Intranasal delivery of erythropoietin plus insulin-like growth factor–I for acute neuroprotection in stroke

Laboratory investigation

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Object. Individually, the cytokines erythropoietin (EPO) and insulin-like growth factor–I (IGF-I) have both been shown to reduce neuronal damage significantly in rodent models of cerebral ischemia. The authors have previously shown that EPO and IGF-I, when administered together, provide acute and prolonged neuroprotection in cerebrocortical cultures against N-methyl-D-aspartate–induced apoptosis. The aim of this study was to determine whether intranasally applied EPO plus IGF-I can provide acute neuroprotection in an animal stroke model and to show that intranasal administration is more efficient at delivering EPO plus IGF-I to the brain when compared with intravenous, subcutaneous, or intraperitoneal administration.

Methods. The EPO and IGF-I were administered intranasally to mice that underwent transient middle cerebral artery occlusion (MCAO). Stroke volumes were measured after 1 hour of MCAO and 24 hours of reperfusion. To evaluate the long-term effects of this treatment, behavioral outcomes were assessed at 3, 30, 60, and 90 days following MCAO. Radiography and liquid scintillation were used to visualize and quantify the uptake of radiolabeled 125I-EPO and 125I-IGF-I into the mouse brain after intranasal, intravenous, subcutaneous, or intraperitoneal administration.

Results. Intranasal administration of EPO plus IGF-I reduced stroke volumes within 24 hours and improved neurological function in mice up to 90 days after MCAO. The 125I-EPO and 125I-IGF-I were found in the brain within 20 minutes after intranasal administration and accumulated within the injured areas of the brain. In addition, intranasal administration delivered significantly higher levels of the applied 125I-EPO and 125I-IGF-I to the brain compared with intravenous, subcutaneous, or intraperitoneal administration.

Conclusions. The data demonstrate that intranasal EPO plus IGF-I penetrates into the brain more efficiently than other drug delivery methods and could potentially provide a fast and efficient treatment to prevent chronic effects of stroke. (DOI: 10.3171/2009.2.JNS081199)

Key Words • cytokine • drug delivery • erythropoietin • intranasal delivery • ischemia • mouse • neuroprotective agent

Stroke is the third most common cause of death in the US after heart disease and all cancer, and is a major contributor to severe disability. As the most common cause of neurological disability, ischemic stroke is often associated with sensorimotor and cognitive impairments due to neuronal degeneration. Intravenous administration of recombinant tissue plasminogen activator is the only FDA-approved medical therapy for treatment of patients with acute ischemic stroke, but recombinant tissue plasminogen activator can only be given within 3 hours of symptom onset, and only 2% of patients are eligible. A number of studies are being conducted to find neuroprotective agents that can improve the outcome and provide a larger window of opportunity for stroke patients. However, the delivery of a wide range of possible therapeutic agents to the brain is hampered by the BBB. Intranasal drug administration could offer a noninvasive method that circumvents the BBB and provides rapid drug absorption for acute brain insults without systemic side effects. A number of different growth factors, pep-
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tides, and hormones have been reported to enter the brain and CSF through the olfactory epithelium and to promote neurogenesis, inhibit neurodegeneration, improve neurological function, and protect against cerebral ischemia.

Erythropoietin has been identified as a neurotrophic and neuroprotective agent in many different animal models of brain injury. Originally identified as a hematopoietic factor, EPO and its receptor are expressed in the mammalian CNS. Brain-derived EPO is upregulated by hypoxia and expression of both EPO and its receptor are specifically modulated during cerebral ischemia. The EPO molecule is an extremely well-studied and well-tolerated compound in humans, and its use in stroke patients has been promising, at least in some studies.

We have previously shown that the efficacy of EPO is increased when combined with IGF-I in an in vitro model. When EPO and IGF-I are given together, they provide acute and prolonged neuroprotection by synergistically activating the phosphoinositide 3-kinase/protein kinase B pathway, thereby preventing apoptosis. We investigate the effects of intranasal administration of EPO plus IGF-I on long-term behavioral outcome in a mouse model for transient ischemia. In addition, we compare intranasal administration to intravenous, subcutaneous, or intraperitoneal administration. Our results indicate that intranasal application of EPO plus IGF-I could provide a novel, noninvasive method for acute neuroprotection after stroke.

Methods

Transient MCAO

All animal experiments were approved by the Institutional Animal Care and Use Committee of The University of Texas Health Science Center at San Antonio. Adult male C57Bl/6 mice weighing between 20 and 25 g were used for the present study. Mice were anesthetized with 1.5–2% isoflurane in a mixture of 70% nitrous oxide and 30% oxygen. The animals’ body temperatures were maintained at 37°C with a heating blanket and feedback system (Harvard Apparatus). Transient focal cerebral ischemia was induced by occlusion of the left MCA by using the intraluminal filament model. Reperfusion was performed by withdrawal of the filament 1 hour after occlusion. Cytokines were administered immediately after withdrawal of the filament. Surface cerebral blood flow was monitored during MCAO by using a laser Doppler flowmeter (Perimed). Mice with surface cerebral blood flow > 20% of baseline were deemed to have unsuccessful occlusions of the MCA and were excluded from the experiment.

Intranasal Administration

A recently described method for intranasal application was used, with slight modifications for mice. Recombinant human EPO (Epoietin-a, 2000 U/ml; Amgen); recombinant human IGF-I (R&D Systems); 125I-EPO ([3-125I]iodotyrosyl)-erythropoietin, human recombinant [high specific activity]; GE Healthcare); and 125I–IGF-I ([3-125I]iodotyrosyl)-insulin-like growth factor–I, human recombinant; GE Healthcare) were dissolved in a vehicle that consisted of 10 mM sodium succinate buffer and 140 mM NaCl (pH 6.2). All treatment solutions had a volume of 12 µl and were applied dropwise (2 µl per drop) over 12 minutes, alternating between each nare. The mouth and the alternate nare were closed during application to allow complete inhalation of the solution into the nasal cavity.

Evaluation of Cerebral Infarction

Mice subjected to 1-hour MCAO received intranasal EPO (100 U), IGF-I (1000 ng), EPO plus IGF-I (100 U plus 1000 ng), or vehicle (16 animals per group). After 24 hours of reperfusion, an observer blinded to the identity of the groups assessed neurological behavior and scored the results as follows: 0, no observable neurological deficit (normal); 1, failure to extend left forepaw upon suspension by tail (mild); 2, circling to the contralateral side (moderate); and 3, leaning to the contralateral side at rest or no spontaneous motor activity (severe). The mice were killed using isoflurane, and their brains were sectioned coronally at 1-mm intervals. The brain sections were incubated in a 2% solution of TTC at 37°C for 5 minutes and fixed by immersion in 4% paraformaldehyde. The area of infarction was measured digitally with the National Institutes of Health 1.62 imaging system. The volume of infarction was calculated by numerical integration of sequential areas, and expressed as a percentage of whole-brain volume after correction for edema, as we have described previously. Differences in stroke volumes were analyzed by ANOVA, followed by a post hoc Scheffé test for multiple comparisons (p < 0.05 was taken as statistically significant).

Liquid Scintillation

Mice received intranasal 125I-EPO (100 U) and 125I–IGF-I (1000 ng) as described above. After 20 or 60 minutes, whole brains (without the olfactory bulb) from untreated and treated mice were collected and homogenized in 1 ml buffer (50 mM Tris-HCl, pH 7.4). Samples were then prepared for liquid scintillation measurement (Perkin Elmer) after the addition of scintillation fluid. To exclude blood contamination, dpm levels from blood samples were subtracted from the total brain values. Differences in dpm levels were analyzed using 1-way ANOVA, keeping significance at a probability < 0.05.

To compare different drug delivery methods, control mice and animals subjected to MCAO received intranasal 125I-EPO (100 U), intranasal 125I–IGF-I (1000 ng), or subcutaneous, intravenous, or intraperitoneal injections each of 125I-EPO (5000 U/kg) or 125I–IGF-I (50 µg/kg). Mice subjected to MCAO received 125I-EPO or 125I–IGF-I after removal of the suture. The brains were collected at various time points (10–720 minutes) following drug administration and prepared for liquid scintillation measurement as described above. The mean dpm values were used to calculate EPO (in units) and IGF-I (in nanograms) concentrations. The Kruskal-Wallis test was used to compare concentrations between the different delivery methods for EPO and IGF-I in normal and poststroke brains, keeping significance at a probability < 0.05. Further post
hock comparisons between the different delivery methods for EPO and IGF-I for different time points in normal and poststroke brain were performed using the Mann-Whitney test, keeping significance at a probability < 0.003 (after the Bonferroni correction).

**Autoradiography Studies**

Mice subjected to MCAO received intranasal $^{125}$I-EPO (100 U) and $^{125}$I–IGF-I (1000 ng). Brains were removed 24 hours after MCAO, frozen in liquid nitrogen-cooled isopentane, and embedded in TissueTek 4583 CRYO-OCT Compound (Sakura Finetek). Embedded brains were sectioned into 20-µm sections and transferred onto gelatin-coated coverslips. Brain sections were exposed to x-ray film for 2–4 days. Sections that were used for autoradiography were stained with cresyl violet for neuronal morphological studies and correlated to the respective autoradiography pattern to identify the morphological location of the radiolabeled cytokines.

**Behavioral Tests**

All behavioral testing was modeled after a previously described method that was modified for this project. Randomly selected mice were grouped as follows (8 per group): Group I animals were subjected to MCAO and treated with vehicle; Group II animals were subjected to MCAO and treated with EPO (100 U); Group III animals were subjected to MCAO and treated with IGF-I (500 ng); and Group IV animals were subjected to MCAO and treated with EPO plus IGF-I (100 U plus 500 ng). An observer blinded to the identity of the groups assessed neurological behavior at 3, 30, 60, and 90 days. Three trials were performed for each behavioral test on each testing day for each mouse. The scores were analyzed using 1-way ANOVA, and post hoc Newman-Keuls tests were used to compare the differences among the treatment groups. To analyze day-by-day differences, repeated-measures ANOVA was performed, followed by the Bonferroni test for multiple comparisons. A probability value < 0.05 was considered significant.

**Sticky Label Test (Modified Schallert Tape Test).** Each mouse was picked up by the scruff of the neck and the tail was fixed between fingers and palm. A cuff of tape measuring 4 × 0.5 cm was placed over the right paw and then measured in a scintillation counter. To exclude blood contamination, dpm levels from blood samples were subtracted from the total brain values. The mean dpm and SD are plotted for 3 mice in each group. *p < 0.01 for treated versus control; †p < 0.01 for level at 20 minutes versus 60 minutes.

**Results**

**Levels of $^{125}$I-EPO and $^{125}$I–IGF-I in the Brain Following Intranasal Administration**

Intranasal administration of $^{125}$I-EPO, $^{125}$I–IGF-I, or their combination yielded high levels of $^{125}$I-EPO and $^{125}$I–IGF-I in the brain. Figure 1 shows that whole-brain lysates collected only 20 minutes after intranasal application had significantly higher levels in the brain compared with controls (p < 0.01). The levels of $^{125}$I-EPO and $^{125}$I–IGF-I were still increasing after 60 minutes and were significantly higher when compared with levels at 20 minutes (p < 0.01).

**Intranasal EPO Plus IGF-I Reduces Infarct Volume After MCAO**

Mice subjected to MCAO received intranasal EPO, IGF-I, EPO plus IGF-I, or vehicle at the start of reperfusion. Infarct volumes were measured 24 hours later and are shown in Fig. 2. Analysis of stroke damage indicates that EPO plus IGF-I reduced infarct volumes more than either cytokine alone. To assess the beneficial effect of intranasal EPO plus IGF-I on neurological outcome, the mice were scored according to the severity of the neurological deficit. The results shown in Table 1 demonstrate that mice treated with EPO plus IGF-I after MCAO had fewer neurological deficits than those treated with either EPO or IGF-I alone.
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To determine whether EPO and IGF-I can reach the injured areas of the brain after stroke, mice that underwent MCAO received intranasal 125I-EPO, 125I-IGF-I, or a combination of these substances after withdrawal of the filament. The brains were removed 20 minutes after intranasal administration. Exogenous 125I-EPO and 125I–IGF-I detected by autoradiography resulted in a darker signal in the areas of infarction compared with the rest of the uninjured brain (Fig. 3), indicating an accumulation of the radiolabeled cytokines in the stroke core and penumbra.

Sticky Label Test

To test for somatosensory deficiencies, the mice in each group were timed to see how long it would take them to remove a piece of tape from their affected forepaw following MCAO. The mice that received intranasal EPO, IGF-I, or EPO plus IGF-I were able to perform the task significantly faster than the vehicle-treated mice on Days 3, 30, 60, and 90 (Fig. 4, p < 0.05). The EPO plus IGF-I–treated mice performed the task significantly better than the EPO-treated mice on all 4 testing days, and animals in this group were better than the IGF-I–treated mice on Days 3, 60, and 90 (p < 0.01). The EPO plus IGF-I–treated mice did not show any improvement from Days 3 to 30, but the group did show significant improvement from Days 30 to 60 (p < 0.01).

Open Field Test

To test the locomotor activity of the mice in each group, an open field test was used. The mice that received intranasal EPO, IGF-I, or EPO plus IGF-I entered more squares than the vehicle-treated mice on Days 3, 60, and 90 (Fig. 4, p < 0.01). The EPO plus IGF-I–treated mice performed the task significantly better than the EPO-treated mice on all 4 testing days, and animals in this group were better than the IGF-I–treated mice on Days 3, 60, and 90 (p < 0.01). The EPO plus IGF-I–treated mice did not show any improvement from Days 3 to 30, but the group did show significant improvement from Days 30 to 60 (p < 0.01).

Comparison of Intranasal Administration to Other Drug Delivery Methods

To determine which drug delivery method is able to deliver higher levels of EPO and IGF-I to the brain, mice

<table>
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<tr>
<th>Group</th>
<th>Stroke Vol (mm³)</th>
<th>Neurological Score†</th>
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<tbody>
<tr>
<td>control</td>
<td>82.7 ± 9.3</td>
<td>2.7 ± 1.3</td>
</tr>
<tr>
<td>IGF-I</td>
<td>64.4 ± 9.2</td>
<td>2.4 ± 0.9</td>
</tr>
<tr>
<td>EPO</td>
<td>56.9 ± 7.1</td>
<td>1.9 ± 0.9</td>
</tr>
<tr>
<td>EPO + IGF-I</td>
<td>37.6 ± 4.7 festivals; ‡</td>
<td>1.2 ± 0.7 festivals; ‡</td>
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</table>

* Values are expressed as the mean ± standard error of the mean, for 16 mice in each group.
† Scores were assigned as follows: 0, no observable neurological deficit (normal); 1, failure to extend left forepaw upon suspension by tail (mild); 2, circling to the contralateral side (moderate); and 3, leaning to the contralateral side at rest or no spontaneous motor activity (severe).
‡ p < 0.05.
received intranasal 125I-EPO (100 U) or 125I-IGF-I (1000 ng), and were compared with mice that received intravenous, intraperitoneal, or subcutaneous injections of 125I-EPO (5000 U/kg) or 125I-IGF-I (50 µg/kg). In addition, mice that underwent 1 hour of MCAO also received the same treatments. Intranasal delivery showed the highest concentrations and earliest peak concentrations compared with subcutaneous, intravenous, and intraperitoneal injections (Fig. 5). Intranasal 125I-EPO and 125I-IGF-I concentrations peaked at 60–120 minutes versus 240–360 minutes for the other delivery systems. The superiority of intranasal delivery was seen for both 125I-EPO and 125I-IGF-I and for normal and poststroke brain (p < 0.01). In the normal brain, intranasal 125I-EPO reached a maximum of 20 U after only 20 minutes (Fig. 5A), and intranasal 125I-IGF-I reached a maximum of 45 ng after 60 minutes (Fig. 5B). In the brain poststroke, intranasal 125I-EPO reached a maximum of 33 U by 120 minutes (Fig. 5C), and intranasal 125I-IGF-I reached a maximum of 54 ng after 60 minutes (Fig. 5D). In contrast, the maximum concentration of 125I-EPO given intravenously, intraperitoneally, or subcutaneously only reached 1 U in the normal brain (Fig. 5A) and 3.5 U in the poststroke brain (Fig. 5C). The maximum concentration of 125I-IGF-I given intravenously, intraperitoneally, or subcutaneously only reached 3.5 ng in the normal brain (Fig. 5B), and 4.5 ng in the brain after stroke (Fig. 5D).

Discussion

The therapeutic potential of systemically applied EPO and IGF-I is limited by their poor ability to cross the BBB, thus limiting their availability in the brain. It has been reported that large doses of EPO administered systemically will enter the CSF through the brain capillary endothelial cells by receptor-mediated endocytosis. However, the need for very high dosages to achieve neuroprotection can potentially lead to elevated hematocrit levels, increased blood viscosity, and perfusion deficits in the brain. The IGF-1 cytokine also has difficulty crossing the BBB, except at specific hypothalamic and anterior thalamic nuclei. In addition, systemically applied IGF-I can be sequestered by IGF-binding proteins, which bind IGF-I with an affinity equal to or greater than that of the IGF-I receptor, thereby limiting the amount of IGF-I that can penetrate into the brain.

Increasing interest has been expressed in the possibility of circumventing the BBB by delivering peptidyl drugs to the CNS through the intranasal pathway. This pathway is believed to involve 2 general mechanisms. The first is internalization of the drug into the primary neurons of the olfactory epithelium, either by endocytic or pinocytotic mechanisms, and intracellular axonal transport to the olfactory bulb. The second is an extracellular pathway that allows for rapid absorption of the drug across the olfactory epithelial cells, either by transcellular or paracellular mechanisms, followed by uptake into the CNS.

In the present study, we demonstrate that 125I-IGF-I (as described by others) and 125I-EPO can gain entry into the CNS within 20 minutes following intranasal application. The rate at which 125I-EPO and 125I-IGF-I appear in the brain suggests an extracellular route across olfactory epithelial cells. It is possible that EPO could also be transported along the trigeminal pathway, as described recently for IGF-I. We measured similar dpm levels in the brains for 125I-EPO and 125I-IGF-I at 20 and 60 minutes after intranasal application. However, when given together, 125I-EPO plus 125I-IGF-I did not have a cumulative effect on dpm levels. This suggests that 125I-EPO and 125I-IGF-I are being taken up by a similar yet unknown mechanism. Further studies will be needed to elucidate the exact uptake mechanism. We also observed that intranasal 125I-EPO and 125I-IGF-I accumulated within the injured areas of the brain after MCAO, which suggests that this application method enables the cytokines to target the ischemic brain tissue.

We had shown previously that EPO plus IGF-I protects neurons in culture against apoptosis-inducing excitotoxic insults to a greater degree than either cytokine alone. Therefore, we hypothesized that EPO plus IGF-I may also be capable of preventing damage after cerebral ischemia/reperfusion injury in vivo. The present study shows that intranasal administration of EPO plus IGF-I reduces stroke damage in mice more efficiently than either cytokine alone. We demonstrate that EPO plus IGF-I can decrease stroke volumes and improve neurological function within 24 hours. Interestingly, we found that a single dosing of intranasal EPO plus IGF-I applied at the beginning of the reperfusion is sufficient to improve neurological outcomes in mice 90 days post-MCAO. Further studies will supply
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information on whether long-term treatment with multiple intranasal applications of EPO plus IGF-I will provide even further improvement after stroke. Moreover, the possibility that intranasal application of EPO plus IGF-I would expand the treatment window if administered several hours after reperfusion needs to be explored.

The intranasal delivery method was significantly more efficient at delivering $^{125}$I-EPO and $^{125}$I–IGF-I to the normal and poststroke brain than the other standard delivery systems evaluated in this study. Intranasal delivery achieved peak brain tissue concentrations 7–31 times higher for $^{125}$I-EPO and 12–20 times higher for $^{125}$I–IGF-I, and at an earlier time interval than the other delivery methods. The peak concentrations for all delivery methods were higher in the poststroke brain than in the normal brain, most likely due to increased BBB permeability from ischemia-induced vascular dysfunction. It was also seen that $^{125}$I–EPO and $^{125}$I–IGF-I concentrations remained high for a longer period in the poststroke brain than in the normal brain, most likely because of vascular dysfunction and impairment of the peptide transport mechanisms from the interstitial to the intravascular space.

Conclusions

Stroke is the most common cause of neurological dis-

ability in the US. Current treatment options for hyperacute and acute stroke are limited, and specific long-term neuroprotective and neurorestorative treatments have not been established. The present study demonstrates a cooperative neuroprotective effect of EPO plus IGF-I in vivo. Our findings suggest that the coadministration of these neuroprotection agents, rather than delivery of a single agent, may provide greater benefit for the acute treatment of patients suffering from stroke. In addition, the intranasal pathway provides a noninvasive method that further enhances the efficiency of EPO and IGF-I by lowering the dosage needed and providing a faster delivery route to the brain. The EPO cytokine is already widely used for the treatment of anemia, and IGF-I has a proven safety record in advanced clinical trials. Thus, treatment with intranasal EPO plus IGF-I could provide a novel therapeutic strategy for a variety of acute neurological events, including stroke.

Disclosure

Drs. Diczaylioglu and Lipton are co-inventors on the patent “Neuroprotective synergy of erythropoietin and insulin-like growth factors, US Patent Application #20050197284,” which is owned by the Burnham Institute for Medical Research, La Jolla, California. The authors report no other conflict of interest concerning the materials or methods used in this study or the findings specified in this paper.
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