Activation of Transient Receptor Potential Vanilloid 1 by Dietary Capsaicin Delays the Onset of Stroke in Stroke-Prone Spontaneously Hypertensive Rats

Xingsen Xu, MD*; Peijian Wang, MD*; Zhigang Zhao, MD; Tingbing Cao, BS; Hongbo He, MD; Zhidan Luo, MD; Jian Zhong, MD; Feng Gao, MS; Zhenyu Zhu, BS; Li Li, MS; Zhencheng Yan, MD; Jing Chen, MD; Yinxing Ni, MD; Daoyan Liu, MPH, PhD†; Zhiming Zhu, MD, PhD, FAHA‡

Background and Purpose—Previous studies show that endothelial nitric oxide synthase (eNOS) plays a prominent role in maintaining cerebral blood flow and preventing stroke. Capsaicin in hot pepper can increase the phosphorylation of eNOS in endothelial cells. We test the hypothesis that chronic dietary capsaicin can prevent stroke through activation of cerebrovascular transient receptor potential vanilloid 1 (TRPV1) channels in stroke-prone spontaneously hypertensive rats (SHRsp).

Methods—SHRsp were fed dietary capsaicin, and their onset of stroke was examined. TRPV1 knockout and transgenic mice were used for determining the function of TRPV1 channels. Expression of eNOS and cerebrovascular reactivity were examined.

Results—Immunofluorescence showed TRPV1 channels and eNOS coexpression in cerebral arterioles. Administration of capsaicin significantly increased phosphorylated eNOS in carotid arteries from wild-type mice but not in TRPV1 knockout mice. Inhibition of eNOS using Nω-nitro-L-arginine methyl ester, removal of endothelium, or mutant TRPV1 significantly reduced capsaicin-induced endothelium-dependent relaxation of basilar arteries in mice. Chronic dietary capsaicin also remarkably increased eNOS expression in carotid arteries from SHRsp. Compared with Wistar-Kyoto rats, SHRsp had impaired endothelium-dependent relaxation of basilar arteries. Administration of capsaicin or L-arginine significantly improved the endothelium-dependent relaxation of basilar arteries in SHRsp. SHRsp had hypertrophy of cerebral arterioles, which was reversed by dietary capsaicin. Importantly, long-term administration of capsaicin significantly delayed the onset of stroke and increased the survival time in SHRsp.

Conclusions—Activation of TRPV1 channels by dietary capsaicin mediated increases in phosphorylation of eNOS and could represent a novel target for dietary intervention of stroke. (Stroke. 2011;42:3245-3251.)

Key Words: cerebrovascular hypertrophy ■ eNOS ■ SHRsp ■ stroke ■ TRPV1

Stroke is a major cause of mortality and severe disability in many countries and significantly increases economic and social burden. Although intravascular thrombolysis, pharmacological therapy, and endovascular interventions show promising results, management of patients with stroke remains far from optimal. In addition, a body of evidence shows that increased fruit and vegetable consumption is associated with a reduced risk of stroke.1 However, the potential mechanism of dietary factors on stroke is unknown. Experimental and clinical studies have clearly demonstrated that endothelial nitric oxide synthase (eNOS) plays an important role in maintaining cerebral blood flow and preventing neuronal injury.2,3 eNOS-deficient mice exhibit larger cerebral infarctions after middle cerebral artery occlusion,4 and cerebral arterioles undergo hypertrophy.5 Further inhibition of eNOS activity reduces cerebral blood flow and increases the size of cerebral infarct in animals.4 Genetic variation at the eNOS locus represents a genetic risk factor for increased susceptibility to human ischemic stroke.6 We recently showed that long-term administration of capsaicin can lower blood pressure in genetically hypertensive rats.7 Capsaicin is a potent agonist for transient receptor potential vanilloid 1 (TRPV1) channels.8 TRPV1 channels are highly expressed in sensory neurons but have also been detected in...
numerous other tissues, including blood vessels and the brain. We found that chronic TRPV1 channel activation by dietary capsaicin increases the phosphorylation of protein kinase A and eNOS and thus the production of NO in endothelial cells. Accordingly, we tested the hypothesis that activation of TRPV1 channels by dietary capsaicin prevents stroke through the improvement of cerebrovascular dysfunction and remodeling in stroke-prone spontaneously hypertensive rats (SHRsp). Our data provide evidence that TRPV1 channels mediate increases in eNOS and may represent a novel target for the therapeutic intervention of stroke.

**Methods**

**Animal Treatments**

C57BL/6J and TRPV1 knockout (TRPV1<sup>−/−</sup>) mice were purchased from Jackson Laboratory (Bar Harbor, ME). TRPV1 transgenic mice were generated previously. Wistar-Kyoto rats and 5-week-old male SHRsp were obtained from Charles River Laboratories (Kingston, NY). Rats and mice were housed under a 12-hour/12-hour day/night cycle; food and water were given ad libitum to all animals. Animals were given the normal standard laboratory chow (control group) or normal chow plus 0.01% capsaicin for mice and 0.02% capsaicin for rats (capsaicin group). Mice were bred for 6 months. Once SHRsp developed stroke, it was euthanized and its brain and carotid arteries were taken. Five SHRsp were given normal chow plus 5% L-arginine for 6 months. Rats were given 1% sodium chloride water to accelerate the hypertension process. All experiments were approved by the Institutional Animal Care and Use Committee of the Third Military Medical University.

**Vascular Relaxation Measurements**

Vascular relaxation was measured in basilar arteries of rats and mice according to previously described methods (see Supplementary Data for detail; http://stroke.ahajournals.org).

**Blood Pressure and Body Weight**

Systolic blood pressure measurement was performed in conscious, restrained rats by tail-cuff photoplethysmography (Model MLT 1030; Power Laboratory; AD Instruments, Sydney, Australia) every month. The body weight of rats had been measured every 2 weeks.

**Detection of Blood Biochemical Parameters**

Blood was drawn from a jugular vein, and the plasma was separated and immediately frozen at −70°C until assayed. Plasma levels of total cholesterol, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, triglycerides, creatinine, uric acid, blood urea nitrogen, blood glucose, and high-sensitivity C-reactive protein were measured using commercially available assay kits.

**Histology and Immunofluorescence**

The intima-media thickness of cerebral arterioles in the brain was determined according to established techniques. 

---

**Figure 1.** Immunofluorescence images of eNOS (green) and TRPV1 (red) in basilar arteries in WT and TRPV1<sup>−/−</sup> mice (A). Immunoblotting shows that capsaicin (0.01% wt/wt) could enhance the levels of phosphorylated eNOS in carotid from WT mice (B–C, n=6), but this effect disappears in TRPV1<sup>−/−</sup> mice (D–E, n=4). eNOS indicates endothelial nitric oxide synthase; TRPV1, transient receptor potential vanilloid 1; WT, wild-type.
cence for eNOS and TRPV1 was performed as previously described (see Supplementary Data for detail).

**Western Blotting**

Immunoblotting of total and phosphorylated eNOS at Ser1177, TRPV1, β-actin, and glyceraldehyde-3-phosphate dehydrogenase were performed using standard techniques as previously reported. See the online Data Supplement for detailed methods.

**Statistics**

Statistical differences between groups were assessed by Student t test or 1-way analysis of variance with Bonferroni multiple comparison post hoc tests as appropriate. The maximum response was calculated from individual agonist concentration–response curves using GraphPad Prism 3.0 (GraphPad Software, San Diego, CA). Survival curves were calculated by the Kaplan–Meier method and compared using the log-rank test. Two-sided probability values of \( P < 0.05 \) were considered statistically significant.

**Results**

**Capsaicin-Induced eNOS Phosphorylation Was Associated With Functional TRPV1 in Carotid Arteries**

Immunofluorescence staining showed TRPV1 and eNOS coexpression in the endothelium of cerebral arteries in wild-type mice, but TRPV1 did not express in TRPV1−/− mice (Figure 1A). Wild-type and TRPV1−/− mice were fed with and without dietary capsaicin for 6 months. Chronic dietary capsaicin significantly increased the level of phosphorylated eNOS in carotid arteries from wild-type mice, but not in TRPV1−/− mice (Figure 1B–E). This result indicates that cerebrovascular eNOS phosphorylation by capsaicin is associated with the presence of functional TRPV1.

**Activation of TRPV1 by Capsaicin Caused the Relaxation of Cerebral Arteries in Mice**

We isolated the basilar arteries from mice and measured the relaxation using myography. Removal of endothelium or TRPV1 knockout significantly reduced capsaicin-induced relaxation of basilar arteries of mice (Figure 2A–B). Capsaicin could improve relaxation of basilar arteries and inhibition of NO synthase using \( N^G \)-nitro-l-arginine methyl ester in wild-type mice, but this effect was absence in TRPV1−/− mice (Figure 2C–D). Furthermore, acetylcholine-induced relaxation of basilar arteries was enhanced in TRPV1 transgenic mice; there was no difference when it was inhibited by \( N^G \)-nitro-l-arginine methyl ester (Supplemental Figure IA). Nitroglycerin-induced relaxations in basilar arteries were not significantly different between wild-type mice and TRPV1−/− transgenic mice (Supplemental Figure IC–D). These results support the notion that capsaicin relaxes the basilar arteries through stimulation of TRPV1 in an endothelium-dependent manner.

**Chronic Dietary Capsaicin Enhanced the Levels of eNOS and Relaxation of Cerebral Arteries in SHRsp**

We examined whether dietary capsaicin increased expression of TRPV1 and eNOS in carotid arteries from SHRsp. It is well documented that eNOS plays a prominent role in maintaining cerebral blood flow and preventing stroke. In this study, we showed that expressions of phosphorylated eNOS, total eNOS, and TRPV1 were significantly increased in the carotid arteries from SHRsp treated with dietary capsaicin compared with untreated SHRsp (\( P < 0.05 \); Figure 3A–D). Compared with normotensive Wistar-Kyoto rats, the endothelium-dependent and endothelium-independent relaxations were significantly reduced in basilar arteries from SHRsp (maximum response, 46.90% ± 3.53% in SHRsp versus 90.91% ± 10.09% in Wistar-Kyoto rats, \( P < 0.01 \); Figure 4A; maximum response, 68.91% ± 5.10% in SHRsp versus 95.42% ± 9.05% in Wistar-Kyoto rats, \( P < 0.01 \); Figure 4C). However, chronic dietary capsaicin or \( l \)-arginine significantly improved the endothelium-dependent relaxation of basilar arteries from treated SHRsp compared with untreated SHRsp.
Thus, eNOS in intact cerebral arteries can be upregulated by chronic capsaicin, which accounts for the improved endothelium-dependent relaxation of basilar arteries in SHRsp. Besides, chronic dietary capsaicin only had a weak effect on the endothelium-independent relaxation of basilar arteries in capsaicin-treated SHRsp compared with untreated SHRsp (maximum response, 68.91%±5.10% in control versus 75.52%±7.32% in the capsaicin group, P=0.10; Figure 4D). Capsazepine could significantly inhibit capsaicin-induced relaxation in basilar arteries from SHRsp (Supplemental Figure II).

**Chronic Dietary Capsaicin Prevented Cerebrovascular Hypertrophy and Delayed the Onset of Stroke in SHRsp**

We further examined whether chronic dietary capsaicin has effects on cerebrovascular hypertrophy and can delay the onset of stroke in SHRsp. Thus, we observed the intima-media thickness changes in intracranial arterioles, which were located on the surface and inside the brain. Notably, the intima-media thickness of intracranial arterioles was significantly decreased in SHRsp treated with dietary capsaicin compared with untreated SHRsp (Figure 5A–B). Compared with control SHRsp, chronic dietary capsaicin showed a remarkable delay in the onset of stroke and significantly increased survival time (100.11±12.11 days versus 76.23±4.32 days, P<0.05; Figure 5C–D).

Collectively, the present results support the notion that long-term consumption of capsaicin delayed the onset of stroke in SHRsp primarily due to a reduction of cerebrovascular hypertrophy and improvement of vasodilatation through TRPV1 activation and subsequently promotes phosphorylation of eNOS in cerebral vessels. Notably, blood pressure and some biochemical parameters were not affected by chronic dietary capsaicin (Table).
The major novel finding of this study is that capsaicin remarkably relaxes basilar arteries and reduces intracranial arterial hypertrophy, which is associated with phosphorylation of eNOS in cerebral arteries of SHRsp. Furthermore, chronic dietary capsaicin significantly delays the occurrence of stroke and increases survival time of SHRsp by activation of TRPV1 channels in cerebral arteries.

Despite substantial advances for the treatment of patients with stroke, effective primary stroke prevention remains the best way for reducing the stroke burden. Identifying high-risk or stroke-prone individuals to target for specific management and interventions is particularly important for public health. Many preventive strategies are available to manage a number of risk factors that increase the risk of a first stroke. High blood pressure has profound effects on the brain and is the major cause of stroke. Hypertension alters the endothelium-dependent relaxation of cerebral blood vessels and leads to cerebrovascular hypertrophy, which increases the susceptibility of the all strokes occurring each year are first strokes, and therefore, primary prevention of stroke remains a great challenge worldwide.

Figure 5. Chronic dietary capsaicin prevents cerebrovascular hypertrophy in SHRsp (A–B). Survival curves and survival time of 2 groups were analyzed (C–D). n=15 in each group. SHRsp indicates stroke-prone spontaneously hypertensive rats.
brain to ischemic injury. Lower blood pressure using various antihypertensive drugs significantly reduces stroke mortality.

In addition to pharmaceutical therapy, diets rich in fruits and vegetables and with increased potassium, but reduced sodium and fat, may reduce the risk of stroke. In the Nurses Health Study and the Health Professionals’ Follow-Up Study, increased consumption of fruits and vegetables was associated with lower stroke risk with a linear relationship of greater consumption to lower risk. For each serving a day of fruit or vegetables, the risk of stroke was 6% lower. Hot peppers are consumed worldwide as vegetables and spices. Capsaicin is the major pungent ingredient in hot peppers and increases the protein level of eNOS in vascular endothelial cells and NO production in rat mesenteric arteries. The effect is independent of a reduction in blood pressure. In addition, this result is similar to relaxation in SHRsp treated with 0.02% capsaicin; SBP, systolic blood pressure; hs-CRP, high-sensitivity C-reactive protein; SD, standard deviation.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Cont</th>
<th>Cap</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tail-cuff SBP, mm Hg</td>
<td>227±14</td>
<td>219±10</td>
<td>0.236</td>
</tr>
<tr>
<td>Total cholesterol, mmol/L</td>
<td>3.54±0.56</td>
<td>4.28±0.41</td>
<td>0.105</td>
</tr>
<tr>
<td>Triglycerides, mmol/L</td>
<td>0.85±0.14</td>
<td>1.12±0.31</td>
<td>0.325</td>
</tr>
<tr>
<td>Low-density lipoprotein cholesterol, mmol/L</td>
<td>1.52±0.10</td>
<td>1.71±0.12</td>
<td>0.424</td>
</tr>
<tr>
<td>High-density lipoprotein cholesterol, mmol/L</td>
<td>1.13±0.20</td>
<td>1.30±0.16</td>
<td>0.297</td>
</tr>
<tr>
<td>Creatinine, μmol/L</td>
<td>53.79±9.12</td>
<td>76.22±18.10</td>
<td>0.368</td>
</tr>
<tr>
<td>Uric acid, μmol/L</td>
<td>57.67±9.66</td>
<td>74.03±19.94</td>
<td>0.061</td>
</tr>
<tr>
<td>Blood urea nitrogen, mmol/L</td>
<td>6.52±1.17</td>
<td>7.53±1.01</td>
<td>0.662</td>
</tr>
<tr>
<td>hs-CRP, mg/L</td>
<td>1.46±0.07</td>
<td>1.91±0.42</td>
<td>0.113</td>
</tr>
</tbody>
</table>

Data are expressed as the mean±SD. SHRsp indicates stroke-prone spontaneously hypertensive rats; Cont, SHRsp control; Cap, SHRsp treated with 0.02% capsaicin; SBP, systolic blood pressure; hs-CRP, high-sensitivity C-reactive protein; SD, standard deviation.

Table. Characteristics of SHRsp Treated With and Without Dietary Capsaicin

Our recent studies show that long-term activation of TRPV1 by capsaicin can increase the phosphorylated levels of eNOS in mesenteric arteries and plasma levels of NO metabolites. The present study shows that chronic dietary capsaicin enhances the expression of eNOS in carotid arteries, which may be related to the increasing relaxation of cerebral vessels. Another interesting result is that long-term administration of capsaicin significantly reduced cerebral vessel hypertrophy. Baumbach et al reported that inhibition of eNOS using N^O-nitro-l-arginine methyl ester resulted in hypertrophy of cerebral arterioles of mice. Furthermore, eNOS-deficient mice developed hypertrophy of cerebral arterioles, which was independent of increasing arterial pulse pressure.

Although diet may influence stroke risk, the optimal dietary habits for stroke prevention are not well established. Dietary trials specifically focused on reducing the risk of stroke are lacking. However, this study demonstrates, for the first time, that chronic dietary capsaicin significantly increases the survival time for SHRsp; furthermore, this effect is independent of a reduction in blood pressure. Previous studies showed that capsaicin or its derivative increases the protein level of eNOS in vascular endothelial cells and NO production in rat mesenteric arteries. The present results indicate that capsaicin treatment prevents stroke in SHRsp through cerebral endothelial cells and NO production in rat mesenteric arteries. The present results indicate that capsaicin treatment prevents stroke in SHRsp through cerebral endothelial cells and NO production in rat mesenteric arteries.

Acknowledgments
We thank Lijuan Wang (Chongqing Institute of Hypertension, China) for technical assistance. We thank Drs Wing Tak Wong and Yu Huang (Chinese University of Hong Kong, China) for critical review of the manuscript.

Sources of Funding
This research was supported by grants from the National Natural Science Foundation of China (30890042) and the National Basic Research Program of China (2011CB503902 and 2012CB517805).

Disclosures
None.

References
Activation of Transient Receptor Potential Vanilloid 1 by Dietary Capsaicin Delays the Onset of Stroke in Stroke-Prone Spontaneously Hypertensive Rats

Xingsen Xu, Peijian Wang, Zhigang Zhao, Tingbing Cao, Hongbo He, Zhidan Luo, Jian Zhong, Feng Gao, Zhenyu Zhu, Li Li, Zhencheng Yan, Jing Chen, Yinxing Ni, Daoyan Liu and Zhiming Zhu

Stroke. 2011;42:3245-3251; originally published online August 18, 2011; doi: 10.1161/STROKEAHA.111.618306

Stroke is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2011 American Heart Association, Inc. All rights reserved.
Print ISSN: 0039-2499. Online ISSN: 1524-4628

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://stroke.ahajournals.org/content/42/11/3245

Data Supplement (unedited) at:
http://stroke.ahajournals.org/content/suppl/2011/08/18/STROKEAHA.111.618306.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Stroke can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Stroke is online at:
http://stroke.ahajournals.org/subscriptions/
Activation of TRPV1 by Dietary Capsaicin Delays the Onset of Stroke in Stroke-Prone Spontaneously Hypertensive Rats

Xingsen Xu, MD*; Peijian Wang, MD*; Zhigang Zhao, MD; Tingbing Cao, BS; Hongbo He, MD; Zhidan Luo, MD; Jian Zhong, MD; Feng Gao, MS; Zhenyu Zhu, BS; Li Li, MS; Zhencheng Yan, MD; Jing Chen, MD; Yinxing Ni, MD; Daoyan Liu, MPH, PhD§ and Zhiming Zhu, MD, PhD, FA
Figure S1. Acetylcholine (ACh)-induced relaxation in the presence or absence of L-NAME (100 μM) in basilar arteries in wild-type (WT) and TRPV1-transgenic (TRPV1-TG) mice (A). Nitroglycerin (NTG)-induced relaxation in basilar arteries in wild-type (WT) and TRPV1-transgenic (TRPV1-TG) mice (B). Nitroglycerin (NTG)-induced relaxation in basilar arteries in control (Cont) and capsaicin (Cap) group in WT (C) and TRPV1<sup>-/-</sup> mice (D).
**Figure S2** Capsazepine significantly inhibited capsaicin-induced relaxation in basilar arteries in SHRsp

![Graph showing relaxation as a function of capsaicin concentration and treatment with or without capsazepine.]

**Figure S3** Tail-cuff SBP in wild-type (WT) and TRPV1 gene knockout (TRPV1−/−) mice during 0.01% dietary capsaicin intervention for six month.

![Graph showing SBP over time for WT and TRPV1−/− mice.]

**Figure S3** Tail-cuff SBP in wild-type (WT) and TRPV1 gene knockout (TRPV1−/−) mice during 0.01% dietary capsaicin intervention for six month.
Figure S4

Figure S4 Chronic dietary capsaicin decreased hemorrhage in brain tissue paraffin section (A), but did not change the maximum area of cerebral punctate hemorrhage under microscope and the amount of neurons around hemorrhage with Nissl's staining (B, C).
**Supplemental methods**

**Vascular relaxation measurements**

Rats or mice were sacrificed after being anesthetized with sodium pentobarbital. The whole brain was quickly removed, and the basilar artery isolated from the brain was gently cleaned of any connective tissue in cold Krebs solution and cut into 2.0-2.5-mm long segments in Krebs solution (NaCl 119 mmol/l; NaHCO₃ 25 mmol/l; glucose 11.1 mmol/l; KCl 4.7 mmol/l; KH₂PO₄ 1.2 mmol/l; MgSO₄ 1.2 mmol/l; and CaCl₂ 2.5 mmol/l, pH 7.4) under a dissecting microscope. Vascular reactivity of freshly isolated basilar arteries was studied using wire myography (Danish Myo Technology, Denmark), as described previously. Basilar arteries were maintained at 37 °C in Krebs solution gassed with 95% O₂ and 5% CO₂. After measurement of passive-tension internal circumference characteristics, tension was set to the estimated *in vivo* internal circumference. We determined the contractile responses by the administration of potassium chloride (KCl, 60 mmol/l) and 5-hydroxytryptamine (5-HT, 10µmol/l) for rat basilar arteries and using KCl (50 mmol/l) and U46619 (400 nmol/l) for mice basilar arteries. Cumulative concentration relaxations using acetylcholine (Ach, 1 nmol/l to 10 µmol/l) and capsaicin (1 nmol/l to 10 µmol/l) were also performed. Capsaicin, 5-HT, U46619, and Ach were purchased from Sigma-Aldrich.

**Histology and immunofluorescence**

The intima-media thickness (IMT) of cerebral arterioles in brain was determined according to established techniques. Brains were fixed in 10% formaldehyde/phosphate-buffered saline and embedded in paraffin. After routine histological procedures, the tissues were stained with hematoxylin and
eosin and studied under ×400 magnification. Pictures were obtained with a
digital camera using a Nikon TE2000 microscope (Nikon Corporation, Tokyo,
Japan). The tunica media was defined as the region between the internal and
external elastic laminae, which was measured at four orthogonal points of the
arterial sections for three consecutive arterial rings per animal using the
imaging software NIS-Elements 3.2 (Nikon Corporation).

Six-µm-thick arteriole sections of basilar arteries were cut on a cryostat
(CM1850; Leica Microsystems) and mounted on slides. The sections were
fixed with 4% paraformaldehyde for 30 minutes. Sections were incubated
overnight at 4°C with the following primary antibodies: rabbit monoclonal
anti-eNOS and goat monoclonal anti-TRPV1 (1:200; Santa Cruz Biotechnology,
Santa Cruz, CA, USA). Appropriate fluorescent dye–conjugated secondary
antibodies (Boster Bio-Engineering, WuHan, China) were incubated in the dark
for 1 hour at 20°C. For negative controls, the primary antibodies were omitted,
and the same staining procedures were followed. The sections were visualized
with a fluorescence microscope, and photomicrographs were recorded and
analyzed with the NIS-Elements 3.2 software (Nikon Corporation).

**Western blotting**

Immunoblotting of total and phosphorylated eNOS at Ser1177 (p-eNOS), β-actin
and GAPDH were performed using standard techniques as previously reported.
Protein supernatants were separated by centrifugation, and protein
concentrations were determined with the Bio-Rad protein assay reagent
(Bio-Rad Laboratories, Hercules, CA, USA). Proteins were separated using
10% sodium dodecyl sulfate polyacrylamide gels and transferred to
Hybond-ECL nitrocellulose membranes (NEN Life Science Products, Boston,
MA, USA) at 100V for 1 h. Membranes were blocked for 8 h at 4 °C with
blocking buffer containing tromethamine hydrochloride-buffered saline and
0.1% polysorbate-20 with 5% wt/vol nonfat dry milk. Membranes were
incubated with primary rabbit monoclonal IgG (1:1000), anti-eNOS, anti-p-eNOS (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 8 h at 4 °C. After washing, membranes were incubated with secondary antibodies (goat anti-rabbit horseradish peroxidase, 1:2000) for 1 h at room temperature and washed extensively. Each sample was processed three to six times.

**Nissl's staining**

The brain was prefixed by perfusion with 4% paraformaldehyde in 0.1 M phosphate buffer (PH 7.4, 4 °C) through the left ventricle. The brain was then removed and further fixed with the same fixative fluid for a week, followed by routine paraffin embedding. Coronal sections of 10 μm were prepared from the optic nerve to middle cerebrum and stained with toluidine blue. In the current study, we investigated the histological alteration of the nervous tissue around hemorrhage by light microscopic study. The amounts of neurons around hemorrhage were analyzed with Nissl's staining by NIS-Elements 3.2 software.