Combining Insulin-Like Growth Factor Derivatives Plus Caffeinol Produces Robust Neuroprotection After Stroke in Rats

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Background and Purpose—Insulin-like growth factor-1 (IGF-1) and caffeinol are both neuroprotective and probably have different mechanisms of action; therefore, they may be more effective in combination.

Methods—We tested the N-terminal tripeptide of IGF-1, Gly-Pro-Glu (GPE), and its analogue, G2MePE, alone and with caffeinol in a rat middle cerebral artery (MCA) suture occlusion model. We randomly assigned rats to 6 groups of 8 to 12 animals: (1) control; (2) GPE, 3 mg/kg per hour; (3) G2MePE, 0.3 mg/kg per hour; (4) caffeinol, a mixture of caffeine (10 mg/kg) with ethanol (0.32 g/kg); (5) GPE with caffeinol (combination of group 2 with 4); and (6) G2MePE with caffeinol (combination of group 3 with 4). Drugs were started 75 minutes after suture occlusion, at the start of reperfusion. Three days after MCA occlusion, neurological deficit (Neurological Deficit Score [NDS]) and lesion volume were measured.

Results—GPE and caffeinol improved NDS by 34% and 36%, respectively (P<0.01), and also decreased cortical but not striatal lesion volume compared with control (GPE cortex, 121 mm³; caffeinol cortex, 134 mm³; and control, 221 mm³; P<0.01). GPE plus caffeinol did not have more efficacy than either GPE or caffeinol alone. G2MePE slightly improved NDS (19.7%, P=0.05) but not lesion volume. However, G2MePE plus caffeinol very significantly improved NDS (64%) and lesion volume in both cortex (combination 95 mm³ versus control 221 mm³) and striatum (combination 74 mm³ versus control 110 mm³) (P<0.001), and was significantly more effective than either caffeinol or G2MePE alone.

Conclusion—Both GPE and caffeinol significantly protect cortex after MCA occlusion. At the doses used in this study, the GPE analogue G2MePE by itself had minimal protective effects, but when combined with caffeinol, it demonstrated robust beneficial effects on cortical and subcortical lesion size and behavioral deficit. Further study of this combination appears justified. (Stroke. 2005;36:129-134.)

Key Words: behavior ■ growth factors ■ ischemia ■ neuroprotection ■ stroke

Insulin-like growth factor-1 (IGF-1) is a polypeptide hormone that has been identified as a potential neuroprotective drug for the treatment of stroke and other forms of neural damage. Preclinical studies over the past decade have demonstrated that IGF-1 can protect against neuronal and glial cell degeneration in animal models of stroke such as hypoxia-ischemia. Although the results to date are encouraging, the size of the IGF-1 molecule has proved problematic and has led researchers to investigate routes of administration that bypass the blood–brain barrier, such as intranasal application, or to focus on fragments of IGF-1 such as the N-terminal peptide Gly-Pro-Glu (GPE). When administered acutely after ischemia, GPE reduced both cortical damage and neuronal loss in CA1–2 subregions of the hippocampus. Protease-resistant analogues (G2MePE) have been developed that prolong the half-life of GPE (usually 1 to 2 minutes).

In animal stroke models, our laboratory has developed and studied caffeinol, a combination of low doses of caffeine and ethanol. This combination is highly effective in reducing ischemic damage, with particularly striking effect on cortex. The widespread use of both caffeine and ethanol in our society, and familiarity with its rapid onset of action and clinical toxicology, has generated substantial interest in this combination, and pilot clinical trials are underway.

There is a general consensus among researchers in the field of “neuroprotection” for stroke that combining 2 or more complementary cytoprotective strategies to increase the potency of the treatment over any single component may be necessary to have a detectable clinical effect. Because IGF
and caffeinol probably have different mechanisms of action (see Discussion), we hypothesized that when given together, the combination would have a more robust benefit than either alone. Therefore, this study aimed to test the effects of combining GPE and G2MePE with caffeinol in reducing the acute damage after middle cerebral artery (MCA) occlusion in rats.

Materials and Methods

Subjects

Adult male Long–Evans rats, weighing 325 ± 25 grams (Harlan, Indianapolis, Ind) at the time of order, were used. The rats were kept on a 12:12-hour light:dark cycle and allowed food and water ad libitum, and the rats were tamed by gentle handling twice daily for 1 week before inclusion in the experiment.

The rats were preselected and pretrained on the behavior tests. Behavior tests and rectal temperature were measured before surgery to exclude abnormal rats. Only rats with <20% foot fault, normal forelimb placing, and rectal temperature at 37.7 ± 0.5°C were included and subjected to MCA occlusion.

MCA Occlusion

Focal brain ischemia was induced by the MCA occlusion intraluminal suture method as previously described by Longa. Briefly, the rats were anesthetized with 2% isoflurane in a mixture of 30% oxygen and 70% nitrous oxide delivered by tracheal intubation. A rectal probe was inserted 4 cm into rectum and the probe was temporarily fixed to the tail. Micro-Renathane tubing (type MRE-040; Braintree Scientific Inc) was cannulated into the jugular vein for intravenous drug delivery.

The right common carotid artery, internal carotid artery, and external carotid artery were exposed through a midline neck incision. A 4-0 poly-1-lysine–coated nylon suture was inserted through a stump of the external carotid artery, and the common carotid artery was kept open and intact. The suture occluder was advanced into the internal carotid artery 19 to 21 mm beyond the carotid bifurcation. Mild resistance indicated that the occluder was properly lodged in the anterior cerebral artery and thus blocked blood flow to the MCA.

The rat was allowed to awaken right after finishing the operation and the Neurological Deficit Score (NDS) was measured 60 to 65 minutes after initiation of the MCA occlusion. Rats with satisfactory deficits on NDS (NDS score 11 to 15) were re-anesthetized with isoflurane with facemask; 75 minutes after starting MCA occlusion, reperfusion was started by withdrawing the suture. The common carotid artery and internal carotid artery were inspected to ensure the return of good pulsation, and the neck incision was closed with silk suture. Drug treatments were started immediately after the start of reperfusion by connecting the precannulated tubing to a perfusion pump to infuse a 3-mL solution into the jugular vein at a speed of 1 mL/h. The rats freely moved around in a cylinder during the drug infusion by using a swivel tether system. After finishing the infusion, the rats were returned to their home cage.

Animal Groups and Doses

Rats with moderate level injury (NDS of 11 to 15 right before reperfusion) were randomly assigned to 6 groups: (1) buffer group (n = 9), 3 mL of saline or 3 mL of succinate buffer (pH 6.0) at 1 mL/h for 3 hours; (2) GPE group (n = 8), 9 mg/kg of GPE in 3 mL of succinate buffer at 1 mL/h for 3 hours; (3) G2MePE group (n = 9), 0.9 mg/kg of G2MePE in 3 mL of saline at 1 mL/h for 3 hours; (4) caffeinol group (n = 12), 10 mg/kg of caffeine (1,3,7-trimethyIxanthon; Acros Organics) and 0.32 g/kg of ethanol (Quantum) in 3 mL of saline at 1 mL/h for 3 hours; (5) GPE plus caffeinol (n = 12), combination of groups 2 and 4 in 3 mL saline at 1 mL/h for 3 hours; and (6) G2MePE plus caffeinol group (n = 9), combination of groups 3 and 4 in 3 mL saline at 1 mL/h for 3 hours.

Rectal Temperature Measurement

Rectal temperature was measured before surgery (without anesthesia), during the process of MCA occlusion, and 72 hours after reperfusion.

Behavioral Measurements

All behavioral tests took place in a quiet and low-light room by an experimenter blinded with respect to the treatment groups. The foot fault and forelimb placing tests were performed according to previously published methods by Bland. The postural reflex and circling tests were performed as described by Bederson. NDS was measured before occlusion, before reperfusion (60 to 65 minutes after initiation of MCA occlusion), and 72 hours after MCA occlusion. NDS (0 to 18) was calculated by combining the score on the following 4 tests.

Postural Reflex Test

The degree of abnormal posture was estimated by suspending rats with their tails 20 cm above a tabletop. Intact rats extended both forelimbs toward the table surface. Rats displaying this behavior were recorded as score 0. Rats with only flexing of the contralateral limb toward the body were recorded as score 2. Rats rotating the contralateral forelimb toward the tail were graded as score 4.

Circling or Side-Walking

Rats that circled or side-walked toward the paretic side on 10 trials were recorded. A score of 2 or 4 was given to each rat according to the severity of their deficits.

Forelimb Placing (Whisker, Forward Tactile, and Lateral Tactile)

Animals were held by their torsos with forelimbs hanging freely. Contra- lateral and ipsilateral forelimb Whisker placing responses were induced by gently brushing the respective vibrissae on the edge of a tabletop for 10 trials. A score of 1 was given each time the rat placed its forelimb on the edge of the tabletop in response to the vibrissae stimulation. Percent successful placing responses were determined (number correct × 10). The lateral tactile placing was similar to the whisker placing, except the placing response was induced by gently contacting the lateral side of the forelimb to the edge of the tabletop, whereas forward tactile placing was induced by contacting the frontal side of the forelimb to the edge of a tabletop. The scale was scored as: 0, immediate and complete placing or more out of 10 trials; 1, delayed and/or incomplete placing >2 out of 10 trials; and 2, no placing.

Foot Fault

Animals were placed on an elevated grid, with openings of 2.3 cm². As the animals traversed the grid, a foot fault was scored each time the contralateral forepaw slipped through an opening in the grid. The total number of steps was also counted. The percent foot fault was calculated as the number of foot faults/total steps × 100. A score of 0 to 4 was given to each rat according to the severity of the deficit by calculating the percent foot faults × 0.04.

Lesion Volume Measurement

On day 3 after MCA occlusion, the rats were deeply anesthetized by intraperitoneal injection of 0.6 mg/kg of chloryl hydrate, intracardiac-perfused with 150 to 200 mL of ice-cold phosphate-buffered saline, then euthanized. The brain was removed within 30 seconds, rinsed in phosphate-buffered saline, and sliced into 2-mm sections. The sections were stained with 2% 2,3,5-triphenyltetrazolium chloride. After 2,3,5-triphenyltetrazolium chloride staining, each section was scanned into a Macintosh computer and analyzed by a computer-interfaced BRAIN imaging system as previously described. The lesion volume was calculated as the sum of 7 slices. Rats that died before 72 hours had postmortem examination of their brains but were not included in lesion volume calculation.
Statistical Analysis

Investigators blinded to treatment assignment performed all behavioral testing and subsequent histological analyses. Statistical analyses were performed using GraphPad Prism version 3.00 for Windows and GraphPad InStat. Behavioral tests and body weight changes were analyzed using 1-way ANOVA, followed by Student-Newman-Keuls multiple comparison post hoc tests with correction for multiple comparisons. A separate ANOVA was performed for each of the time points to see at what time point the NDS scores became significantly different. Pearson correlation coefficients were calculated between the infarct volume and behavioral tests at the 72-hour time point. All data were expressed as mean±SD. * P<0.05; **P<0.01; ***P<0.001 versus buffer control.

Results

Mortality
MCA suture occlusion × 75 minutes caused a relative high mortality (12%); 11 rats out of 92 that were subjected to MCA occlusion mainly related to cerebral edema and hemorrhagic conversion of the infarct died.19–22 This mortality rate and its causes, in fact, are similar to what is seen in human stroke patients with MCA occlusions. There was no significant difference in mortality between any of the study groups.

Physiological Signs
There were no significant differences in baseline body weight between the groups (P=0.71). G2MePE combined with caffeineol significantly prevented body weight loss at 72 hours (P<0.05) compared with the buffer group, but there were no significant differences among all the other groups (Figure 1). Rectal temperature did not differ between the groups.

Lesion Volume
GPE significantly reduced the lesion volume in cortex (125.7 mm3; P<0.01), but not in striatum (89.5, P=0.14), compared with the buffer group (221.1 and 110.3). Caffeineol also significantly reduced cortical lesion volume (134.2, P<0.01), but not in striatum (87.0; P=0.06). G2MePE did not reduce lesion volume in either cortex or striatum (cortex 192.5, P=0.25; striatum 92.0, P=0.08).

GPE combined with caffeineol reduced cortical infarct volume to 100.50 (P<0.01) and striatal volume to 82.13 (P<0.05), but the additive effect of the combination was not significantly different from either GPE or caffeineol alone.

G2MePE combined with caffeineol displayed an additive effect compared with either G2MePE or caffeineol alone. The protective effect of the combination on lesion volume compared with buffer control was shown in the cortex (94.8, P<0.001) and also in the striatum (74.0; P<0.001) (Figure 2). When the G2MePE plus caffeineol was compared with its individual components, the combination was significantly better than G2MePE alone (P<0.05, in cortex and striatum), and also significantly better than caffeineol alone in cortex (P<0.05), but not in striatum (P=0.32).

Behavioral Test Results
Sensory motor deficits were improved in parallel with the changes seen in cortical lesion volume. NDS changes from before reperfusion to 72 hours for the individual tests are shown in Figure 3. The total NDS at 72 hours after reperfusion is shown in Figure 4.

GPE significantly decreased the NDS by 34% (8.35 versus 12.6; P<0.01) (Figure 4). Caffeineol reduced the NDS by 36%...
Discussion

We found that the IGF derivative, GPE, was neuroprotective in a rat stroke model, and that one of its analogues, G2MePE, in combination with caffeinol, was even more neuroprotective, even when administration was delayed until 75 minutes after MCA occlusion.

Both GPE and caffeinol decreased cortical lesion volume and improved neurological functional recovery, consistent with previous reports from different stroke models. Although there was a trend, the combination of GPE with caffeinol did not show an additive effect over GPE alone. G2MePE alone did not have much protective effect on lesion volume or NDS but had very significant additive effects when combined with caffeinol. Whereas there was a positive trend, the failure of G2MePE alone to substantially reduce damage may be related to the low dose (0.9 mg/kg).

Multiple deleterious factors have been implicated in the pathology of ischemic damage, including neurotransmitter release, ion imbalance, free-radical formation, mitochondrial dysfunction, gene expression, protein synthesis impairment, inflammation, and programmed cell death. The treatments tested in these experiments may target several of these pathways.

Special attention during the search for an effective stroke treatment has been devoted to the role of NMDA, GABA, and adenosine receptors, which are directly implicated in modulation and execution of ischemia-evoked excitotoxic damage. Adenosine modulates neuronal excitability, as well as the release of many neurotransmitters. These include the excitatory amino acids (EAA), which, through activation of specific ionotropic receptors (primarily NMDA but also certain AMPA and kainate receptors), play an instrumental role in mediating neuronal damage.
role in ischemia-induced Ca\(^{2+}\)-mediated excitotoxic damage.\(^{26-29}\)

In vivo, caffeine and ethanol are readily absorbed and distributed to all body fluids, including the cerebrospinal fluid. The protective mechanism of caffeine or the biological activities of caffeine and ethanol in stroke are still not clear. The biological activity of caffeine is highly dose-dependent and is expressed through its ability to induce the intracellular release of Ca\(^{2+}\) (interaction at the level of IP3 and ryanodine receptor), inhibit phosphodies-
terase, and block GABA and adenosine receptors.\(^{30-33}\) The biological activities of ethanol include inhibition of excit-
tory NMDA receptors and activation of inhibitory GABA receptors.\(^{33}\)

IGF-1 has substantial neuroprotective properties but does not cross the blood–brain barrier because of its size. GPE, the active peptide component cleaved from IGF-1, can cross the blood–brain barrier with excellent brain penetration. However, the mechanism of its neuroprotective effect is still unknown. At relatively high doses (μM range), GPE binds to the NMDA receptor (most probably at the glutamate binding site) at a concentration >10 μmol/L, where it probably acts as a partial agonist, mimicking (with lower efficacy) the effects of glutamate at 10 to 300 μmol/L, and antagonizing the effects of glutamate at >300 μmol/L. In situations of excessive glutamate release, such as occurs during stroke and brain injury, GPE acting as an NMDA receptor antagonist might inhibit the neurotoxic effects of glutamate overload. However, NMDA receptor activity of GPE probably does not explain the neuroprotective effect seen in our study because only nM concentrations of GPE are achieved in cerebrospinal fluid after infusion of neuroprotective doses. The cytoprotec-
tive effect of GPE may be mediated through its own unique receptor, but this novel binding site is still undetermined.

The main limitation of this study, besides the uncertain action of the drugs, is that we tested only one dose of GPE or G2MePE. Other doses should now be evaluated to optimize results. Also, longer duration to starting treatment should be analyzed.

In conclusion, we found that derivatives of the active segment of IGF-1 reduce acute ischemic damage and, in combination with caffeine, have greater effect than either treatment alone. The analogue of GPE, G2MePE, in combination with caffeine, is a potentially effective neuroprotective approach that deserves further study.

Acknowledgments

This work was supported by grants to James Grotta (R01 NS23979) and Jarek Aronowski (R01 NS039378). We thank Dr Timothy Schallert for advice on the behavioral tests. The GPE and G2MePE were donated by NeuronZ Limited, Auckland, New Zealand.

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Stroke. 2005;36:129-134; originally published online November 29, 2004;
doi: 10.1161/01.STR.0000149624.87661.18

Stroke is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0039-2499. Online ISSN: 1524-4628

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