti-inflammatory activity attributed to inhibition of cyclooxygenase, estrogenic activity, and antiplatelet activity (*3*, *4*). Recently, resveratrol was found to prevent atherosclerosis, lipid peroxidation, and myocardial infarction (*5*−*8*).

Cerebral ischemic stroke is the most common neurodegenerative disease in the aged population. Cortex, corpus striatum, and hippocampus are the most vulnerable areas to injury due to transient global ischemia (*9*). Ischemia-induced neuronal loss is initiated by the release of excitatory neurotransmitters (*10*, *11*), which leads to membrane depolarization, increase in intracellular calcium concentration, and production of nitric oxide (NO) and reactive oxygen species (ROS), that is, superoxide anion radical, hydrogen peroxide and highly cytotoxic byproduct hydroxyl radical (*12*, *13*). NO is synthesized from L-arginine by nitric oxide synthase (NOS) and plays dual roles concerning ischemic injury. There are three isoforms of NOS that are named after the tissue from which they are first cloned. Neuronal NOS (nNOS) and endothelial NOS (eNOS) are constitutively expressed and are calcium dependent. Inducible NOS (iNOS) is expressed after immunologic challenge and neuronal injury and is calcium independent under most circumstance. Resveratrol stimulates the eNOS activity and appears to have protective and vasorelaxing effects (*14*, *15*); however, resveratrol down-regulates the iNOS activity via NF-κB binding activity inhibition and post-transcriptional modification (*16*, *17*).

A growing body of evidence supports the hypothesis that chronic administration of resveratrol could protect a variety of tissues against ischemic injury by reducing the free radical production (*18*−*20*). The aim of the present study was to elucidate the acute effects of resveratrol on cerebral ischemia-

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induced hippocampal neuron loss and the role of NO in neuroprotection effects of resveratrol.

MATERIALS AND METHODS

Animals. Adult male Wistar rats (300−350 g, $n = 60$) were purchased from the Animal Center of the National Taiwan University (Taipei, Taiwan). The animals were housed five in a group at an ambient temperature of 22 ± 1 °C, with a 12-h light/dark cycle. Pelleted rat chow and tap water were available ad libitum. The phytoestrogen levels of rat chows are normal. Adequate anesthesia was maintained to abolish the corneal reflex and pain reflex induced by tail pinching throughout all experiments (∼10 h) after a single intraperitoneal (i.p.) dose of urethane (1.4 g/kg of body weight). All of the procedures were approved by the Animal Care and Use Committee. The care and handling of the animals were in accord with internationally recognized guidelines for ethical animal research. At the end of the experiments, rats were killed with an overdose of pentobarbital (100 mg/kg, i.p.). All efforts were made to minimize the animal numbers, which are required to produce meaningful experimental data. Rats were randomly assigned to three different groups of 60 animals. They received one of the following treatments: sham operation (control), cerebral ischemia induced by bilateral cerebral artery ligation (ischemia), and cerebral ischemia combined with resveratrol administration [20 mg/kg, intravenous (i.v.)] (ischemia + resveratrol).

Cerebral Ischemia Induction. Rats were randomly divided into three groups. One group is the sham operation (control). The second group of animals underwent cerebral ischemia induced by bilateral cerebral artery ligation (ischemia) (*21*). After the general anesthesia, the femoral artery and vein of rats were cannulated with polyethylene tubing (PE 50) for blood pressure and heart rate monitoring and drug administration. Core temperature was monitored and regulated to 37 °C by electric heating pad. The common carotid arteries were then gently exposed, and 3-0 silk ligatures threaded through polyethylene tubing were placed around them. The third group of animals was subjected to ischemia combined with resveratrol administration (20 mg/kg, i.v., provided by R.Y.Y.C.) (ischemia + resveratrol). Resveratrol was dissolved in 20% alcohol and injected intravenously during or immediately after occlusion of both common carotid arteries.

Measurement of Cerebral Blood Flow. Local cerebral blood flow (CBF) in the hippocampus was monitored with a laser Doppler flowmeter (MBF3D, Moor Instrument). The animals were positioned in a stereotaxic apparatus (Stoelting model 51390; Stoelting Co.) to insert a 24-gauge stainless steel needle probe (diameter, 0.5 mm; length, 50 mm) into the hippocampus with the following coordinates: anterior, 4.8 mm; lateral, 1.5 mm; horizontal, 3.0 mm from the top of the skull (*22*).

Evaluation of Neuronal Damage Score. At the end of the experiments, the brain was quickly removed and fixed in 10% neutral buffered formalin. Serial (15 μm) sections through the hippocampus were stained with hematoxylin and eosin. Microscopic evaluations were scored on a scale of 0−3, modified from the grading system of Pulsinelli et al. (*23*), in which 0 is normal, 1 is about one-third of the neurons are damaged, 2 indicates that approximately two-thirds of the neurons are damaged, and 3 indicates that all neurons are damaged. Each hemisphere was evaluated independently without the examiner knowing the experimental conditions.

Measurement of Extracellular Free Radical. A 2 mm burr hole was made in the right parietal bone over the hippocampus for the placement of the microdialysis probes. The dura was removed, and a microdialysis probe with a 4 mm long dialysis membrane was implanted vertically into the right hippocampus with the following coordinates (*22*): anterior, 4.80 mm; lateral, 1.5 mm; horizontal, 3.0 mm from the top of the skull. Hydroxyl radical concentrations were measured by the production of 2,3-dihydroxybenzoic acid (DHBA) and 2,5-DHBA, which resulted from the hydroxylation of sodium salicylate by hydroxyl radicals. The microdialysis probe was perfused with Ringer's solution (0.860 g of NaCl, 0.030 g of KCl, and 0.033 g of $CaCl_2$ per 100 mL), which contains 2 mM sodium salicylate at a constant flow rate of 1.5 μL/min by means of a microinjection pump (CMA 102). Of each dilaysate, 25 μL was injected into a high-performance liquid chromatography (HPLC) system (Bioanalytical System Inc., West Lafayette, IN) equipped with an electrochemical detector and a phase II ODS-3 column (length, 10 cm; diameter, 3.2 mm; particle size, 3 μM). Elution of DHBA was accomplished with a 100 mM monochloroacetic acid buffer with 0.5 mM EDTA and added 1% methanol and 1% tetrahydrofuran.

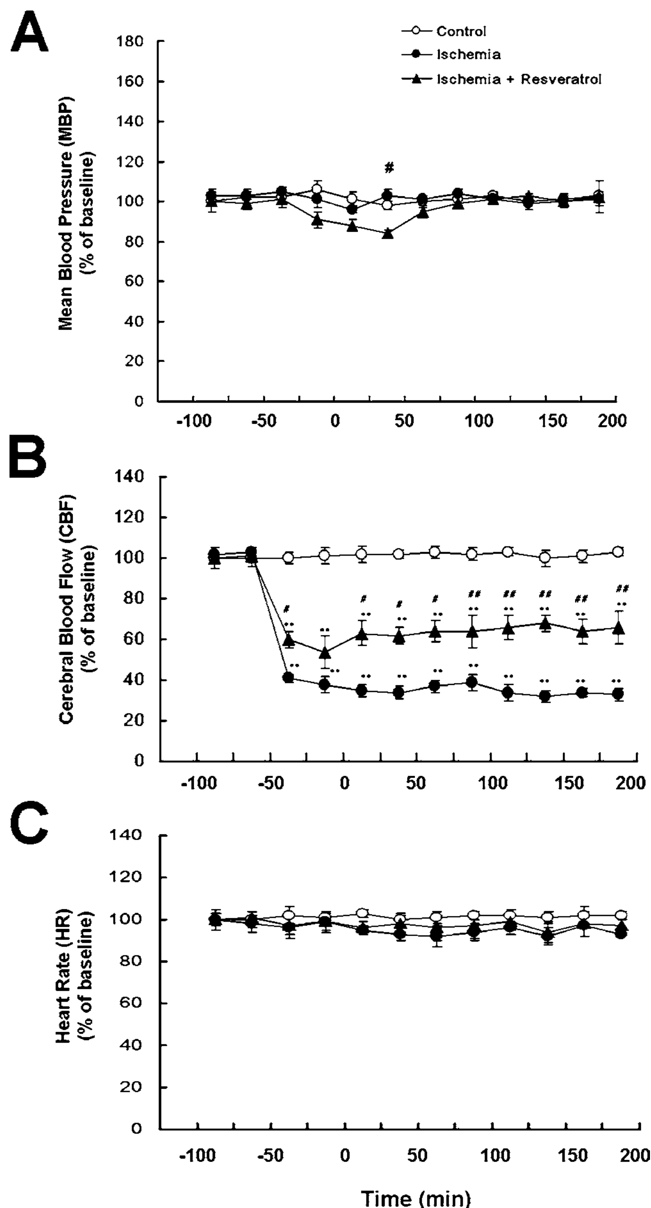

Figure 1. Graphs show time course changes of mean arterial blood pressure, cerebral blood flow, and heart rate in control or rats treated with saline or resveratrol during ischemia: (○) values in controls; (●) values in ischemia animals. Another 12 rats served as resveratrol administration groups (▲). Points represent mean ± SEM values. *, $P < 0.05$, significantly different from control values; **, $P < 0.01$, significantly different from control values; #, $P < 0.05$, significantly different from ischemia values; ##, $P < 0.01$, significantly different from ischemia values.

Detection of Nitric Oxide. Nitrite is the major end-product of nitric oxide, which was undertaken to monitor NO production during ischemia. Nitrite levels in dialysate samples were measured by Griess reaction (*24*−*26*). The samples were mixed with 1% NH_4Cl and reacted with Griess reagent [1 part 1% sulfanilamide and 1 part 0.1% *N*-(naphththylenediamine)] to yield an azo dye after incubation at 40 °C. Subsequently, the sample was analyzed by spectrophotometry for absorbance at 546 nm.

Assay of Superoxide Dismutase (SOD). After the experiments, the brains were quickly removed and dissected into hippocampus. The SOD

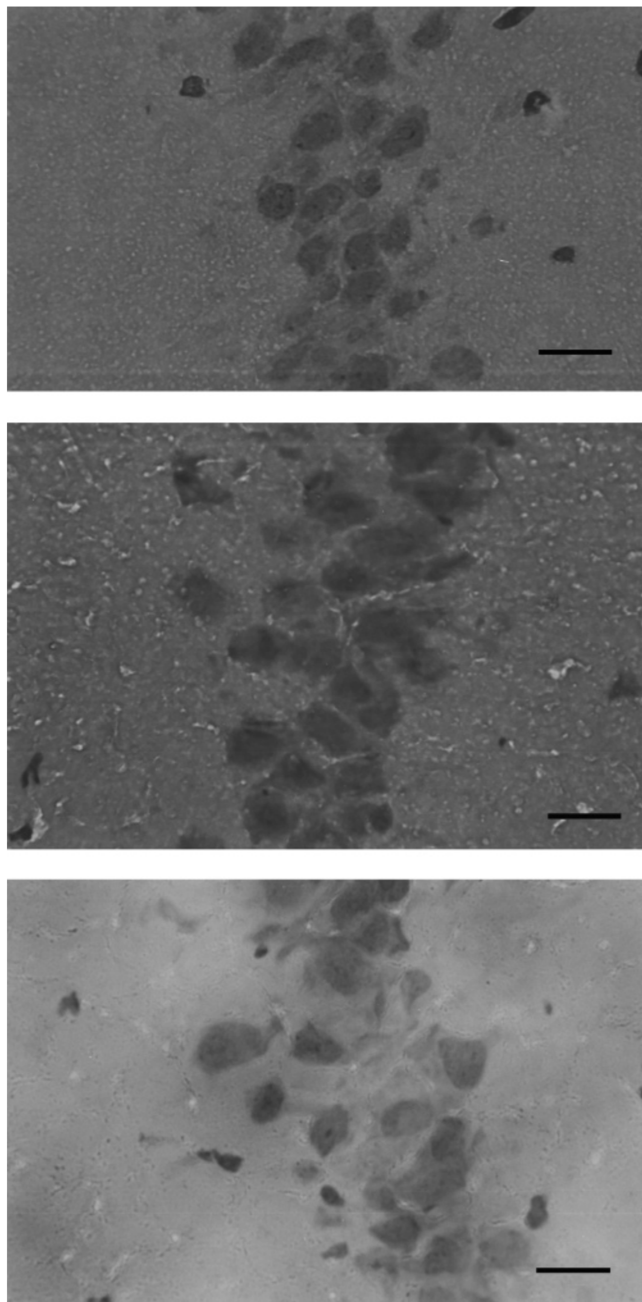

Figure 2. Photomicrographs showing morphology of hippocampal neurons in control rats (top), rats with ischemia (middle), or rats treated with resveratrol [20 mg/kg, IV (bottom)]. As compared to the control group, rats with ischemia showed neuronal swelling and shrinkage, followed by neuronal loss at 6 h after ischemia. Administration of resveratrol significantly attenuated this ischemia-induced neuronal loss.

activity was measured using the method of Misra and Fridovich (*27*). Hippocampus was homogenized in 10 volumes (w/v) of 16.6 mM potassium phosphate buffer, containing 0.033 mM ethylenediamine-tetraacetic acid (EDTA) (pH 7.8). The homogenate was centrifuged again at 50000*g* for 30 min, and 20−40 μL of the homogenate was added to 2 mL of reaction mixture consisting of 5 mM potassium phosphate buffer, 0.033 mM EDTA (pH 7.0), 0.25 mM dianisidine, and 12.5 mM riboflavin (pH7.0). The absorbance of the sample at 460 nm was determined before and after illumination using a 20 W fluorescent tube in a box; the change in absorbance observed in the blank (with 200 μg of bovine serum albumin but no homogenate) was subtracted from each sample. SOD activity was calculated by dividing the change in optical density of the sample by the change in each unit of SOD from the standard curve.

Table 1. Neuronal Damage Scores of Hippocampus from Control Rats, Ischemia Rats, and Ischemia Rats Combined with Resveratrol Administration

group	neuronal damage score[a] (0–3) hippocampus
control groups	0.5 ± 0.3
ischemia groups	2.6 ± 0.5*
ischemia with resveratrol administration group	1.2 ± 0.4†

[a] Values are mean ± SEM of eight rats per group obtained 6 h after the ischemia induction. *, $P < 0.05$, significant difference from control values by ANOVA; †, $P < 0.05$, significant difference from ischemia values by ANOVA.

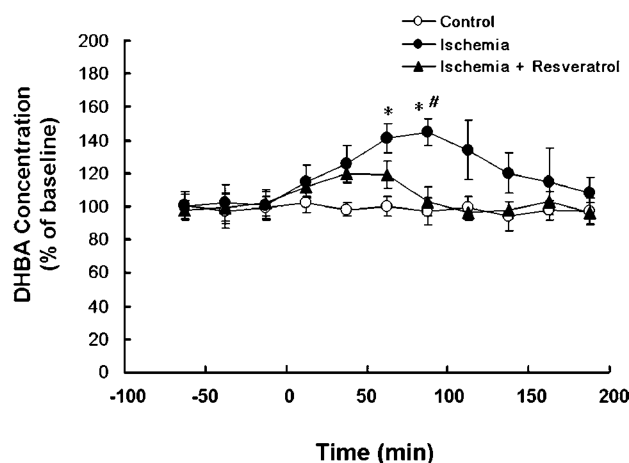

Figure 3. Effects of resveratrol administration on DHBA concentration in the hippocampus: (○) values in controls; (●) values in ischemia animals. Another 12 rats served as resveratrol administration groups (▲). Points represent mean ± SEM values. *, $P < 0.05$, significantly different from control values; #, $P < 0.05$, significantly different from ischemia values.

Statistical Analysis. Data from experiments were expressed as mean ± standard error of the mean (SEM). Statistical analysis was conducted by ANOVA with Student *t* test. A *P* value of <0.05 was considered to be statistically significant.

RESULTS

The first experiment evaluated the effects of ischemia and resveratrol administration on mean arterial blood pressure (MBP), hippocampal blood flow (CBF), and heart rate (HR) in 10 rats. As shown in **Figure 1**, there were no significant differences of mean arterial blood pressure and heart rate among groups. However, the CBF decreased during carotid artery ligation, and administration of a single dose of resveratrol (20 mg/kg, i.v.) could increase the CBF (from 40 to 60%, $P < 0.05$) significantly.

The histological photographs of neuronal damage are shown in **Figure 2**, and the neuronal damage scores are summarized in **Table 1**. Compared with the control rats, rats subjected to ischemia displayed higher neuronal damage scores in the hippocampus. Rats treated with resveratrol had considerably reduced hippocampal neuronal damage scores.

The effect of resveratrol administration on extracellular DHBA concentrations in hippocampus is shown in **Figure 3**. The DHBA value is the summation of 2,3-DHBA and 2,5-DHBA, which could reflect the levels of free radicals. It was evidenced that DHBA increased ∼1.5-fold during ischemia and

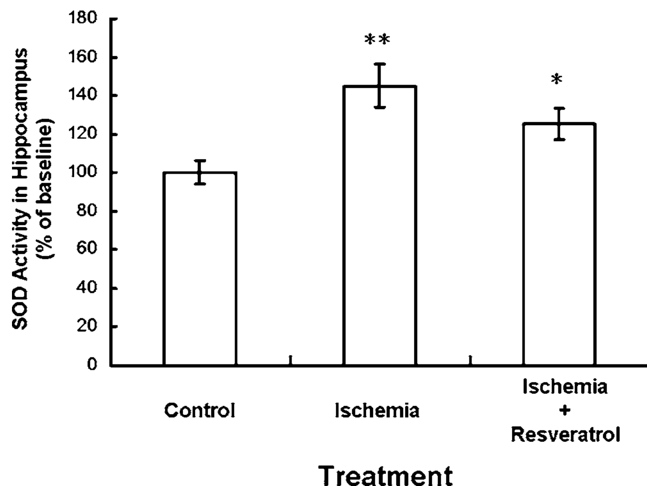

Figure 4. Effects of ischemia and resveratrol administration on the SOD activity in the tissue homogenates of the hippocampus. Bars represent mean ± SEM values ($n = 12$). **, $P < 0.01$, significantly different from control values; ANOVA.

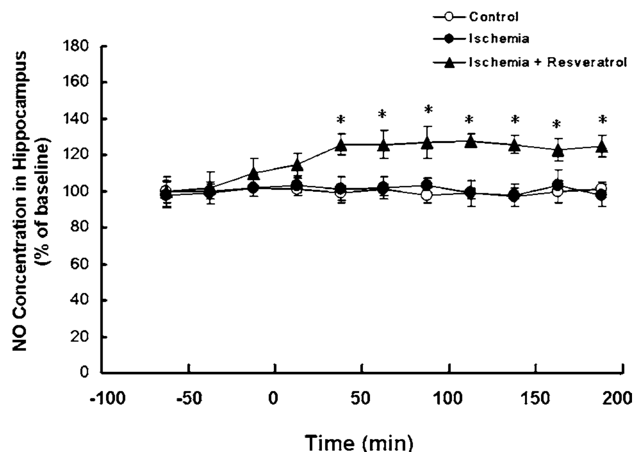

Figure 5. Effects of resveratrol administration on NO concentration in the hippocampus: (○) values in controls; (●) values in ischemia animals. Another 12 rats served as resveratrol administration groups (▲). Points represent mean ± SEM values. *, $P < 0.05$, significantly different from control values; #, $P < 0.05$, significantly different from ischemia values.

that resveratrol administration could significantly attenuate the DHBA overloading.

SOD plays an important role in free radical scavenging. To elucidate the effects of resveratrol on SOD, SOD activities among different groups were measured and are shown in **Figure 4**. Common carotid artery ligation increased the hippocampal SOD activity (150% of baseline), and resveratrol administration reduced hippocampal SOD activity (130% of baseline).

NO is a potent vasodilator; the effect of CBF elevation elicited by resveratrol may be mediated by NO production. To evaluate the neuroprotective effect of resveratrol on NO production, we continuously collected and measured NO production among groups. In this study, consecutive detection of nitrite, a major end-product of NO, was undertaken to measure NO production. In the ischemia group, there was no significant increase in nitrite production compared to the sham operation group. Resveratrol administration significantly increased the nitrite production in the hippocampus (**Figure 5**).

DISCUSSION

In the present study, we provide evidence that a single administration of resveratrol (20 mg/kg, i.v.) attenuated the ischemia-induced neuronal cell loss through scavenging free radicals and CBF elevation, which is mediated by NO production. We suggested that resveratrol may elicit neuroprotective effects by stimulating NO formation or release, which increases cerebral blood flow and prevents peroxynitrite formation during ischemia.

Resveratrol has been proved to be a major constituent responsible for cardiovascular benefits associated with moderate wine consumption (*15*, *28*). Several studies attributed this beneficial effect of resveratrol to its potent antioxidant activity (*18*, *19*). Direct neuroprotective effects of resveratrol against oxidative stress have been studied in the PC12 cells and animals (*29*, *30*). Chronic resveratrol treatment significantly decreased the infarct volume by increasing the neuronal endogenous defensive ability to combat ischemia-induced oxidative stress (*18*). Neurons are vulnerable to the reactive oxygen species generated by ischemia−reperfusion (*31*−*33*). Reactive oxygen species produced by transient oxygen−glucose deprivation have been shown to cause mitochondrial membrane depolarization and permeability transition. A number of studies have demonstrated the antioxidant properties of resveratrol, for example, its ability to protect against oxidative DNA damage in stroke-prone hypertensive rats (*34*), to suppress lipid peroxidation (*35*), and to inhibit cerebral mitochondrial ROS production (*36*).

The hydroxylation of sodium salicylate by hydroxyl radicals leads to DHBA, which could be detected by HPLC and used to reflect the level of hydroxyl radicals. In the present study, we observed that the DHBA level significantly increased after ischemia and peaked at 1.5 h and that resveratrol administration significantly reduced the hippocampal DHBA. Two possible mechanisms may account for the results. Resveratrol has been found to possess free radical scavenging properties (*31*). The first mechanism concerns the ability of resveratrol to serve as a free radical scavenger to protect against oxidative stress. Thus, it could directly scavenge the free radical during cerebral ischemia. The second mechanism concerns the ability of resveratrol to inhibit cerebral mitochondrial ROS production (*36*). Thus, it could suppress the formation of the free radical during cerebral ischemia. It is evidenced that the glutamergic mechanism may be a trigger for the hydroxyl radical production during ischemia (*37*). The hippocampus contains the most abundant glutamergic neurons in the brain, and this could explain the present results. Superoxide radicals are produced by glutamate *N*-methyl-D-aspartate (NMDA) receptor activation in cultured cerebellar granular cells (*37*, *38*). In vivo studies also demonstrated that local administration of glutamate or NMDA is associated with the hydroxyl radical formation in the brain (*39*, *40*). During ischemia, the NMDA receptor activation induced intracellular calcium content elevated and oxygen free radical could generate through arachidonic acid release (*41*), which is evidence that arachidonic acid is a major contributor to free radical formation (*42*). It is also reported that NMDA activation is associated with NO generation (*43*) and that NO also provides an additional linkage between excitatory amino acid activity and free radical generation, which supports the assumption that hydroxyl radical generation induced by NMDA administration is blocked by nitric oxide synthase inhibition (*40*).

NO plays dual roles in the ischemia-induced neuronal death. NO is a free radical and also form peroxynitrites, a potent oxidant that can potentially cause membrane lipid peroxidation leading to neuronal death or myocardial dysfunction (*44*−*47*). However, NO is a potent vasodilator, which could be neurprotective against cerebral ischemia through vasodilation and significantly increase the blood flow in the penumbra area of

ischemia (*48*). We found that resveratrol administration significantly increased the hippocampal NO production, which led to cerebral blood flow elevation and protected animals from ischemia-induced neuron loss. The effect of resveratrol on NOS is controversial. It is reported that resveratrol suppressed the iNOS activity in macrophage (*17*, *49*) and NO release, which induce leukemic B cells apoptosis (*50*). However, Hattori et al. suggested that the antioxidant property of resveratrol may be explained by its ability to enhance iNOS expression (*51*). In addition, resveratrol is also demonstrated to stimulate the eNOS activity in artery and elicit the cardioprotective or neuroprotective effects. The effects of resveratrol on NOS activity need to be further investigated. The present study showed that resveratrol administration scavenged free radicals to prevent the peroxynitrite formation, which resulted from interaction between NO and superoxide anion.

From this study, we could conclude that resveratrol administration induced NO production, which led to cerebral vasodilation and CBF elevation, attenuated the free radical level during ischemia, and significantly protected animals from free radical overloading-induced neuronal death.

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