Resveratrol neuroprotective effects during focal cerebral ischemia injury via nitric oxide mechanism in rats

Sheng-Kou Tsai, MD, PhD,a,b Li-Man Hung, PhD,c Yuan-Tsung Fu, MD,d Henrich Cheng, MD, PhD,e Mao-Wei Nien, Master,a,b Hsin-Yi Liu, BS,f Friedrich Bo-Yuan Zhang, BS,f and Shiang-Suo Huang, PhD,f Taipei, Tao-Yuan, and Taichung, Taiwan

Background: Our prior study showed that resveratrol could suppress infarct volume and exert neuroprotective effect on rats subjected to focal cerebral ischemia (FCI) injury. Recently, it has been reported in some literature that resveratrol protects the spinal cord, kidney, and heart from ischemia-reperfusion injury through upregulation of nitric oxide (NO). Therefore, this study was designed to investigate the role of nitric oxide on the neuroprotective mechanisms of resveratrol on rats after FCI injury.

Methods: The FCI injury was induced by the middle cerebral artery (MCA) occlusion for 1 hour and then a 24-hour reperfusion followed in the anesthetized Long-Evans rats. Resveratrol was intravenously injected after 1 hour MCA occlusion.

Results: Treatment of resveratrol (0.1 and 1 µg/kg) decreased the lactate dehydrogenase (LDH) in plasma and malondialdehyde (MDA) in FCI injury brain tissue, whereas the level of NO in plasma was increased. In addition, resveratrol downregulated protein and mRNA expression of inducible nitric oxide synthase (iNOS), and upregulated protein and mRNA expression of endothelial nitric oxide synthase (eNOS), while the expression of protein and mRNA of neuronal nitric oxide synthase (nNOS) was unchanged. Pretreatment with N^o^-nitro-L-arginine methyl ester (L-NAME, the nonselective NOS inhibitor) or L-N^o^-5-(1-iminoethyl)-ornithine (L-NIO, the eNOS selective inhibitor) completely blocked the effect of resveratrol in decreasing infarction volumes.

Conclusions: This study demonstrated the important role of NO in the neuroprotective effect of resveratrol in FCI injury.


Clinical Relevance: Ischemic brain damage is the major cause of permanent disability in young adults. It has been suggested that up to 80% of all strokes result from ischemic damage in the middle cerebral artery area. The potential for clinical application of pharmacological agents has generated enormous interest in identifying the underlying intracellular signaling pathways and to develop therapeutic strategies that can benefit ischemic stroke injury in patients. This study explored the possible involvement of nitric oxide in neuroprotective effect of resveratrol on focal cerebral ischemia injury.

Nitric oxide (NO), a cellular signaling molecule, is synthesized during the stoichiometric conversion of L-arginine to L-citrulline in the presence of oxygen and nicotinamide adenine dinucleotide phosphate (NADPH), which is catalyzed by nitric oxide synthase (NOS). Three isoforms of NOS that were named by the tissue are first cloned. Neuronal NOS (nNOS; 157 kDa) and endothelial NOS (eNOS; 140 kDa) are constitutively expressed and calcium-dependent, whereas inducible NOS (iNOS; 135 kDa) is expressed after immunologic challenge and neuronal injury and is calcium-dependent under most circumstances. NO has a variety of physiological and pathological effects in biological systems. Under normal physiological conditions, NO is an important endogenous vasodilator that can regulate cerebral blood flow and mediate vascular response to a diverse group of stimulations. In addition, NO can inhibit platelet aggregation and inhibit both platelet and polymorphonuclear neutrophil (PMN) adhesion to the vascular endothelium. Several lines of evidence have reported that NO donor or L-arginine exerts neuroprotective effect in stroke models. The findings indicate that NO donor, sodium nitroprusside, improves blood flow and reduces tissue damage after focal cerebral ischemia. In both normotensive and hypertensive animals, L-arginine induces sustained cerebral blood flow increases in normal brain as well as in a marginally perfused brain region distal to middle cerebral artery (MCA) occlusion and decreases infarction volume after MCA occlusion. Resveratrol, a polyphenol phytoalexin (trans 3,4',5'-trihydroxystilbene), is abundantly available in a wide variety of plants.
of plant species. It is present in the skin and seeds of grapes, and constitutes one of the major components of red wine. Resveratrol has been reported to have a host of physiological effects, including the prevention of lipid peroxidation in human LDL, inhibition of arachidonic acid metabolism, and platelet activity. Resveratrol was also found to stimulate nitric oxide (NO) production in endothelial cells to exert vasodilatory effect on blood vessels and to interfere with the release of inflammatory mediators by activated human polymorphonuclear leukocytes (PMN), and down-regulate adhesion-dependent thrombogenic PMN functions. Resveratrol is considered as a potential neuroprotective agent in treating acute scenarios of PMN functions. Recently, resveratrol was found to protect spinal cord, kidneys, and heart from ischemia-reperfusion injury through upregulation of NO.

On the basis of these findings, the purpose of this experimental study was to determine the role of NO in the neuroprotective effect of resveratrol on rats subjected to FCI injury.

**MATERIALS AND METHODS**

**Animals.** The present investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health (NIH publication No. 85-23, revised 1996). Male Long-Evans rats (National Laboratory Animal Breeding and Research Center) weighing 250 to 320 g were used throughout this study. These animals were housed in a room with controlled temperature (24 ± 1°C) and humidity (55 ± 5%) under a 12:12 h light-dark cycle. They were allowed free access to food and water.

**Surgical procedure.** Each rat was anesthetized with halothane (1% to 3.5% in a mixture of 70% N2O and 30% O2) with the use of a mask. Body temperature was maintained during surgery at 37 ± 0.5°C with a heating pad servo-controlled by a rectal probe. The right femoral artery was cannulated with PE-50 polyethylene catheters for continuous monitoring of heart rate and mean arterial blood pressure (MABP) by Statham P23 XL transducer and displayed on a Gould RS-3400 physiological recorder (Gould, Cleveland, Ohio), and blood sampling for analysis of blood gases by a blood gas analyzer (GEM-5300 Instrumentation Laboratory Company, Lexington, Mass). In addition, the right femoral vein was cannulated for drug administration.

Focal ischemic infarcts were made in the right lateral cerebral cortex in the territory of the MCA. Both common carotid arteries were exposed by midline anterior cervical incision. The animal was placed in a lateral position, and a skin incision was made at the midpoint between the right lateral canthus and the anterior pinna. The temporal muscle was retracted, and a small (3-mm diameter) craniectomy was made at the junction of the zygoma and squamosal bone using a drill (Dremel Multipro +5395, Dremel, Racine, Wis) cooled with saline solution. Using a dissecting microscope (OPMI-1, ZEISS, Carl Zeiss, Germany), the dura was opened with fine forceps, and the right MCA was ligated with 10-0 monofilament nylon ties. Both common carotid arteries were then occluded by microaneurysm clips for 1 hour. After removing the clips, return of flow was visualized in the both common carotid arteries. However, the right MCA ligation remained in place permanently.

**Infarct volume analysis.** Twenty-four hours after cerebral infarction, animals were anesthetized and killed by rapid decapitation. Brain were removed, inspected visually for the anatomy of the MCA and for signs of hemorrhage or infection, immersed in cold saline solution for 10 minutes, and sectioned into standard coronal slices (each 2-mm thick) using a brain matrix slicer (Jacobowitz Systems, Zivc–Miller Laboratories Inc, Allison Park, Pa). Slices were placed in the vital dye 2,3,5-triphenyltetrazolium chloride (TTC, 2%; Sigma, Sigma-Aldrich, St. Louis, Mo) at 37°C in the dark for 30 minutes, followed by 10% formalin at room temperature overnight. The outline of right and left cerebral hemispheres as well as that of infarct tissue, which was clearly visualized by a lack of TTC staining, was outlined on the posterior surface of each slice using an image analyzer (color image scanner, EPSON GT-9000, connected to an image analysis system (AIS software, Imaging Research Inc, Brock University, Ontario, Canada) run on a personal computer, AMD K6-2 3D 400. Infarct volume was calculated as the sum of infarct area per slice multiplied by slice thickness. Both the surgeon and image analyzer operator were blinded to the treatment given each animal.

**Plasma lactate dehydrogenase (LDH) and NO analysis.** Cellular damage was evaluated by measuring the LDH in plasma. Samples of arterial blood were drawn from the femoral catheter at the end after 1 hour MCA occlusion and 24 hour reperfusion, collected in heparinized tubes. The blood was kept at 4°C until it was centrifuged at 2000 × g for 15 min. The plasma was recovered and aliquots were used for determination of LDH activity. LDH activity was measured spectrophotometrically, according to the method of Bergmeyer and Brent, by following the rate of conversion of NADH to NAD+, at 340 nm.

The deproteinized plasma samples were frozen and kept until analysis. For measurement of NO, we employed the NO/ozone chemiluminescence technique (NOA 280, Sievers Instruments, Boulder, Colo). The detection of plasma NO level is based on its reaction with ozone, which leads to the emission of red light. The photons from this reaction are detected and transformed to an electrical signal by a photomultiplier tube (PMT). Due to the use of filters in front of the PMT, NO/O3 chemiluminescence recorded with the Sievers NOA 280 is highly specific for NO. The current from the PMT is A/D converted and fed into a PC running the Asyst software (Sievers NO Analysis Liquid Program). The amount of light produced by NO/O3 chemiluminescence is proportional to the amount of NO.
sampled. Hence, the calculated area under the curve of the PMT current for each determination is proportional to the amount of NO. This was verified before each experiment by standard curves (1, 5, 10, 20, 40, 100 μmol/L), which were produced using freshly prepared solutions of sodium nitrite in distilled water, which was reduced to NO in an equimolar manner by the reducing agent. We chose to measure the level of nitrite or nitrate on blood sample by using a reaction vessel containing a reducing system (Vanadium [III] dissolved in 1 M HCl), which was sampled and NO was generated from nitrite or nitrate in an equimolar manner. A continuous stream of helium (99.999%) purged the resultant NO from the reaction vessel to the chemiluminescence chamber.

**Western blot analysis.** Rats were perfused with saline, and the brains were prepared for Western blot analysis. Brains tissue was homogenized in Laemmli lysis buffer containing protease inhibitors (10 μg/mL/0.2 g tissue weight, Sigma, St. Louis, Mo). Protein concentrations in each sample were determined using a protein assay kit (BCA kit; Pierce, Rockford, Ill), and the samples were stored at −80°C until use. Aliquots containing 120 μg of protein were subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membrane (IPVH00010; Millipore Corp, Bedford, Mass). Western blot analysis of NOS protein was performed as previously described.

**Reverse transcription polymerase chain reaction (RT-PCR).** nNOS, eNOS, and iNOS mRNA were detected in the occluded zone of the brain by RT-PCR, as previously described. Total RNA was extracted from the brain tissue with RNase Maxi kits (Qiagen, Valencia, Calif). First strand cDNA synthesis was then performed with the use of 5 μg of total RNA, oligo (dT) primer (BRL), and Superscript III Reverse Transcriptase (Invitrogen, Carlsbad, Calif) according to manufacturer’s instructions. RT-PCR was carried out in O’ in 1DNA polymerase solution at 50°C for 60 minutes followed by enzyme inactivation at 72°C for 15 minutes. The primer sequences are shown in Table 1. The amplification procedure consisted of initial denaturation at 95°C for 5 minutes, following cycle parameters of denaturation at 95°C for 1 minute, annealing at 60°C for 1 minute, and extension at 72°C for 1 minute for 35 cycles. The amplified products were separated gel electrophoresis in 1.5% agarose gel containing 0.5 mg/ml ethidium bromide. Each set of PCRs included control samples run without RNA or in which the RT step was omitted. The RT-PCR procedure was highly reproducible under the present experimental conditions.

**Malondialdehyde assay.** Malondialdehyde (MDA) levels were measured by using thiobarbituric acid method described by Okhawa. Briefly, brain tissues were homogenized with 10 times (w/v) sodium phosphate buffer. The reagents of 1.5 ml 20% of acetic acid, 0.2 ml 8.1% of sodium dodecyl sulfate, 1.5 ml 0.8% of thiobarbituric acid was added to 0.1 ml tissue sample. The mixture was then heated at 100°C for 60 minutes. After cooling, 1.0 ml distilled water and 5.0 ml of the mixture of n-butanol and pyridine was added and centrifuged. The organic layer was withdrawn and measured at 532 nm. MDA concentrations in the samples were calculated by a standard calibration curve by using 1,1,3,3-tetramethoxypropane prepared in the same manner. Each measurement was performed in duplicate. MDA concentration was expressed as nanomoles per gram tissue weight.

**Drug administration.** Resveratrol (Sigma) was dissolved in 40% (v/v) propylene glycol to the desired concentrations in normal saline. Final concentration of propylene glycol in the resveratrol solution was 4 × 10⁻³% (v/v). At this concentration, propylene glycol had no effect on the infarct size of focal cerebral ischemia. In this study, resveratrol solution of 0.3 ml was administered at three

### Table I. Primer sequences of NOS isoforms and β-actin

<table>
<thead>
<tr>
<th>Primer sequence</th>
<th>Primer sequence</th>
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<tr>
<td>nNOS forward primer</td>
<td>5’-TTCCGAAAGCTTTCTGCGACACGCAATTT-3’</td>
</tr>
<tr>
<td>nNOS reverse primer</td>
<td>5’-AGATCTAAGGCGGTTGGTACCTTC-3’</td>
</tr>
<tr>
<td>iNOS forward primer</td>
<td>5’-TCAGCAGCCTTTCCACCACAA-3’</td>
</tr>
<tr>
<td>iNOS reverse primer</td>
<td>5’-CCATCCTCTCGACACCTTTCCCT-3’</td>
</tr>
<tr>
<td>eNOS forward primer</td>
<td>5’-TGCCAGCATCACCTACGA-3’</td>
</tr>
<tr>
<td>eNOS reverse primer</td>
<td>5’-CCCGGAGCTCAATACCCT-3’</td>
</tr>
<tr>
<td>β-actin forward primer</td>
<td>5’-TGCCAGGAAGGAGGATCTCG-3’</td>
</tr>
<tr>
<td>β-actin reverse primer</td>
<td>5’-GCCGATAGTGATGACCTGACG-3’</td>
</tr>
</tbody>
</table>

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different doses ($10^{-6}$, $10^{-7}$, and $10^{-8}$ g/kg) via femoral vein when the common carotid arteries clips were removed. Rats injected with 0.3 ml vehicle, $4 \times 10^{-3}$/% (v/v) propylene glycol in normal saline, were used as controls. Sham operated animals underwent all surgical procedure except MCA ligation. For examining which NOS was responsible for the neuroprotective effect of resveratrol in FCI injury, we administrated a nonselective NOS inhibitor (L-NAME, 1 mg/kg), a selective nNOS inhibitor (7-nitroindazole, 50 mg/kg), a specific iNOS inhibitor (S-methylisothiourea sulfate, 3 mg/kg), and a selective eNOS inhibitor (L-NIO, 10 mg/kg) 15 minutes before MCA occlusion to examine their antagonistic effect with resveratrol. Animals were randomly allocated to each drug treatment and control groups.

**Statistics.** Data are expressed as mean ± standard error of mean (SEM). Statistical analysis of differences in plasma LDH and NO levels, physiological measurements, infarct volume between vehicle and drug treated groups were carried out by one-way analysis of variance (ANOVA) for combined data and followed by unpaired, two-tailed Student $t$ test. $P < .05$ was considered to be statistically significant.

**RESULTS**

**Plasma LDH levels.** Cellular damage was evaluated by measuring the LDH level in plasma at the end period of focal cerebral ischemia. The effects of resveratrol on LDH activity are shown in Table II. Low LDH activity was seen in sham-operated animals (91.5 ± 28.7 U/L) (n = 7) before occlusion. In the operated animals without resveratrol treatment, large increase in the LDH activity was observed in the plasma (267.7 ± 36.3 U/L) (n = 7). In contrast, administration of resveratrol attenuated LDH release during 1 hour MCA occlusion and 24 hour reperfusion. Resveratrol at the dose of 1 mg/kg reduced the LDH activity to 105.4 ± 23.8 U/L (n = 7). The effect of (259.8 ± 41.6 mm$^3$ [n = 7]) and L-NIO (233.8 ± 28.5 mm$^3$ [n = 7]), but had no influence when pretreatment with 7-nitroindazole (138.0 ± 29.7 mm$^3$ [n = 7]) or S-methylisothiourea sulfate (160.2 ± 31.2 mm$^3$ [n = 9]).

**Table II.** LDH (U/L) and NO ($\mu$mol/L) release in the plasma during focal cerebral ischemia

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>LDH</th>
<th>NO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>7</td>
<td>91.5 ± 28.7</td>
<td>7.4 ± 1.4</td>
</tr>
<tr>
<td>Vehicle</td>
<td>12</td>
<td>267.7 ± 36.3</td>
<td>12.3 ± 1.2</td>
</tr>
<tr>
<td>Resveratol 0.01 µg/kg</td>
<td>7</td>
<td>188.6 ± 23.5</td>
<td>13.9 ± 1.8</td>
</tr>
<tr>
<td>0.1 µg/kg</td>
<td>7</td>
<td>99.2 ± 19.4*</td>
<td>20.3 ± 1.6*</td>
</tr>
<tr>
<td>1 µg/kg</td>
<td>10</td>
<td>105.4 ± 29.3*</td>
<td>25.7 ± 4.3*</td>
</tr>
<tr>
<td>L-NAME 1 mg/kg + resveratol 1 µg/kg</td>
<td>9</td>
<td>259.8 ± 41.6</td>
<td>11.2 ± 0.8</td>
</tr>
<tr>
<td>7-nitroindazole 50 mg/kg + resveratol 1 µg/kg</td>
<td>11</td>
<td>138.0 ± 29.7*</td>
<td>14.0 ± 1.9</td>
</tr>
<tr>
<td>S-methylisothiourea 3 mg/kg + resveratol 1 µg/kg</td>
<td>9</td>
<td>160.2 ± 31.2*</td>
<td>16.2 ± 3.0</td>
</tr>
<tr>
<td>L-NIO 10 mg/kg + resveratrol 1 µg/kg</td>
<td>9</td>
<td>233.8 ± 28.5</td>
<td>14.8 ± 1.4</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SEM of seven rats. Vehicle is 4×10$^{-3}$/% (v/v) propylene glycol in normal saline. NO release was measured by the presence of nitrite and nitrate in the plasma.

* $P < .05$ compared with vehicle group.

**Plasma NO.** The effects of resveratrol on decreased LDH release was blocked by pretreatment with L-NAME NO contents are shown in Table II. NO release was measured by the presence of nitrite ($NO_2^-$) and nitrate ($NO_3^-$) in plasma. The NO content in sham-operated rats was 7.35 ± 1.41 µmol/L (n = 7). In the operated animals without resveratrol treatment, the NO content in plasma of rats was 12.26 ± 1.21 µmol/L (n = 7). Administration of resveratrol increases the NO release in a dose-dependent manner during 1 hour MCA occlusion and 24 hour reperfusion. Resveratrol at the dose of 1 µg/kg, the plasma NO was increased to 25.70 ± 4.28 µmol/L (n = 7). However, L-NAME, 7-nitroindazole, S-methylisothiourea sulfate, and L-NIO decrease the effect of resveratrol on upregulation of NO release.

**Protein levels of NOS.** It was noticed that the levels of iNOS, eNOS, and nNOS proteins in either nonoccluded zone or occluded zone of sham-operated rats were similar. As shown in Fig 1, the density of NOS protein expression was normalized with β-actin from the same samples. Focal cerebral ischemia induced iNOS expression and administration resveratrol at the dose of 1 µg/kg significantly suppressed iNOS expression in the occluded zone. In contrast, administration 1 µg/kg resveratrol significantly increased the eNOS expression in the occluded zone compared with vehicle-treated group. The protein level of nNOS expression was not significantly different with or without resveratrol treatment after focal cerebral ischemia between nonoccluded and occluded brain tissue. Fig 1 is representative of seven different experiments.

**NOS mRNA expression.** Resveratrol at the dose of 1 µg/kg reduced the iNOS mRNA signal in occluded zone compared with the vehicle-treated group. However, administration of 1 µg/kg resveratrol increased eNOS mRNA expression in occluded zone compared with the vehicle-treated group. There was no influence on nNOS mRNA expression in non-occluded zone and occluded zone between resveratrol-treated group and vehicle-treated group (Fig. 2). Fig 2 is representative of seven different experiments.
MDA levels. MDA levels in brain tissue after 1 hour of MCA occlusion and 24-hour reperfusion are shown in Fig. 3. Focal cerebral ischemia resulted in significantly increased levels of MDA in the group treated with vehicle (394.0 ± 38.6 nmol/g wet tissue) compared with sham group (127.8 ± 26.8 nmol/g wet tissue). MDA levels in the resveratrol treated groups were decreased in a dose-dependent manner. Resveratrol at the dose of 1 μg/kg significantly reduced the MDA level to 157.3 ± 31.3 nmol/g wet tissue. Each group is seven rats.
The effects of resveratrol on infarct volume are shown in Fig. 4. The middle cerebral artery was occluded for 1 hour and then reperfused for 24 hours; the infarct volumes in the rats were 150.9 ± 15.1 mm³ (n = 12). If 1 μg/kg resveratrol was treated immediately after release of the common carotid arteries clips, the infarct volume in rats significantly reduced to 42.8 ± 11.8 mm³ (n = 10). The effect of resveratrol on decreased infarct volume was completely blocked by pretreatment with L-NAME (147.8 ± 31.8 mm³ [n = 9]) and L-NIO (110.5 ± 21.1 mm³ [n = 9]), but had no influence when pretreatment with 7-nitroindazole (58.6 ± 16.1 mm³ [n = 11]) or S-methylisothiourea sulfite (71.9 ± 17.0 mm³ [n = 9]).

**DISCUSSION**

In this study, we demonstrated that treatment with resveratrol, at the doses of 0.1 and 1 μg/kg, significantly decreased LDH levels in the femoral blood on rats after FCI injury. The observation in Table I showed that the resveratrol at the dose of 0.1 μg/kg seemed more effective than 1 μg/kg, but the difference did not reach statistically significant level. The LDH activity in plasma was an indicator of cellular damage. The decreasing of LDH activity after resveratrol administration suggested that resveratrol might reduce the neuronal damages elicited after FCI injury. This result was consistent with the finding of our previously study, which showed that resveratrol suppressed the total infarct volume in rats subjected to FCI injury.16 The result indicated that resveratrol possessed robust neuroprotective effect against FCI in rats.

In addition, our results revealed that the NO contents in plasma were significantly increased with a dose-dependent manner in resveratrol-treated group compared with vehicle-treated group in rats after FCI injury. NO is a small, gaseous, biologically active messenger with a wide range of physiological and pathological actions.5 NO possesses vasodilator effect,6 anti-inflammatory activity,7 and anti-platelet activity.8,9 All these physiological effects of NO seem to have a beneficial role during FCI injury. However, it has been reported that the interaction between NO and superoxide radicals generates peroxynitrite, a potent free radical that could induce lipid peroxidation of cellular membranes.3 Current studies indicate that NO plays a dual role, and may have protective or toxic effect on brain tissue under FCI injury, depending on its source. NO is produced by a family of isoenzymes termed of NOS.4 The eNOS and nNOS are present under physiological conditions, whereas the iNOS is expressed in response to immunological stimulation. Among the three isoforms of NOS, eNOS produces NO with beneficial effects, such as vasodilative effect, anti-platelet aggregation effect, and inhibit PMN adhesion, whereas nNOS and iNOS produce NO with deleterious effects. Overproduction of NO from either nNOS or iNOS leads to neurodegeneration.29 This receives further support from the evidence of the reduction of brain edema and infarction volume in nNOS or iNOS knockout mice after FCI injury.30,31 In this study, we found that iNOS protein was induced in the rat brain tissue after FCI injury. Treatment with resveratrol suppressed iNOS protein expression in the same condition. Whereas, treated with resveratrol significantly increased eNOS protein expression in brain tissue after FCI injury compared with vehicle-treated group in rats. But there was no significant difference in nNOS protein expression between resveratrol-treated group and vehicle-treated group. Further study showed that a similar result on mRNA expression. Resveratrol downregulated iNOS mRNA expression, upregulated eNOS mRNA expression, and had no influence on nNOS mRNA expression. These results suggest that the effects of resveratrol on iNOS and eNOS expression were through transcription. Although the iNOS produces more NO compared with eNOS, we found that the NO content in plasma was significantly increased in resveratrol-treated group in this study. It may due to the delayed appearance of iNOS in cerebral ischemic injury. Unlike eNOS and nNOS, iNOS is not a constitutive enzyme, and is produced after the cytokine stimulation of neutrophils resulting from ischemia. Messenger RNA of iNOS is detectable 12 hours after ischemia.32 Resveratrol is a naturally polyphenolic compound that possesses antioxidant properties. Indeed, we found that administration of resveratrol decreased MDA, a lipid peroxidation product is considered a presumptive marker for oxidative stress, in a dose-dependent manner in brain tissue after FCI injury. It is suggested that resveratrol reduced the oxidative stress on rats due to FCI injury. A number of studies have demonstrated the role of free radicals on the injury of FCI.32 The antioxidant activity of resveratrol may
act through suppressed superoxide radicals to prevent the injury from the interaction of superoxide anion and NO in dose-dependent manner. NO is known as a vasodilator, inhibits platelet aggregation, and adds platelet and PMN to the vascular endothelium, which are beneficial for the improvement of FCI injury.

To investigate which NOS was responsible for the neuroprotective effect of resveratrol in rats after FCI injury, we administrated a nonselective NOS inhibitor (L-NAME), a selective nNOS inhibitor (7-nitroindazole), a specific iNOS inhibitor (S-methylisothiourea sulfate), and a selective eNOS inhibitor (L-NIO), 15 minutes prior to MCA occlusion to examine their antagonistic effects with resveratrol. Whether 7-nitroindazole inhibit both nNOS and eNOS have generated considerable debate, the IC50 values for inhibition of nNOS and eNOS are similar in vitro. But 7-nitroindazole does not influence the endothelium-dependent relaxation of blood vessels in vivo. Coert reported that 7-nitroindazole in a dose range from 0.1 to 100 mg/kg inhibited nNOS without alterations in MABP. Thus, it is considered that 7-nitroindazole does not affect eNOS activity in vivo. Based on this finding, many investigators use 7-nitroindazole as a nNOS inhibitor. In this study, the dose of 7-nitroindazole was 50 mg/kg, which is considering as a selective nNOS inhibitor. On the other hand, Wolff reported that L-NIO was the most potent in inhibiting the eNOS with an IC50 value of 0.08 µM, a value approximately four-fold lower than that observed for the iNOS and nNOS. L-NIO reduces resting cyclic GMP levels and constricts pial arteries, both consistent with eNOS contributes to resting vascular tone. In addition, L-NIO significantly inhibits the hypertensive responses to acetylcholine and bradykinin in anesthetized rats. Based on this finding, several studies use L-NIO as an eNOS inhibitor. Our studies found that nNOS and iNOS inhibitors did not act against resveratrol to augment the infarct volume. However, administration of nonselective NOS inhibitor or selective eNOS inhibitor completely blocked the decreased infarction effect of resveratrol. The results suggested that eNOS and iNOS, but not nNOS, may be involved in the neuroprotective effect of resveratrol in rats subjected to FCI injury and NO plays a crucial role in the effects.

In this study, we present the evidence that resveratrol increases NO production, attenuates the free radicals, and significantly protects rats brain tissue against FCI injury.

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**AUTHOR CONTRIBUTIONS**

Conception and design: S-KT, L-MH, S-SH
Analysis and interpretation: L-MH, HC, M-WN, H-YL, S-SH
Data collection: HC, M-WN, H-YL, FB-YZ

Writing the article: S-KT, L-MH, Y-TF, S-SH
Critical revision of the article: S-KT, L-MH, HC, S-SH
Final approval of the article: S-KT, L-MH, S-SH

**REFERENCES**