Humanin Is a Novel Neuroprotective Agent Against Stroke

Xingshun Xu, MD; Chu C. Chua, PhD; Jinping Gao, MD; Ronald C. Hamdy, MD; Balvin H.L. Chua, PhD

Background and Purpose—Humanin (HN) is a 24-amino acid peptide best known for its ability to protect neurons from damage caused by Alzheimer disease-related proteins. This study examines the neuroprotective effects of HNG (a potent form of HN) on focal cerebral ischemia/reperfusion injury in mice.

Methods—Mice underwent middle cerebral artery occlusion for 75 minutes followed by 24-hour reperfusion. Mice were pretreated with 0.1 μg HNG (intracerebroventricularly) 30 minutes before ischemia; posttreated at 0, 2, 4, and 6 hours after ischemia; or pretreated with 1 μg HNG (intraperitoneally) 1 hour before ischemia. Neurological deficits and cerebral infarct volume were evaluated. Neuronal apoptosis and activated poly(ADP-ribose) polymerase expression were measured by TUNEL and Western blot analysis, respectively. Activated ERKs were examined by Western blot analysis.

Results—Pretreatment with 0.1 μg HNG (intracerebroventricularly) 30 minutes before ischemia reduced cerebral infarct volume from 56.2±3.0% to 26.1±1.4% (P<0.01). HNG posttreatment after 4 hours of reperfusion reduced cerebral infarct volume to 45.6±2.6% (P<0.05). Pretreatment with 1 μg HNG (intraperitoneally) 1 hour before ischemia or posttreatment after 2 hours of reperfusion reduced cerebral infarct volume significantly. HNG also significantly improved neurological function and inhibited both neuronal apoptosis as well as poly(ADP-ribose) polymerase activation. A significant decrease of phospho-ERK was observed in mice treated with HNG, whereas phospho-JNK and phospho-p38 levels were not altered.

Conclusions—Our results demonstrate that HNG protects against cerebral ischemia/reperfusion injury in mice. HNG offers neuroprotection in vivo at least in part by inhibiting ERK activation. These findings suggest a potential therapeutic role for HNG in the treatment of stroke. (Stroke. 2006;37:2613-2619.)

Key Words: cerebral ischemia ■ humanin ■ MAP kinase ■ neuroprotection ■ stroke
group (I/R group); and (4) HNG-treated I/R group (I/R+HN group).
In the I/R+HN group, the animals were divided into several subgroups: pretreatment group and posttreatment 0-hour, 2-hour, 4-hour, and 6-hour groups. HNG-treated groups were administered 0.1 μg HNG (Peptide International Inc) in 10 μL saline intraventricularly or 1 μg in 100 μL saline intraperitoneally (IP). Vehicle-treated groups were administered saline 10 μL intraventricularly or 100 μL IP.

Middle Cerebral Artery Occlusion Model
Middle cerebral artery occlusion (MCAO) was induced as described by Clark et al.9 Briefly, a 6-0 nylon monofilament (Ethilon, Ethicon Inc) coated with silicon resin (Heraeus) was introduced into the right common carotid artery and advanced 9 to 11 mm until a faint resistance was felt, signifying MCAO. Reperfusion was achieved by withdrawing the suture after 30 minutes or 75 minutes of occlusion. Body temperature was maintained at 36.5 to 37.5°C by means of a heating blanket and a lamp throughout the procedure from the start of the surgery until the animals recovered from anesthesia. Monitoring of occlusion and reperfusion of the middle cerebral artery was achieved using a laser Doppler blood flowmeter (Periflux 5010; PERIMED) positioned 1 mm posterior and 3 mm lateral to the bregma bilaterally. To record physiological parameters, a polyethylene catheter was inserted into the femoral artery. Arterial blood pressure, heart rate, pH, pO2, pCO2, temperature, and plasma glucose were measured 30 minutes before ischemia or after 75 minutes of ischemia and 30 minutes of reperfusion.

Intraventricular Administration of HNG
A small burr hole was made in the left parietal region (0.5 mm posterior and 1.0 mm lateral to the bregma on the left side). A 28-G needle was inserted into the left lateral ventricle (2.5 mm in depth), and 10 μL of HNG or saline was injected over a period of 5 minutes.

Neurological Deficit Scoring Evaluation
Neurological deficits were measured 24 hours after MCAO. The scoring system was based on the five-point scale system described by Yang et al.10

Detection of Infarction Volume
After neurological evaluation of the mice, brains were removed and sliced into 1-mm sections. Slices were incubated for 30 minutes in 0.1% solution of 2,3,5-triphenyltetrazolium chloride (TTC; Sigma) at 37°C, fixed in 10% buffered formalin phosphate (Fisher Scientific) and photographed. The infarct area in each brain slice was quantified using light microscopy at 40x magnification, and 10 fields for each section were selected from the cortex. The number of TUNEL-positive cells in each field was counted and expressed as cell number/mm² through a scale calibration.

Western Blot Analysis
The animals were killed after 30 minutes of ischemia followed by 30 minutes, 2 or 4 hours of reperfusion. A period of 30 minutes of ischemia was chosen because minimal cell death was detected by TTC staining. The whole right hemisphere was quickly removed and pulverized into powder in liquid nitrogen. After protein extraction and determination of protein concentration, proteins (75 μg) were electrophoresed and electrotransferred to nitrocellulose membrane. Blots were incubated with primary antibodies overnight at 4°C. After washing, blots were incubated with HRP-conjugated secondary antibody for 1.5 hours. Blots were developed with the ECL chemiluminescence system (GE Healthcare) and were captured on autoradiographic films (Kodak). Films were scanned and densitometric analysis of the bands was performed with AlphaEase Image Analysis Software.

Anti-phospho-(Thr180/Tyr182)-p38, rabbit polyclonal anti-p38, rabbit polyclonal anti-JNK, anti-ERK1/2, anticleaved PARP, and β-tubulin antibodies were purchased from Cell Signaling Technology, Inc. Mouse monoclonal anti-phospho-(Thr202/Tyr204)-ERK1/2 and mouse monoclonal anti-phospho-(Thr183/Tyr185)-JNK antibodies were obtained from Santa Cruz Biotechnology Inc.

Statistical Analysis
All data are expressed as mean±SEM. Differences between groups were determined with the Student t test for infarct volume; differences among groups were compared by one-way analysis of variance followed by Tukey multiple-comparison test if there was a significant difference between groups. Differences were deemed statistically significant if P<0.05.

Results
HNG Improves Neurological Deficits After Ischemia/Reperfusion Injury
Figure 1 shows neurological scores after 75 minutes of MCAO and 24 hours of reperfusion in the different experimental groups. There were no significant neurological deficits in the sham group and HN group. Severe neurological deficits were observed in the I/R group, including circling movements, postural abnormalities, severe paw flections, and less spontaneous movements. HNG treatment (0.1 μg per
mouse) significantly improved the neurological deficits in I/R+HN group (P<0.01), as evidenced by fewer disturbances in posture and circling movements. I/R+HN group mice had significantly improved neurological scores compared with sham group mice (P<0.01). Physiological parameters were measured 30 minutes before and after MCAO. HNG administration had no significant effect on arterial blood pressure, heart rate, pH, pO2, pCO2, temperature, or plasma glucose (Table).

### Physiological Parameters of Control and HNG-Treated Mice

<table>
<thead>
<tr>
<th></th>
<th>Saline-Treated Mice</th>
<th>HNG-Treated Mice</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before Ischemia</td>
<td>After Ischemia</td>
</tr>
<tr>
<td>Mean arterial blood pressure, mm Hg</td>
<td>76.2±2.4</td>
<td>77.0±2.6</td>
</tr>
<tr>
<td>Heart rate, beats/min</td>
<td>474±23</td>
<td>461±17</td>
</tr>
<tr>
<td>pH</td>
<td>7.29±0.02</td>
<td>7.27±0.02</td>
</tr>
<tr>
<td>pO2, mm Hg</td>
<td>31.4±1.5</td>
<td>33.4±2.1</td>
</tr>
<tr>
<td>pCO2, mm Hg</td>
<td>108.2±6.6</td>
<td>109.6±8.1</td>
</tr>
<tr>
<td>Temperature, °C</td>
<td>37.0±0.1</td>
<td>37.0±0.1</td>
</tr>
<tr>
<td>Glucose, mg/dL</td>
<td>92.9±8.2</td>
<td>103.5±11.0</td>
</tr>
</tbody>
</table>

Values are expressed as mean±SEM. These parameters were measured at 30 minutes before ischemia or after 75 minutes of ischemia and 30 minutes of reperfusion (n=6 in each group).

### HNG Reduces Infarct Volume

There was no detectable infarction in the sham and HN groups, whereas there was a large infarct volume in the I/R group as shown in Figure 2A. The I/R+HN group had a total infarct volume of 26.1±1.4%, which was 54% lower than the volume of the I/R group (56.2±3.0%; P<0.01) (Figure 2B). HNG posttreatment at 0 hour, 2 hours, and 4 hours after MCAO decreased infarct size, but this effect was not significant when HNG was given 6 hours after MCAO (Figure 2C).

---

Figure 2. The protective effects of HNG treatment on cerebral infarct volume in ischemia/reperfusion (I/R) injury. Mice were treated with HNG or saline. After 75 minutes of ischemia and 24 hours of reperfusion, cerebral infarct volume was determined by TTC staining. (A) Representative photographs of TTC-stained brain sections. Mice were treated with 0.1 µg HNG (intracerebroventricularly [ICV]) or saline 30 minutes before ischemia. (B) Quantitative analysis of cerebral infarct volume in I/R group and I/R+HN group. Mice were treated with 0.1 µg HNG (ICV) or saline 30 minutes before ischemia. (C) Quantitative analysis of cerebral infarct volume after HNG posttreatment. Mice were treated with 0.1 µg HNG (ICV) or saline at 0 hour, 2 hours, 4 hours, and 6 hours after 75 minutes of ischemia. (D) Quantitative analysis of cerebral infarct volume after HNG treatment (intraperitoneally). Mice were treated with 1 µg HNG (intraperitoneally) or saline 1 hour before ischemia or at 2 hours and 4 hours after 75 minutes of ischemia. Bars represent means±SEM of six to eight brains. *P<0.01 vs I/R group, **P<0.05 vs I/R group.
We analyzed infarct size in the cortex and striatum separately to verify whether the protective effect of HNG on infarct volume is similar in these parts of the brain. In the I/R group, the infarct volumes of cortex and striatum were 46.5±2.6% and 8.8±0.7%, respectively. In the I/R+HN group, the infarct volumes of cortex and striatum were 21.3±1.1% and 4.8±0.5%, respectively. Overall, HN treatment decreased the infarct volume in cortex by 54% and the infarct volume in striatum by 45%. This data indicates that HN reduces infarct size in both cortex and striatum (P<0.01).

Because intraperitoneal administration of HNG would be more feasible relevant than intraventricular administration in a clinical setting, we administered 1 μg HNG (IP) 1 hour before occlusion. In this experiment, infarct volume decreased from 54.1±3.2% in the I/R group to 34.8±1.3% in the I/R+HN group (P<0.01, Figure 2D). HNG posttreatment (IP) 2 hours after MCAO also decreased infarct size, but this protective effect was not seen when HNG was given 4 hours after MCAO.

**HNG Attenuates Neuronal Apoptosis**

To explore the effects of HNG on apoptosis, TUNEL staining was used to identify apoptotic cells in the parietal cortex 24 hours after MCAO. Control sections incubated without TdT enzyme showed no staining (results not shown). Brain sections from the sham and HN group showed very few TUNEL-positive nuclei, whereas there was an increase in TUNEL-positive cells in the I/R group. The positively stained apoptotic nuclei were observed in the infarct area and penumbra of the ischemic hemisphere. The increase in TUNEL-positive cells was inhibited in the I/R+HN group (Figure 3A). Quantitative analysis of TUNEL-positive cells (Figure 3B) revealed that TUNEL-positive nuclei in the ischemic cortex were reduced from 678.1±48.1/mm² in the I/R group to 234.3±32.3/mm² in I/R+HN group (P<0.01).

**HNG Decreases Cleaved Poly(ADP-ribose) Polymerase**

Poly(ADP-ribose) polymerase (PARP) is a substrate for caspase-3, and cleaved PARP is an important marker for caspase-3 activity.13 Western blot analysis was performed after 1 hour of ischemia and 24 hours of reperfusion to measure cleaved PARP. The I/R group showed a robust increase of cleaved PARP (89 kDa) in the ischemic hemisphere. In contrast, cleaved PARP was attenuated in the I/R+HN group (Figure 3C). As shown in Figure 3D, quan-

---

**Figure 3.** The protective effects of HNG on neuronal apoptosis and cleaved PARP. Neuronal apoptosis was detected by TUNEL in the parietal cortex after 30 minutes of ischemia and 24 hours of reperfusion. (A) Representative photomicrographs of TUNEL staining in four groups. (B) TUNEL-positive nuclei in parietal cortex were quantified by random counting of 10 fields per section and converted to cell number per mm². Cleaved PARP (89 kDa) was examined by Western blot analysis in ischemic whole right hemisphere tissue after 1 hour of ischemia and 24 hours of reperfusion. (C) Representative Western blots of cleaved PARP in four groups. β-tubulin (60 kDa) was used as a loading control. (D) Quantitative analysis of cleaved PARP in four groups. Bars represent means±SEM of six brains. *P<0.01 vs ischemia/reperfusion group.
titative analysis revealed that HNG significantly decreased cleaved PARP levels after MCAO compared with the I/R group (P < 0.01).

**HNG Inhibits ERK Activation**

The mitogen-activated protein kinase family consists of ERK, JNK, and p38. In this study, phospho-ERK1/2 (p-ERK) reached a peak at 30 minutes of reperfusion, and levels remained high even 4 hours after reperfusion. As shown in Figure 4A, p-ERK was elevated at 30 minutes after reperfusion, and this elevation was attenuated in I/R HN group mice. Quantitative analysis of the ratio of p-ERK to total ERK revealed that there was a significant difference between the I/R HN group and I/R group at 30 minutes, 2 hours, and 4 hours (P < 0.01, Figure 4B through 4D).

**HNG Has No Effects on JNK and p38 Activation**

We also examined the activation of the other two members of the mitogen-activated protein kinase family (JNK and p38) by Western blot analysis. Phospho-JNK (p-JNK) and phospho-p38 (p-p38) increased to peak levels 2 hours after reperfusion (results not shown). HNG had no effect on p-JNK levels (P > 0.05, Figure 5A and 5B) or p-p38 levels in the I/R HN group (Figure 5A and 5C).

**Discussion**

This study demonstrates for the first time that HNG protects against cerebral I/R injury in a mouse MCAO model as evidenced by reduced infarct volume and decreased neuronal apoptosis. In addition, this study suggests that HNG is able to cross the blood–brain barrier (BBB). Finally, inhibition of ERK by HNG is likely involved in the protective mechanism of HNG.

**Neuroprotective Effect of HNG Against Cerebral Ischemia/Reperfusion Injury**

There is increasing evidence that severe ischemic injury may cause high glutamate levels, acute Ca²⁺ overload, and free radicals, all of which lead to necrosis and apoptosis. Apoptosis is actively executed by several members of the caspase family, including caspase-3, which is involved in the final execution phase of apoptosis. Increased neuronal caspase-3 expression is observed in transient ischemia injury. In this study, I/R injury increased neuronal apoptosis and the level of cleaved PARP, a caspase-3 substrate and marker for caspase-3 activity. HNG administration attenuated neuronal apoptosis in the cortex and the level of cleaved PARP in the ischemic hemisphere (Figure 3).

**HNG Crosses the BBB in the Ischemic Brain**

As a result of their size and charge, most peptides and protein drugs have limited access to the brain for central nervous system diseases. Although HNG is a small peptide with MW 2860, there is no significant amount of evidence as to whether HNG can cross the BBB. Krejcova et al showed that administration of HNG (IP) in rats reversed the impairment of spatial memory induced by 3-quinuclidinyl benzilate, suggesting that HNG can cross the BBB in the absence of a disrupted BBB. It is not clear exactly how HNG crosses the BBB. HNG may cross the BBB through transporters or HN
receptors in the endothelial cells in a manner similar to erythropoietin, which crosses the BBB by binding to the abundant erythropoietin receptors present in brain capillaries. In our studies, we showed that HNG administration (IP) reduces infarct volume. In and of itself, this result could mean that the disruption of BBB endothelial cells by I/R injury facilitated the crossing of HNG through the BBB. Alternatively, it is possible that HNG can cross the BBB without disruptions such as ischemic brain damage.

The Protective Effect of HNG Is Mediated by a Receptor-Dependent Inhibition of ERK Activity, but It Is Not Mediated by JNK or p38 Activity

Recent evidence suggests that activated ERK1/2 contributes to neuronal cell death and that inhibition of ERK1/2 activation provides beneficial effects after focal I/R injury. In this study, we observed a marked decrease of p-ERK by HNG after 30 minutes of ischemia followed by 30 minutes, 2 hours, or 4 hours of reperfusion (Figure 4). It is not clear exactly how inhibition of p-ERK by HNG is related to HNG’s neuroprotective effects. It has been reported that inhibition of p-ERK attenuates phosphosynapsin I, a substrate of ERK that is necessary for the maintenance of synaptic vesicle contact with actin filaments. Maintenance of synaptic vesicle contact results in a decreased release of excitotoxic amino acids such as glutamate, thus protecting against cerebral I/R injury. Alternatively, inhibition of the ERK1/2 pathway after focal cerebral ischemia may also lead to a reduction of some proapoptotic transcriptional gene products such as c-fos.

JNK activation plays an important role in stress-induced apoptosis. Inhibiting JNK activity may offer an effective antiapoptotic strategy in vitro and in vivo. Hashimoto et al reported that HN inhibits amyloid precursor protein-induced neuronal cell death by suppressing JNK activation and ASK1/JNK pathway in vitro. However, in our study, HNG did not affect p-JNK levels after I/R injury (Figure 5A and 5B). In addition, phospho-p38 levels were not altered by HNG after cerebral I/R injury in mice. This suggests that p38 is not involved in HNG’s neuroprotective mechanism. Whether HN peptide acts from the outside or the inside of cells remains controversial. Hashimoto et al has argued that HN must be secreted from cells or added exogenously to bind to cell surface receptors, thereby triggering cell survival signal pathways. Reed’s group demonstrated that HN acts inside the cell without requiring secretion and that HN sequesters Bax, Bid, or BimEL in the cytoplasm to suppress apoptosis. In addition, only membrane-permeable HN containing poly-arginine provided protection against apoptosis. However, our experiments demonstrate that HNG, which is unable to get inside the cells, reduces the infarct volume and rescues neuronal cells from apoptosis induced by cerebral I/R injury. These results support the contention that HNG binds to its surface receptors and initiates survival pathways to protect against cerebral I/R injury.

In summary, we demonstrate that HNG protects against I/R injury in a mouse MCAO model. As a result of its ability to cross the BBB in the ischemic brain, HNG may have therapeutic potential as a protective agent against stroke. The therapeutic potential of this agent warrants future study.

Sources of Funding

This study was supported by grants from American Heart Association–Southeast Affiliate and VA Merit Review.
Disclosures
None.

References
Humanin Is a Novel Neuroprotective Agent Against Stroke
Xingshun Xu, Chu C. Chua, Jinping Gao, Ronald C. Hamdy and Balvin H.L. Chua

*Stroke*. published online September 7, 2006;
*Stroke* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2006 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/early/2006/09/07/01.STR.0000242772.94277.1f.citation

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/