Kallikrein Gene Transfer Protects Against Ischemic Stroke by Promoting Glial Cell Migration and Inhibiting Apoptosis

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Abstract—Kallikrein/kinin has been shown to protect against ischemia/reperfusion-induced myocardial infarction and apoptosis. In the present study, we examined the potential neuroprotective action of kallikrein gene transfer in cerebral ischemia. Adult, male Sprague-Dawley rats were subjected to a 1-hour occlusion of the middle cerebral artery followed by intracerebroventricular injection of adenovirus harboring either the human tissue kallikrein gene or the luciferase gene. Kallikrein gene transfer significantly reduced ischemia-induced locomotor deficit scores and cerebral infarction after cerebral ischemia injury. Expression of recombinant human tissue kallikrein was identified and localized in monocytes/macrophages of rat ischemic brain by double immunostaining. Morphological analyses showed that kallikrein gene transfer enhanced the survival and migration of glial cells into the ischemic penumbra and core, as identified by immunostaining with glial fibrillary acidic protein. Cerebral ischemia markedly increased apoptotic cells, and kallikrein gene delivery reduced apoptosis to near-normal levels as seen in sham control rats. In primary cultured glial cells, kinin stimulated cell migration but inhibited hypoxia/reoxygenation-induced apoptosis in a dose-dependent manner. The effects of kinin on both migration and apoptosis were abolished by icatibant, a bradykinin B2 receptor antagonist. Enhanced cell survival after kallikrein gene transfer occurred in conjunction with markedly increased cerebral nitric oxide levels and phospho-Akt and Bcl-2 levels but reduced caspase-3 activation, NAD(P)H oxidase activity, and superoxide production. These results indicate that kallikrein gene transfer provides neuroprotection against cerebral ischemia injury by enhancing glial cell survival and migration and inhibiting apoptosis through suppression of oxidative stress and activation of the Akt–Bcl-2 signaling pathway. (Hypertension. 2004;43[part 2]:1-8.)

Key Words: ischemia ■ stroke ■ kinins ■ gene transfer ■ apoptosis

Stroke is the third leading cause of death and the most common cause of disability in the United States. Although drug therapy remains the sole choice of treatment for stroke patients, there has been no conclusive evidence of long-lasting motor and cognitive improvement with any of the current drugs. Therefore, a search for suitable regimens to rescue the central nervous system after ischemia has been a major research endeavor.

Reperfusion injury is thought to play a critical role in the pathophysiology of cerebral ischemia. Reactive oxygen species (ROS) are implicated in reperfusion injury after cerebral ischemia. Increased oxidative stress during reperfusion exacerbates cerebral ischemia–induced neuronal apoptosis. Tissue kallikrein is capable of cleaving low-molecular-weight kininogen to form vasoactive kinin peptides. The ability of kinin to dilate cerebral arterial vessels is due in part to the release of endothelium-derived relaxing factor/nitric oxide (NO). Other than vasodilation, recent studies have shown that kallikrein/kinin also plays a vital role in angiogenesis and apoptosis in response to hindlimb or cardiac ischemia. NO has been known to play a complex role in cerebral ischemia.

Excitotoxic or ischemic conditions excessively activate neuronal NO synthase (NOS), resulting in production of NO that is toxic to surrounding neurons. Inducible NOS, which is not normally present in healthy tissues, is induced shortly after ischemia and contributes to secondary late-phase damage. Conversely, NO generated from endothelial NOS (eNOS) is critical in maintaining cerebral blood flow and reducing infarct volume. Upregulation of eNOS resulted in neuroprotection against cerebral ischemia and reduction of stroke damage. Taken together, these results suggest that the tissue kallikrein/kinin system, through activation of eNOS and subsequent NO production, might modulate endothelial function and promote cell survival and could have protective effects on the brain in the setting of ischemic stroke.

Our previous study demonstrated that adenovirus-mediated gene transfer of human tissue kallikrein ameliorated a salt-induced blood pressure rise and cerebral damage and reduced the stroke-induced mortality rate in Dahl salt-sensitive rats. To determine whether kallikrein/kinin has neuroprotective effects against ischemic stroke, we used a rat model of focal cerebral ischemia by 1-hour occlusion of the middle cerebral...
artery (MCA) followed by reperfusion and immediate intracerebroventricular injection of adenosivirus carrying the human tissue kallikrein gene. We examined the potential effects of kallikrein gene transfer on cerebral cell survival and angiogenesis in the brain after ischemia/reperfusion (I/R). Our results show that kallikrein/kinin protects against ischemic stroke, characterized by a significant reduction in motor deficits, cerebral infarction, and apoptosis, as well as promotion of glial cell survival and migration.

Methods

Animals and Treatments

Twenty-four male, Sprague-Dawley rats (225 to 250 g body weight; Harlan Sprague-Dawley, Indianapolis, Ind) were used in this experiment. All procedures complied with standards for the care and use of animal subjects, as stated in the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Resources, National Academy of Sciences, Bethesda, Md).

Cerebral I/R surgery was performed as previously described. In brief, the rats were anesthetized intraperitoneally with ketamine/xylazine (90 mg/10 mg per kg body weight). A nylon filament (Ethicon 4-0, with the tip diameter tapered to the size of a 26-gauge needle) was inserted from the right external carotid artery by way of the internal carotid artery to the base of the right MCA to stop the blood flow. After 1 hour of ischemia, the filament was withdrawn, blood reperfusion was established, and the skin was sutured. Adenosivirus containing human tissue kallikrein cDNA (Ad.CMV-TK) or control virus (Ad.CMV-Luc) (1×1011 plaque-forming units in 10 μL) was stereotactically delivered into the right lateral cerebral ventricle (1.5 mm lateral, 1.0 mm posterior to the bregma and 4.5 mm deep from the skull surface) immediately after reperfusion (n=9). Body temperature of the animals was maintained at 37°C during surgery until they recovered from anesthesia. An additional group of animals (n=6) underwent sham surgery as controls.

Behavioral Test

Rats were tested for neurologic deficits after cerebral I/R. The scoring scale was as follows: 0=no observable neurologic deficits; 1=failure to extend right forepaw (mild); 2=circling to the contralateral (moderate); and 3=loss of walking or righting reflex (severe). Rats with neurologic deficit scores ≥2 after full recovery from anesthesia and surgery were used in the experiment.

Cerebral Infarct Staining With TTC

At day 9 after surgery, the animals were again anesthetized intraperitoneally with ketamine/xylazine (90 mg/10 mg per kg body weight) and perfused transcardially with 0.9% saline, and the brains were removed and weighed. Serial coronal brain sections (~2 mm thick) were immersed in normal saline containing 2% 3,3,5-triphenyltetrazolium chloride (TTC) for 30 minutes at 37°C. To minimize artifacts produced by postischemic edema in the infarcted area, the infarct volume was calculated with an alternate technique, as previously described.

In brief, the infarcted area in the ipsilateral hemisphere from the total intact area of the contralateral hemisphere was determined by counting ~400 to 500 glial cells in 5 randomly chosen fields.

Immunohistochemistry and Double Immunofluorescence

Sections (5 μm) of paraffin-embedded brain were subjected to immunohistochemistry with use of a staining kit (Universal Elite ABC, Vector) and performed according to the manufacturer’s instructions. An affinity-purified anti-human kallikrein antibody (5 μg/mL) and an anti-glial fibrillary acidic protein (GFAP, Chemicon) antibody were used for immunostaining. For double immunofluorescence analyses, sections were incubated with a mixture of anti-human kallikrein (5 μg/mL) and anti-monocyte/macrophage (ED1) antibodies (Chemicon), followed by a mixture of anti-mouse IgG–tetramethylrhodamine B isothiocyanate and anti-rabbit IgG–fluorescein isothiocyanate antibodies (Sigma). Cerebral apoptosis was analyzed by TdT-mediated dUTP nick end-labeling (TUNEL) assay. The TUNEL-positive (apoptotic) cells were counted in 10 microscopic fields of each brain section.

ELISA for Human Tissue Kallikrein

The levels of immunoreactive human tissue kallikrein in rat brain were measured by ELISA.

Primary Cultured Glial Cells and Migration Assay

In brief, brains were removed from fetal Sprague-Dawley rats on embryonic day 15 and incubated in Hanks’ solution chilled on ice. The tissues were chopped into small pieces and incubated with Ca2+-free Hanks’ solution containing trypsin (0.05 mg/mL) and collagenase (0.01 mg/mL) at 37°C for 30 minutes, followed by the addition of soybean trypsin inhibitor (0.1 mg/mL) and DNase (0.1 mg/mL). The tissue was then centrifuged for 5 minutes (1000 rpm), and the pellet was resuspended in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum and D-glucose (4.5 mg/mL). Cells were plated on 60-mm tissue-culture dishes. The culture dishes were kept in a humidified incubator under 5% CO2 and 95% air at 37°C for 14 to 21 days. After 14 to 21 days, glial cells were stained by GFAP antibody for confirmation.

Cell migration assays were performed in 24-well modified Boyden transwell microchambers (Costar) containing 8-μm pore size polyelectylen eatheralate. The lower surface of each filter was coated with phospho-buffered saline containing 6.5 μg/mL rat fibronectin (30 minutes at 37°C) and then blocked with 1% bovine serum albumin in serum-free DMEM for 30 minutes at 37°C. For each assay, 104 cells were resuspended in 200 μL of serum-free DMEM and plated on the upper chamber, and the lower wells were filled with either serum-free DMEM (background migration), DMEM with bradykinin (0.1, 1, or 5 μM/L), in the absence or presence of icatibant (5 μg/mL), or DMEM supplemented with 10% fetal calf serum (maximal migration control) and incubated at 37°C for 6 hours. Cells remaining in the upper surface of the filter were removed with a cotton swab, and cells that had migrated (lower surface) were fixed and stained with a Diff-Quik stain set (Dade Behring). The number of cells that had migrated through the filter was counted under an inverted microscope and expressed as the percentage of migrated cells measured in the presence of 10% fetal calf serum.

Hypoxia/Reoxygenation-Induced Glial Cell Apoptosis

Cultured glial cells were growth-arrested in serum-free medium for 18 hours at 37°C before the experiments. Cells were incubated for 12 hours under hypoxic conditions (95% N2 and 5% CO2), followed by 24-hour reoxygenation (95% O2 and 5% CO2). Before hypoxia/reoxygenation (H/R), glial cells were incubated with bradykinin (0.1 and 1 μM/L) with or without icatibant (10 μM/L) for 60 minutes. Apoptotic glial cells were identified (fixed in 4% paraformaldehyde) by TUNEL staining (Roche), as previously described. The positive cells were determined by counting ~400 to 500 glial cells in 5 randomly chosen fields.

Western Blot Analyses of Bel-2, Akt, and Cleaved Caspase-3

Brain extracts (80 to 120 μg) were subjected to Western blot analyses. Antibodies to Bel-2 (Santa Cruz Biotech), phospho-Akt, total Akt (Cell Signaling Technology), and cleaved caspase-3 (Cell Signaling Technology) were used in the study.

Caspase-3 Activity Assay

Brain extracts were incubated at 37°C for 30 minutes with caspase-3 fluorogenic substrate II (Calbiochem). To measure nonspecific hydrolysis of caspase-3 substrate II, a preparation of an inhibitor-
treated brain extract was used. Caspase-3 activity was quantified in a Perkin-Elmer fluorometer with excitation at 380 nm and emission at 460 nm.

Measurements of Nitrate and Nitrite Levels, NAD(P)H Oxidase Activity, and Superoxide Formation
Nitrate and nitrite (NOx) levels in brain extracts were measured as previously described.22 NADH/NADPH oxidase activity was measured by lucigenin-enhanced chemiluminescent detection of superoxide in a luminometer (TD-20/20, Turner Designs).23 Superoxide production was measured by ferricytochrome c reduction, and the superoxide dismutase–inhibitable reduction of cytochrome c was measured at 550 nm.24

Statistical Analysis
Results are expressed as mean±SEM. Comparisons among groups were made by ANOVA followed by Fisher protected least significant difference test or by unpaired Student t test. Differences were considered significant at P<0.05.

Results
Kallikrein Gene Transfer Reduced Neurologic Deficit Scores and Cerebral Infarction
Neurologic deficit scoring was performed at days 1 and 9 after gene transfer. At day 9, neurologic deficit scores were significantly decreased in the kallikrein group compared with those in the luciferase group after cerebral I/R (0.44±0.24 versus 1.67±0.49; n=9, P<0.05; Figure 1A). However, there was no difference in neurologic deficit scores between the 2 groups at 1 day after gene delivery (data not shown). Figure 1B shows representative images of serial coronal brain sections stained with TTC from each group. Brain sections from the sham rat without infarction are stained red. Focal cerebral infarction in the right hemisphere in the rat from the Ad.CMV-Luc group after cerebral I/R is stained white. After kallikrein gene delivery, brain sections appeared reddish and relatively normal. The total infarct volume in the Ad.CMV-TK group was significantly reduced compared with that of the Ad.CMV-Luc group (11.6±0.7 versus 8.4±1.8 mm3/rat; P<0.05, n=4; Figure 1C).

Expression and Localization of Human Tissue Kallikrein in Ischemic Rat Brain After Gene Transfer
Recombinant human kallikrein in the rat brain after gene delivery was identified by double immunostaining and ELISA. Figure 2A shows representative immunostaining of human tissue kallikrein in the ischemic brains of rats that had received kallikrein gene transfer. Figure 2B shows double immunofluorescence of human tissue kallikrein and ED1 in the ischemic brains of rats. Human tissue kallikrein and ED1 were colocalized near areas of tissue damage after gene transfer. Human kallikrein levels in the rat brain reached a level of 0.29±0.12 ng/mg protein (n=3), as measured by ELISA 9 days after kallikrein gene delivery but were not detected in control rats.

Effects of Kallikrein Gene Transfer on Glial Cell Survival and Cerebral Apoptosis
Figure 3A shows immunostaining of GFAP, a marker for glial cells. Increased staining of GFAP surrounding the ischemic core was observed in the Ad.CMV-Luc group, indicating activation of glial cells or reactive gliosis. In the Ad.CMV-TK group, GFAP staining not only accumulated in the penumbral but also appeared inside the ischemic core.
These results indicate that kallikrein enhanced glial cell survival in the penumbra and migration into the ischemic core. Figure 3B shows representative TUNEL-positive apoptotic cells in the rat brains. Kallikrein gene transfer markedly reduced I/R-induced cerebral apoptosis. In the Ad.CMV-Luc group, there was a significantly increased number of TUNEL-positive cells compared with the sham group (28.66±4.29 versus 0.14±0.24 TUNEL-positive cells per field at 300×; n=4, P<0.001). Kallikrein gene transfer significantly reduced apoptosis in response to cerebral ischemia (3.40±0.84 versus 28.66±4.29 TUNEL-positive cells per field at 300×; n=4, P<0.01 versus the Ad.CMV-Luc group).

### Effects of Kinin on Migration of and H/R-Induced Apoptosis in Cultured Glial Cells

To confirm the effect of kinin as a chemoattractant on glial cells, we used the chemotaxis assay in modified Boyden chambers. Kinin significantly enhanced the migration of primary cultured glial cells in a dose-dependent manner, and the effect was blocked by icatibant, indicating a bradykinin B1 receptor-mediated event (Figure 4A). Figure 4B shows representative TUNEL-positive glial cells.

Kinin reduced H/R-induced glial cell apoptosis, and the effect was blocked by icatibant. Figure 4C shows the results of quantitative analysis of apoptotic glial cells. Kinin significantly inhibited H/R-induced apoptosis in cultured glial cells, but kinin’s protective effect was abolished by icatibant.

### Effects of Kallikrein Gene Transfer on Cerebral NOx, Superoxide Formation, and NAD(P)H Oxidase Activity

Figure 5A shows that kallikrein gene delivery significantly increased cerebral NOx production compared with rats that had received the luciferase gene (1.47±0.06 versus 1.17±0.07 nmol/mg protein; n=9, P<0.001). In addition, kallikrein gene delivery significantly reduced cerebral NADPH and NADH oxidase activities (5.30±0.27 versus 7.16±0.46, and 5.59±0.76 versus 12.75±1.18 RLU/mg protein per minute, respectively; n=9, P<0.01) and cerebral superoxide production (1.94±0.50 versus 7.24±0.91 nmol/mg protein per minute; n=9, P<0.05) in the brain extracts compared with those of the Ad.CMV-Luc group (Figure 5B through 5D).
Effects of Kallikrein Gene Transfer on Akt–Bcl-2 Signaling, Cleaved Caspase-3 Levels, and Caspase-3 Activity

Figure 6A through 6C shows Western blot analyses (upper panel) and quantitative analyses (lower panel). Kallikrein gene delivery in rats with cerebral I/R significantly increased phosphorylation of Akt but not total Akt compared with the values in rats that had been injected with the luciferase gene (0.82±0.14 versus 0.34±0.09; n=3, P<0.05; Figure 6A). Kallikrein gene delivery also markedly increased Bcl-2 levels in the ischemic brains of rats compared with those that had received the luciferase gene (1.21±0.11 versus 0.72±0.05 arbitrary units; n=3, P<0.05; Figure 6B) but reduced cleaved caspase-3 protein levels (433.3±56.3 versus 1041±186.7 arbitrary units; n=3, P<0.001) (Figure 6C) and activity (15.9±3.7 versus 27.2±5.4 pmol/mg per minute; n=4 or 5, P<0.05) (Figure 6D) compared with those in rats injected with the luciferase gene.

Discussion
This is the first study to demonstrate that tissue kallikrein protects against neurologic deficit and cerebral infarction induced by ischemic stroke. The protective effects of kallikrein gene transfer were correlated with the enhancement of
glial cell survival and migration and attenuation of I/R-induced cerebral apoptosis and oxidative stress. The protective effects were not mediated by the blood pressure–lowering effect of tissue kallikrein, because the blood pressures of these rats after gene transfer remained normal (data not shown). A previous study showed that neuronal damage induced to the MCA becomes apparent at day 1 and reached maximal levels from 2 to 7 days after reperfusion. After intracerebroventricular kallikrein delivery, expression and localization of recombinant human tissue kallikrein could be detected in the ischemic rat brain 9 days after gene transfer. In addition, our previous results showed expression of human tissue kallikrein in various rat brain regions at 1 to 7 days after intracerebroventricular delivery of the kallikrein gene. These observations taken together suggest that expression of recombinant kallikrein in the ischemic brain could rescue the ischemic penumbra and subsequently reduce I/R-induced infarction after gene transfer. Most important, we observed that after occlusion of the MCA, I/R-induced locomotor deficit scores were significantly ameliorated in rats that had received the kallikrein gene transfer. It has been documented that spontaneous partial recovery of motor and/or cognitive dysfunction in stroke patients often occurs, but the factors that affect such functional improvement have not been well elucidated. In this study, we have shown that kallikrein gene transfer promotes a near-total recovery from stroke-induced locomotor disability.

It is well documented that ROS are generated and play a detrimental role in cerebral I/R injury. Induction of oxidative stress during reperfusion exacerbates cerebral ischemia–induced neuronal apoptosis. Our results show that expression of kallikrein in the ischemic brain resulted in markedly increased cerebral NOx levels in conjunction with reduced NAD(P)H oxidase activity and superoxide production. NO is known to be a potent antioxidant, because it inhibits NADPH oxidase activity and thus, ROS formation in neutrophils. Moreover, a recent study showed that NO plays a vital role in neuroprotection after MCA occlusion, indicating that up-regulation of eNOS could protect against cerebral ischemia. Production of NO in the ischemic brain was capable of reducing generation of ROS, such as superoxide, by inhibiting NAD(P)H oxidase activity, and it subsequently facilitated the protective effects on ischemia-induced cerebral apoptosis. Furthermore, increased ROS production during reperfusion might also contribute to the induction of caspase-8, thereby exacerbating apoptosis after focal cerebral ischemia. Therefore, these results indicate that kallikrein/kinin, through NO formation, suppresses oxidative stress in ischemic stroke.

Our present study shows that kallikrein gene transfer significantly reduced I/R-induced cerebral apoptosis, which was accompanied by increased phospho-Akt and Bcl-2 levels but reduced caspase-3 activation. Akt, a serine/threonine kinase, is believed to promote cell viability in various cell types. Akt phosphorylation was upregulated in the early stage of MCA occlusion in mice, suggesting that prosurvival cell signaling is initiated in an active fashion before cell death pathways are activated in the ischemic penumbra. In addition, Bcl-2 was downregulated in neurons after cerebral ische-
Therefore, the antiapoptotic effect of kallikrein gene transfer on cerebral ischemia is most likely mediated by activation of Akt and increased Bcl-2 levels, which subsequently resulted in reduced activation of caspase-3 activity. NO has also been shown to inhibit apoptosis by inhibiting caspase-3 activation. Taken together, these findings indicate that kallikrein gene transfer protected against ischemia-induced stroke mainly through activation of the Akt–Bcl-2 signaling pathway, which could lead to increased eNOS activity and thus, cerebral NO formation and neuroprotection.

We observed that kallikrein gene transfer resulted in the appearance of glial cells in the ischemic core. This might be attributed to migration of glial cells from the ischemic penumbra. In in vitro studies, we showed that kinin was able to enhance the migration of primary cultured glial cells and to inhibit glial cell apoptosis induced by H/R in a dose-dependent manner. These effects of kinin on cell migration and apoptosis were blocked by icatibant, indicating a bradykinin B2 receptor–mediated event. These findings reinforce the notion that enhancement of glial cell survival and migration in the ischemic penumbra and core through activation of bradykinin B2 receptors is crucial in neuroprotection against cerebral ischemia. However, a recent study showed that early treatment with a bradykinin B2 receptor antagonist (0.25 and 6.25 hours after the onset of ischemia) attenuated the damage of transient cerebral ischemia by inhibiting edema formation. This result indicated that kinin might have a detrimental effect in the early stage of ischemic onset. In contrast, we have shown that kallikrein gene transfer protected against neurologic deficits and cerebral infarction after focal cerebral injury. One explanation is that the expression of recombinant tissue kallikrein was delayed until 3 to 5 days.

after local gene transfer to reach a high level. Moreover, the observed protective effect of kallikrein on cerebral infarction and apoptosis was examined 9 days after the onset of ischemia. Taken together, these results suggest that kallikrein-kinin is beneficial during the late stage of ischemic stroke by promoting glial cell survival and migration and inhibiting apoptosis.

**Perspectives**

The present study demonstrates that kallikrein/kinin protects against ischemia-induced neurologic dysfunction and cerebral infarction by enhancing glial cell survival and migration and inhibiting apoptosis. These effects occurred in conjunction with suppression of oxidative stress and activation of the Akt–Bcl-2 signaling pathway. A continuous supply of kallikrein/kinin by gene transfer or protein infusion might offer a therapeutic target for the treatment of ischemic stroke.

**Acknowledgments**

This work was supported by National Institutes of Health grant HL29397.

**References**


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Hypertension. published online December 29, 2003;
Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2003 American Heart Association, Inc. All rights reserved.
Print ISSN: 0194-911X. Online ISSN: 1524-4563

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http://hyper.ahajournals.org/content/early/2003/12/29/01.HYP.0000110905.29389.e5.citation

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