Background: The Nogo-66 antagonistic peptide (NEP1-40) is a potential candidate for therapeutic intervention of neuronal injury. However, delivery of the proteins across the blood–brain barrier is severely limited by its size and biochemical properties. The current study was designed to evaluate the transducible effects of the trans-activator of transcription (TAT) transduction system for NEP1-40 to cross the blood–brain barrier and to clarify whether intraperitoneal administration of TAT-NEP1-40 could protect cerebral neurons from ischemic injury.

Methods: Adult male Sprague-Dawley rats were submitted to a 120-min focal ischemia and received an intraperitoneal injection of No-TAT-NEP1-40, TAT-NEP1-40, TAT-β-galactosidase, or vehicle. The existence of the proteins in the brain was analyzed with immunofluorescence and Western blot techniques at 6 h after injection. Brain ischemic injury was evaluated by neurologic deficit scores, infarction volumes, terminal deoxynucleotidyl transferase-mediated dUDP-biotin nick end labeling staining, and assay of caspase-3 activity.

Results: Western blot analysis and immunofluorescence staining confirmed the presence of TAT-NEP1-40 protein in the brains 6 h after injection. Intraperitoneal injection of TAT-NEP1-40 could attenuate the numbers of terminal deoxynucleotidyl transferase-mediated dUDP-biotin nick end labeling-positive cells and activated caspase-3 positive cells, and increase the viability of the cells in the ischemic border zone, compared with that treated with No-TAT-NEP1-40, TAT-β-Gal, or vehicle. Furthermore, treatment with TAT-NEP1-40 significantly improved the neurologic outcomes and reduced the size of the infarction in rats.

Conclusions: The results demonstrate that the TAT-NEP1-40 could be efficiently delivered into the rat brains and improve ischemia-induced neurologic outcomes through attenuating cell apoptosis in ischemic brains.

ACUTE cerebral ischemia–reperfusion injury results in severe neurologic deficit and even leads to death. Finding effective neuroprotective medicines and therapeutic measures that are able to improve prognosis of the patients with stroke has long been a hot field in medical research.1 Although some of treatment regimens, primarily based on physical therapy, have partly improved the recovery of these patients, there are no drugs available that could effectively repair the brain tissue damage resulting from ischemia or mechanical injury.2 Axonal damage is a key pathologic manifestation in a variety of injuries of the central nervous system (CNS), such as stroke and traumatic brain injury. An attractive drug discovery strategy to treat brain injuries is to search for agents that can promote CNS axonal regeneration.1,3 CNS myelin is a primary source for axonal growth inhibitor in the adult brain, and three proteins, Nogo-A,4 myelin-associated glycoprotein,5 and oligodendrocyte myelin glycoprotein,6 seem to be the main constituents responsible for the inhibition of axonal growth. The identification of the Nogo-66 receptor (NgR),7 which interacts with one of two Nogo-A inhibitory domains as well as with myelin-associated glycoprotein and oligodendrocyte myelin glycoprotein, has established its crucial role in mediating the inhibitory effects of CNS myelin elements. This discovery provides an unprecedented opportunity to manipulate adult CNS axonal regeneration. Therefore, it is possible that blockage of NgR could promote the functional recovery and reverse the devastating consequences of CNS injuries.8

Nogo-A has two inhibitory domains: 66-amino acid region (Nogo-66) and N-terminal region (Amino-Nogo). Further molecular analyses reveal that a 40-residue peptide (Nogo extracellular peptide 1-40 [NEP1-40]) behaves as an antagonist of NgR. The blockage of the Nogo-66 interaction with NgR using NEP1-40 increases functional recovery and axonal regeneration after spinal cord injury.9,10 Recently, some studies have demonstrated that the interruption of the Nogo-NgR pathway might enhance axonal sprouting and thereby promote the re-
covery after focal brain injury.\textsuperscript{11–15} Although NEP1-40 peptide seems to be a potential candidate for therapeutic intervention after CNS injury, for many years delivery of these therapeutic proteins into the brain parenchyma has been limited because of the obstacle of the blood–brain barrier (BBB). Kilic et al.\textsuperscript{14} have demonstrated that systemic delivery of neuroprotective protein Bcl-xL does not lead to protection after focal cerebral ischemia. However, systemic administration of recombinant fusion protein of Bcl-xL containing the trans-activator of transcription (TAT) protein transduction domain (PTD) attenuated ischemic brain injury and neuronal apoptosis.\textsuperscript{15,16} Therefore, a primary obstacle to clinical use of NEP1-40 is the lack of an efficient, noninvasive method for transporting a biologically relevant amount of the respective protein across the BBB.

It is now known that PTD can serve as a transporter for macro molecular-like full-length proteins to cross cell membrane to cytoplasm or cross BBB to brain parenchyma. PTD-mediated protein transduction technique represents a novel and promising strategy for the therapy of a majority of neurologic diseases, including ischemic stroke.\textsuperscript{17,18} It has been demonstrated that fusion proteins containing TAT domain enable them to cross cell membranes and the BBB after systemic administration.\textsuperscript{14,17} Intraperitoneal injection of the 120-kd \( \beta \)-galactosidase (\( \beta \)-Gal) protein fused to the TAT protein results in a successful delivery of a biologically active fusion protein in various kinds of tissues, including the mouse brain.\textsuperscript{19,20} In fact, several examples of TAT fusion proteins, such as Bcl-xL, glial cell line–derived neurotrophic factor, superoxide dismutase, and X chromosome–linked inhibitor of apoptosis, have been tried and shown evident neuroprotective effects in adult models of cerebral ischemia.\textsuperscript{19,21–25}

Because of the potential neuroprotective effects of NEP1-40 protein, the current study was designed to assess the possible beneficial effect of TAT-NEP1-40 fusion protein in focal cerebral ischemic injury. First, to evaluate the transducible effects of the TAT transduction system, the coding sequence of NEP1-40 was cloned into the pTAT-HA vector, and TAT-NEP1-40 fusion protein was expressed in \textit{Escherichia coli}. We then tested the ability of transduction of TAT-NEP1-40 across the BBB. Finally, we investigated whether an intraperitoneal administration of TAT-NEP1-40 fusion protein may functionally alleviate brain injury after transient focal ischemia in rats.

Materials and Methods

Materials

The experimental protocol used in this study was approved by the Ethics Committee for Animal Experimentation of the Fourth Military Medical University. The animals were provided by the Experimental Animal Center of the Fourth Military Medical University and housed under controlled condition with a 12-h light–dark cycle, temperature at 21\(^\circ\)C ± 2°C, and humidity in 60–70\% for at least 1 week before drug treatment or surgery. The rats were allowed free access to standard rodent diet and tap water.

Experimental Grouping

To determine the transducible effect of TAT-NEP1-40 in the brain, nine adult male Sprague-Dawley rats weighing 280–320 g were submitted to a 120-min focal ischemia induced by intraluminal thread occlusion of the middle cerebral artery and received intraperitoneal injections of 40 nmol No-TAT-NEP1-40, TAT-NEP1-40, or TAT-\( \beta \)-Gal at the onset of reperfusion. These animals were killed 6 h after injection. Brains were used for the immunofluorescence and Western blot analysis of TAT-\( \beta \)-Gal, No-TAT-NEP1-40, TAT-NEP1-40 transduction (\( n = 3 \) per group).

To assess the protective effect of TAT-NEP1-40 fusion against focal cerebral injury, additional animals were randomly assigned to four groups. Intraperitoneal injections of either 4.0 ml phosphate-buffered saline (PBS; 0.1 \( \mathrm{m} \)) or 40 nmol TAT-NEP1-40, No-TAT-NEP1-40, or TAT-\( \beta \)-Gal dissolved in 4.0 ml PBS (0.1 \( \mathrm{m} \)) were performed imme-
diately after a 120-min episode of ischemic injuries induced by middle cerebral artery occlusion (MCAO) followed by different durations of reperfusion, according to the indicators to be observed.

In a separate experiment, the physiologic variables in five additional rats weighing 280–320 g were measured during ischemia and up to 30 min after the onset of reperfusion. The animals were anesthetized with 2% isoflurane delivered by a facemask at a flow rate of 2 l/min oxygen. The right femoral artery was cannulated for continuous monitoring of blood pressure and arterial blood sampling. A rectal probe was inserted to monitor core temperature. Arterial blood gases and plasma glucose were measured at the onset of ischemia, 60 min after ischemia, and 30 min after reperfusion.

Expression and Purification of Recombinant Fusion Proteins

The NEP1-40 coding sequence (amino acid residues: -RIYKVQIAQKSDEGHPFRAYLEVAISEELVQKYNS-) was amplified from the Nogo-A complementary DNA (Kazusa DNA Research Institute, Kisarazu, Chiba, Japan) by polymerase chain reaction with the primer pairs 5′-CTCCATGGTTAGGATATACAAGGGTGTGATC-3′ and 5′-ACCTCGAGTCAAGAATTACTGTACTTCTGAAC-3′ (the underlined were NcoI and XhoI sites, respectively; the polymerase chain reaction cycling program was 94°C for 5 min for degeneration in advance, 25 cycles at 94°C for 30 s, 55°C for 60 s, 72°C for 30 s, and finally, 72°C for 10 min for elongation). Amplified fragments were digested with NcoI/XhoI and cloned into the corresponding sites of pTAT-HA vector (kindly provided by Steven F. Dowdy, Ph.D.). Therefore, the final expression plasmid pTAT-NEP1-40 was generated. To prove the transduction of TAT fusion protein, the pNo-TAT-NEP1-40 plasmid as a control protein was obtained from pTAT-NEP1-40 by cutting down TAT sequence with BamH1 digestion (due to the presence of two BamHI restriction sites at both ends of TAT sequence). The plasmids pTAT-NEP1-40 and pNo-TAT-NEP1-40 were identified by automated DNA sequencing. To prove the function of TAT-NEP1-40, TAT-β-Gal vector present in the supernatant of bacterial lysate was purified by the Ni2+-nitrilotriacetic acid affinity column.24,25 The concentrations of proteins were determined by the bicinchoninic acid protein assay kit and stored in 10% glycerol–PBS at −80°C until use.

Establishment of Transient Focal Cerebral Ischemia

Transient focal cerebral ischemia was induced by MCAO in the rats as described in our previous study.26,27 Briefly, the rats were fasted for 12 h but were allowed free access to water before surgery. Anesthesia was induced with 4% isoflurane and was maintained with 2% isoflurane delivered by a mask. The right common carotid artery and the right external carotid artery were exposed through a ventral midline neck incision and were ligated proximally. A 5-0 nylon monofilament suture (Ethicon Nylon Suture; Ethicon Inc., Osaka, Japan) with its tip rounded by heating near a flame was inserted through an arteriotomy in the common carotid artery just below the carotid bifurcation and then advanced into the internal carotid artery approximately 17–18 mm distal to the carotid bifurcation until a mild resistance was felt, thereby occluding the origins of the anterior cerebral artery, the middle cerebral artery, and the posterior communicating artery. Reperfusion was accomplished by withdrawing the suture after 120 min of ischemia. The incision sites were infiltrated with 0.25% bupivacaine hydrochloride for postoperative analgesia. Rectal temperature was monitored with a probe (Spacelabs Medical Inc., Redmond, WA) and maintained at 37.0°–37.5°C by surface heating and cooling during surgery, which usually lasts for 10–15 min.

Western Blotting Analysis of TAT-NEP1-40

Transduction into Brain

Brains were dissected, complemented with lysis buffer, homogenized, and centrifuged, and supernatants were used for sodium dodecylsulfate–polyacrylamide gel electrophoresis. Equal amounts of protein were diluted in 7X sample buffer, boiled, and loaded onto 15% polyacrylamide gels. Proteins were transferred onto polyvinyl diflouride membranes. Membrane was incubated in blocking solution containing 0.05% Tween-20 and 5% nonfat dry milk for 1 h, and immersed with monoclonal mouse antibody against 6-His (1:2,000) in antibody dilution buffer (Tweeen-20 containing 1% nonfat dry milk) for 2 h in room temperature. The membrane was rinsed, incubated in horseradish peroxidase–labeled goat anti-mouse IgG (1:12,500) for 1 h in room temperature, washed, immersed in electrochemiluminescent reagent, and exposed to Electrochemiluminescence-Hyperfilm (Amersham Biosciences, Piscataway, NJ).

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Immunofluorescence Analysis of TAT-NEP1-40 Transduction into Brain

For analysis of TAT-NEP1-40 transduction, after 6-h injection intervals, the rats were perfusion fixed with 4% paraformaldehyde in 0.1 M PBS (pH 7.4) during deep anesthesia. The brains were removed and were postfixed in the same fixatives for 3 h and immersed in 20% sucrose in 0.1 M phosphate buffer at 4°C overnight. Sagittal sections thickened in 14 μm were cut with a Leica CM1800 Cryostat (Leica Microsystems, Wetzlar, Germany). The sections were washed three times for 5 min for each in PBS and immersed in PBS containing 0.3% triton and 10% normal goat serum for 30 min. After three further risings in PBS, sections were incubated overnight at 4°C with monoclonal mouse antibody against 6-His (1:1,000) dissolved in PBS containing 0.3% triton and 2% normal goat serum. On the next day, sections were washed three times 5 min for each in PBS and incubated for 2 h with FITC-conjugated anti-mouse IgG (1:500) diluted with PBS. After 3 further rinses in PBS, the distribution of fluorescence was analyzed under a BX-60 fluorescence microscope (Olympus Corporation, Tokyo, Japan).

Neurobehavioral Evaluation

After withdrawing the suture and recovery from anesthesia, the rats were put back to their cages with free access to food and water. Twenty-four hours after reperfusion, the animals were neurologically assessed by an investigator who was unaware of the animal grouping. A six-point scale modified from that described by Longa et al.28 was used for neurologic assessment: 0 = no deficit; 1 = failure to extend left forepaw fully; 2 = circling to the left; 3 = falling to the left; 4 = no spontaneous walking with a depressed level of consciousness; 5 = dead.

Measuring Infarction Volume by 2,3,5-Triphenyltetrazolium Chloride Staining

Twenty-four hours after reperfusion, the rats were reanesthetized with 4% isoflurane in oxygen and decapitated. The brains were rapidly removed and cooled in iced saline for 10 min. Six 2-mm-thick coronal sections were cut with the aid of a brain matrix. Sections were immersed in 2% 2,3,5-triphenyltetrazolium chloride at 37°C for 30 min. Unstained areas were defined as infarct and measured by using Adobe Photoshop 7.0.1 (Adobe Systems Incorporated, San Jose, CA). The demarcation between infarcted and noninfarcted tissue was outlined, and the area of infarction in the section was calculated by subtracting the area of the nonlesioned ipsilateral hemisphere from the total area of the contralateral hemisphere. The total volume of infarction was calculated by integration of the lesion areas from all six sections measured.

TUNEL Staining

After 12 h of reperfusion, nuclear damage after ischemia was assessed in situ by TUNEL staining. The protocol for detecting DNA fragmentation was based on the method by Wang et al.29 with slight modification for use in frozen sections. Briefly, air-dried sections were postfixed in 4% paraformaldehyde at room temperature for 15 min and washed twice in 50 mM Tris-Cl/NaCl on ice. After the sections were exposed to 2% H2O2 in methanol to block the endogenous peroxidase activity and washed three times for 2 min with TBS, the enzyme reaction was conducted at 37°C for 2 h with the following reaction mixture: 1 μl terminal deoxynucleotidyl transferase, 1 μl Digit-d-UTP, and 18 μl terminal deoxynucleotidyl transferase labeling buffer using a total volume of 20 μl for each section. After blocking with 2% bovine serum albumin for 30 min, sections were incubated with biotinylated Digit Ab (1:100) at room temperature for 30 min and then washed with TBS three times. Sections were subsequently incubated with streptavidin–biotin complex (1:100) at 37°C for 1 h and then washed with TBS five times, 4 min for each. Finally, the sections were incubated in 3,3-diaminobenzidine and counterstained with hematoxylin. In the TUNEL staining studies, the results were quantitatively evaluated with the method described by Wang et al.29 Briefly, 32 pixels of 0.10 mm² were placed by light microscope with 100× magnification; 16 pixels were from the boundary zone of middle cerebral artery and anterior cerebral artery, and the others were from that of middle cerebral artery and posterior cerebral artery. Then, the total number of positively stained cells in these pixels was counted and expressed as cells/mm².

Immunofluorescence Analysis of Caspase-3 Activation

For analysis of activated caspase-3, after 12 h of reperfusion, the rats were perfusion-fixed with 4% paraformaldehyde in 0.1 M PBS (pH 7.4) during deep anesthesia. The brains were removed and were postfixed for 4 h in 4% paraformaldehyde in 0.1 M PBS and immersed in 20% sucrose in 0.1 M PBS at 4°C overnight. Sagittal 14-μm sections were cut with a Leica CM1100 Cryostat. The sections were washed three times for 5 min in 0.1 M PBS and immersed for 30 min in 0.1 M PBS containing 0.3% triton and 10% normal goat serum. After three further rinses in 0.1 M PBS, sections were incubated overnight at 4°C in PBS containing 0.3% triton, 2% normal goat serum, and rabbit anti-cleaved caspase-3 (Asp175) antibody (1:1,000). Subsequently the sections were washed three times for 5 min in 0.1 M PBS and incubated for 2 h in 0.1 M PBS containing 2% normal goat serum and FITC-conjugated anti-rabbit second antibody, diluted to 1:500. After three further rinses in PBS, caspase-3 activation was evaluated under a BX-60 fluorescence microscope. The number of activated caspase-3–positive cells

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was quantified as described by Wang et al.\(^29\) and expressed as cells/mm\(^2\).

**Statistical Analysis**

Brain sections were examined by two independent and blinded investigators. The software, SPSS 11.0 for Windows (SPSS Inc., Chicago, IL), was used to conduct statistical analyses. All values, except for neurologic scores, are presented as mean ± SD. The physiologic data were analyzed using one-way analysis of variance with repeated measures. Differences in physiologic variables between groups were analyzed using analysis of variance with post hoc Student–Newman–Keuls test. The infarct volumes and the numbers of TUNEL-positive cells, viable cells, and activated caspase-3–positive cells were analyzed by one-way analysis of variance, and between-group differences were detected with post hoc Student–Newman–Keuls test. The neurologic deficit scores were expressed as median (range) and were analyzed with Kruskal–Wallis test followed by Mann–Whitney \(U\) test with Bonferroni correction. Values of \(P < 0.05\) were considered as statistically significant.

**Results**

**Assessment of In Vivo Transduction of TAT-NEP1-40 Fusion Protein into Rat Brains**

As shown in figure 1A, Western blotting showed a visible band at 14.4-kd in TAT-\(\beta\)-Gal–treated animals 6 h after reperfusion. Similarly, TAT-\(\beta\)-Gal was detected as well in TAT-\(\beta\)-Gal–treated animals. However, no protein transduction was detectable in the animals treated with No-TAT-NEP1-40. Concomitantly, existence of TAT-NEP1-40 in the brain was also demonstrated by using immunofluorescence. As shown in figure 1B, immunofluorescent staining revealed that TAT-\(\beta\)-Gal and TAT-NEP1-40 fusion proteins were seen in the brain, whereas no fluorescence signals were detected in the brain of rats treated with No-TAT-NEP1-40. These results indicate that TAT-mediated NEP1-40 fusion was transduced into brains.

**Physiologic Variables**

Physiologic variables are summarized in table 1. There were no significant differences for the variables among different time points (at the onset of ischemia, 60 min

### Table 1. Physiologic Variables (n = 5)

<table>
<thead>
<tr>
<th>Variable</th>
<th>Baseline</th>
<th>60 min ischemia</th>
<th>30 min reperfusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>T, °C</td>
<td>37.3 ± 0.38</td>
<td>37.2 ± 0.36</td>
<td>37.2 ± 0.32</td>
</tr>
<tr>
<td>MAP, kPa</td>
<td>14.7 ± 1.1</td>
<td>14.5 ± 0.9</td>
<td>14.6 ± 1.1</td>
</tr>
<tr>
<td>pH</td>
<td>7.39 ± 0.06</td>
<td>7.36 ± 0.05</td>
<td>7.34 ± 0.04</td>
</tr>
<tr>
<td>(P_{\text{aCO}_2}), kPa</td>
<td>32.3 ± 5.9</td>
<td>31.1 ± 7.5</td>
<td>30.5 ± 8.1</td>
</tr>
<tr>
<td>(P_{\text{aCO}_2}), kPa</td>
<td>5.2 ± 0.6</td>
<td>5.7 ± 0.8</td>
<td>6.1 ± 1.0</td>
</tr>
<tr>
<td>Glucose, mM</td>
<td>6.32 ± 0.63</td>
<td>6.81 ± 0.70</td>
<td>6.87 ± 0.75</td>
</tr>
</tbody>
</table>

Physiologic data were obtained from five animals at each time point. Variables are presented as mean ± SD. Between-time point analysis did not detect significant differences.

MAP = mean arterial pressure; \(P_{\text{aCO}_2}\) = arterial carbon dioxide partial pressure; \(P_{\text{aO}_2}\) = arterial oxygen partial pressure; T = rectal temperature.
after ischemia, and 30 min after reperfusion). Arterial blood gases (partial pressure of oxygen, partial pressure of carbon dioxide, pH), mean arterial pressure, body temperature, and plasma glucose remained in the normal range during the experimental period observed.

**Neurologic Assessment**

The neurologic deficit scores of four groups are shown in Table 2. Twenty-four hours of treatment with TAT-NEP1-40 improved the neurologic deficit scores to 1 (0–2), significantly different from 2 (1–3) in the control, No-TAT-NEP1-40, and TAT-β-Gal groups (P < 0.004, 0.010, and 0.004, respectively). There were no statistical differences in the neurologic deficit scores among the latter three groups.

**Infarction Volume**

Focal cerebral ischemia was produced in 24 rats by transient MCAO. All animals were subjected to 120 min of ischemia and 24 h of reperfusion. The areas of MCAO-induced ipsilateral cerebral infarction were determined by loss of triphenyltetrazolium chloride staining (fig. 2A). The infarct volumes of four groups are shown in figure 2B. There was a significant reduction in infarct size in the rats that received TAT-NEP1-40 treatment in comparison with the PBS–, No-TAT-NEP1-40–, and TAT-β-Gal–treated rats (P < 0.01 for each comparison). There were no differences in infarction volume among the control, No-TAT-NEP1-40, and TAT-β-Gal groups.

**TUNEL Staining**

Ischemia-induced brain injury and the neuroprotective effect of TAT-NEP1-40 treatment were also assessed by observation of the level of cell apoptosis in the brain with TUNEL staining and hematoxylin counterstaining. As shown in figure 3, TUNEL staining was negative in the brain sections at the contralateral hemisphere of ischemic groups and in the whole brain of sham animals at 12 h after reperfusion (data not shown). However, the TUNEL staining was strong in the ipsilateral hemisphere of control group 12 h after reperfusion (figs. 3A and E). TUNEL-positive cells were distributed in the cerebral cortex and dorsal caudate. A large number of stained cells in the ischemic bounder zone of rat brain were seen in the No-TAT-NEP1-40 (figs. 3C and G) and TAT-β-Gal groups (figs. 3D and H), whereas in contrast, only small TUNEL-positive cells in the TAT-NEP1-40–treated group were observed (figs. 3B and F). The treatment with TAT-NEP1-40 significantly reduced the number of TUNEL–positive cells at the time 12 h after reperfusion, compared with PBS–, No-TAT-NEP1-40–, and TAT-β-Gal–treated animals (P < 0.05). There was no difference among the latter three groups. On the other hand, the number of viable cells was significantly increased in

![Fig. 2. Reduction of infarct volumes by TAT-NEP1-40 in middle cerebral artery occlusion (MCAO)-induced focal cerebral ischemic injury brain injury was assessed by 2,3,5-triphenyltetrazolium chloride staining. (A) Representative 2,3,5-triphenyltetrazolium chloride–stained sections outlining the infarcted areas in rats of four groups. Unstained areas were defined as infarct. (B) The bar graph showing the statistical analysis for infarct volumes in four groups (n = 8). Note that TAT-NEP1-40 significantly reduces the infarct volume. Values given are mean ± SD. * P < 0.05 versus control group. β-Gal = β-galactosidase; NEP1-40 = Nogo extracellular peptide 1-40; TAT = trans-activator of transcription.](http://anesthesiology.pubs.asahq.org/pdfaccess.ashx?url=/data/journals/jasa/931055/ on 04/01/2017)
TAT-NEP1-40 in comparison with PBS–, No-TAT-NEP1-40–, and TAT-/H9252/Gal–treated animals (P/H11021 0.05), as revealed by hematoxylin staining, but there was no difference among the latter three groups (fig. 3I).

Activity of Caspase-3

As shown in figure 4, immunohistochemistry analysis revealed that caspase-3 immunofluorescent cells were distributed primarily in the border zone of cortical infarction at 12 h after reperfusion. In ischemic animals treated with the vehicle (fig. 4A), No-TAT-NEP1-40 (fig. 4C), and TAT-β-Gal (fig. 4D), a considerable number of cells immunoreactive to activated caspase-3 were found in the border zone of cortical infarction 12 h after reperfusion. In contrast, the amounts of caspase-3 immunofluorescent cells greatly decreased in animals treated with TAT-NEP1-40 (P/H11021 < 0.01; fig. 4B).

Discussion

In the current study, by using a recombinant technique, we produced a biologically active NEP1-40 fusion protein containing the TAT-PTD that allows an in vivo delivery of the fusion protein across the BBB. We found that when TAT-NEP1-40 was injected intraperitoneally, the protein was efficiently transduced into brain parenchyma within 6 h. Furthermore, the entrance of TAT-NEP1-40 to brain significantly decreased the infarct sizes and improved the neurologic outcomes 24 h after reperfusion followed by 120 min of focal ischemia. The ischemia-induced neuronal apoptosis and activity of caspase-3 in the brain were also markedly attenuated by treatment with TAT-NEP1-40. These results provide the first evidence that the engineered TAT-NEP1-40 protein transduced into the brain still keeps biologic activity and protects the brain against ischemia–reperfusion injury through inhibition of neuronal apoptosis.

The NgR is thought to be the primary target of Nogo-66 action, though additional receptor molecules might interact with the other Nogo-A domains. Studies have demonstrated that NgR is also a functional receptor for two other known myelin-derived axonal outgrowth inhibitors, myelin-associated glycoprotein and oligodendrocyte myelin glycoprotein. Therefore, NgR is a central...
point for the convergence of three myelin inhibitors. Research on NEP1-40 peptide has provided evidence that CNS axon regrowth can be promoted by manipulating the inhibitory components of myelin. Because the NEP1-40 peptide specifically blocks the Nogo-NgR interaction, it seems that the peptide might be a potential candidate for therapeutic intervention after CNS injury. However, a primary obstacle for clinical use of NEP1-40 is the lack of an efficient, noninvasive method for delivery of this protein across the BBB in biologically relevant amounts. It would be of great importance to find out specific measures that transfer this biologically active protein across the BBB into the brain.

It is well known that the delivery of therapeutic proteins into tissues and across the BBB is severely limited by the size and biochemical properties of the respective proteins. Therefore, therapeutic compounds, peptide mimetics, and proteins may pass cell membranes only when the molecules are small, i.e., less than 600 Da. Diffusion of bioactive peptides across the BBB is also restricted to short (≤6 amino acids) and highly lipophilic peptides. As mentioned previously, cerebral ischemia induces disruption of the BBB, allowing large molecules including proteins to enter the brain. However, this disruption occurs after several hours of ischemia, whereas the therapeutic time window for most neuroprotective agents is less than 6 h. Therefore, to be more effective to achieve its therapeutic action, such neuroprotective agents must cross the BBB when it is still “intact.” Interestingly, it has been demonstrated that the PTD of TAT protein is a promising technique for disease therapy because of its ability to deliver a variety of full-length protein–peptides through the plasma membrane into cells and, most importantly, across the BBB into brain tissue in vivo. In this study, the translocation capability of TAT-NEP1-40 to the brain was evaluated in a rat model of focal cerebral ischemia by using Western blotting and immunohistochemical techniques. Our results showed that a translocation of TAT proteins into brain tissue including the ischemic and the contralateral hemisphere within 6 h after an intraperitoneal injection of TAT-NEP1-40 or TAT-β-Gal. Similar results were reported by other researchers, in whose studies 120-kd β-Gal protein and FITC peptide fused to TAT protein were injected intraperitoneally. They found a successful delivery of TAT-FITC peptide and TAT-β-Gal into various tissues in mice, including the brain. Taken together, these studies suggest that these TAT fusion proteins not only rapidly enter the brain parenchyma, but also keep their biologic activity, confirming that this method is effective for the therapy of brain diseases.

Recently, TAT-mediated delivery of proteins with therapeutic potential has been achieved in several models of nerve trauma and ischemia. Several groups have published positive results using PTD for the delivery of therapeutic proteins in relevant animal models of human ischemic stroke. Intravenous delivery of several TAT fusion proteins, such as Bcl-xL, glial cell line–derived neurotrophic factor, superoxide dismutase, and X chromosome–linked inhibitor of apoptosis and intraperitoneal administration of PTD-Bcl-xL fusion protein, efficaciously reduced brain injury in adult models of cerebral ischemia. Moreover, some studies have demonstrated that the blockade of the Nogo-NgR pathway can enhance axonal sprouting and thereby promote recovery after focal brain infarction. In rats with
MCAO, both motor skills and corticofugal axonal plasticity are promoted by intracerebroventricular administration of a functional NgR blocking fragment. Behavioral improvement is achieved when the therapy is initiated 1 week after arterial occlusion. Recently, after distal MCAO in stroke-prone renovascular hypertensive rats, the expression of Nogo-A in oligodendrocytes persistently increased, and its localization became redistributed around damaged axons and dendrites. Intracerebroventricular administration of NEP1-40 down-regulated the expression of Nogo-A, reduced axonal injury, and enhanced axonal regeneration, suggesting that inhibition of Nogo-A/NgR signal using NEP1-40 can reduce neuronal damage in the thalamus after distal MCAO. Similarly, NEP1-40 promoted regrowth of lesioned entorhinal hippocampal axons in vitro by blocking of Nogo-66/NgR signaling. Summarized from above, Nogo-66 and NgR have a central role in limiting axonal regeneration after CNS injury, and interruption of the Nogo-NgR pathway can enhance functional recovery and axonal regeneration. As a matter of course, the NEP1-40 is a potential therapeutic agent for neuronal damage.

The death-suppressing effect of TAT-NEP1-40 fusion proteins was initially confirmed in cultures of PC12 cells in our previous study, in which TAT-NEP1-40 but not TAT-β-Gal inhibited oxygen and glucose deprivation-induced apoptotic death (additional information regarding this is available on the ANESTHESIOLOGY Web site at http://www.anesthesiology.org). Because of its cytoprotective effects, we chose to study the effects of TAT-NEP1-40 fusion protein in vivo in the current study. In a highly reproducible focal cerebral ischemia model induced by MCAO, we demonstrated that intraperitoneal administration of TAT-NEP1-40 at a dose of 40 nmol resulted in reduction of infarct sizes and improvement of neurologic function 24 h after focal ischemia and reperfusion. This efficacy likely results from the direct protection by TAT-NEP1-40, because the protection occurred without altering body temperature or other physiologic parameters during or after ischemia. At 12 h after reperfusion, the TUNEL labeling appeared intense and abundant, whereas the intensity of hematoxylin staining was significantly reduced in these damaged structures as visualized by TUNEL staining and counterstained with hematoxylin staining, indicating a decreased viability of the ischemic cells as a result of the ischemic injury. Intraperitoneal administration of TAT-NEP1-40 at the onset of reperfusion significantly reduced the number of TUNEL-positive cells and increased the viability of the ischemic cells in ischemic areas after 12 h reperfusion, indicating that the protective effect of TAT-NEP1-40 against ischemic injury may be mediated by an antiapoptotic mechanism.

Activation of caspase-3 is a key step in the apoptosis pathway. Decreased apoptosis was observed in caspase-3 knockout mouse or treatment with caspase-3 inhibitors (Z-VAD-FMK), whereas increased caspase-3 activity has been demonstrated in transient and permanent focal ischemia. In a rat focal ischemia model, caspase-3 enzymatic activity in the penumbral cortex peaked at 6–12 h after ischemia. Consistent with other reports, the results from the current study showed that ischemia induced an increase in caspase-3 activity, whereas treatment with TAT-NEP1-40 reduced the caspase-3 activity at 12 h after reperfusion. The reduction of caspase-3-activity could be attributed to an overall reduction of neuronal injury, thereby reducing the amount of toxic agent that induces caspase-3 activation, but not direct inhibition of the enzyme. Therefore, we deduce that TAT-NEP1-40 transferred into brain parenchyma exerts its neuroprotective effect through reduction of brain damage and as a consequence, caspase-3 activity is decreased.

Previous studies have demonstrated that cellular expression of Nogo-A and NgR are significantly up-regulated in the ischemic cortex where neuronal damage occurs, suggesting a role of Nogo-A and NgR in cerebral pathologic damage. When Nogo-A binds and activates NgR, NgR is then coupled with P75 neurotrophin receptor. Activation of the NgR-P75 leads to activation of the Rho and Rho kinase pathway, which induces caspase-3 activation and results in neurons apoptosis, suggesting an important role of the complex signaling of NgR pathway in ischemia-induced neuronal apoptosis. Although we did not explore the detailed mechanism underlying the protective effect of TAT-NEP1-40 observed, we speculate that blockage of the interaction of Nogo-A with NgR using TAT-NEP1-40 might inhibit the activation of the NgR-P75 complex, suppress the downstream effector RhoA activation, and reduce caspase-3 activity and neuronal apoptosis through a variety of downstream effectors.

In the current experiment, protection of brain tissue was seen when NEP1-40 was conjugated with TAT and administered after ischemia. The result demonstrates that the TAT system as a carrier is a powerful tool for delivery of curative protein into the brain. Because the manipulation of the method is simple and the result is elegant, no surgical interventions (such as trephination of the skull) are required, and the proteins reliably reach their site of action. Therefore, fusion proteins may be a useful tool in transporting large molecules across the BBB.

In conclusion, the current study shows that intraperitoneal delivery of TAT-NEP1-40 has a neuroprotective effect on focal cerebral ischemic injury. The mechanism might be associated with attenuation of caspase-3 activity and neuronal apoptosis. Our experimental results also indicate that the TAT-mediated protein transduction can be used to deliver biologically active neuroprotective agents across the BBB, providing a potential application in treatment of stroke, traumatic brain injury, and other neurologic diseases. However, the long-term effect of TAT-NEP1-40 on brain plasticity after focal ischemia.
deserves further investigation, and the associated molecular mechanisms underlying the TAT-NEP1-40 neuroprotective effect need to be further explored.

The authors also thank Steven F. Dowdy, Ph.D. (Professor, Howard Hughes Medical Institute and Department of Cellular and Molecular Medicine, University of California San Diego, School of Medicine, La Jolla, California), for providing the pTAT-HA and pTAT-HA-β-galactosidase vectors.

References


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