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Intranasal administration of IGF-1 attenuates hypoxic-ischemic brain injury in neonatal rats

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Abstract

To determine whether intranasal administration (iN) of recombinant human insulin-like growth factor-1 (rhIGF-1) provides neuroprotection to the neonatal rat brain following cerebral hypoxia-ischemia (HI), two doses of rhIGF-1 (50 µg at a 1 h interval) were infused into the right naris of postnatal day 7 (P7) rat pups with or without a prior HI insult (right common carotid artery ligation, followed by an exposure to 8% oxygen for 2 h). Our result showed that rhIGF-1 administered via iN was successfully delivered into the brain 30 min after the second dose. In the following studies rhIGF-1 was administered to P7 rat pups at 0, 1 or 2 h after HI at the dose described above. Pups in the control group received cerebral HI and vehicle treatment. Pups that underwent sham operation and vehicle treatment served as the sham group. Brain pathological changes were evaluated 2 and 15 d after HI. Our results showed that rhIGF-1 treatment up to 1 hr after cerebral HI effectively reduced brain injury as compared to that in the vehicle-treated rats. Moreover, rhIGF-1 treatment improved neurobehavioral performance (tested on P5-P21) in juvenile rats subjected to HI. Our results further showed that rhIGF-1 inhibited apoptotic cell death, possibly through activating the Akt signal transduction pathway. rhIGF-1 enhanced proliferation of neuronal and oligodendroglial progenitors after cerebral HI as well. These data suggest that iN administration of IGF-1 has the potential to be used for clinical treatment.

Keywords

insulin-like growth factor-1; intranasal administration; cerebral hypoxia-ischemia; newborn; Akt; proliferation

Introduction

Insulin-like growth factor-1 (IGF-1) has an important role in normal brain development, promoting neuronal growth, cellular proliferation and differentiation (D'Ercole et al., 1996; Popken et al., 2004). Exogenous IGF-1 has been shown to protect against ischemic brain damage in both the adult (Dempsey et al., 2003; Schäbitz et al., 2001) and newborn animal (Brywe et al., 2005; Cao et al., 2003; Guan et al., 2000) when injected directly into the brain. Consistent with these findings, our previous study also showed that intracerebral ventricular (icv) injection of IGF-1 attenuated the white matter damage in a P4 neonatal rat model of

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cerebral HI (Lin et al., 2005). However, the icv injection might not be practical in humans, as it requires surgery with potential risks of infection and may cause other complications. Therefore, the development of less invasive techniques capable of delivering IGF-1 to the central nervous system would clearly aid in its effective clinical use, particularly in the treatment of chronic conditions where repeated dosing might be necessary.

The olfactory region of nasal passages has unique anatomic and physiologic attributes that provide both extracellular and intracellular pathways into the CNS bypassing the blood brain barrier. The dendritic processes of the olfactory sensory neurons are directly exposed to the external environment in the upper nasal passage while their axons project through perforations in the cribriform plate of the ethmoid bone to synaptic glomeruli in the olfactory bulb (OB). A direct extracellular pathway between the nasal passages and the brain was first conclusively demonstrated for horseradish peroxidase (Balin et al., 1986). Recently, IGF-1 has been found to diffuse into the adult rat brain and spinal cord along olfactory and trigeminal pathways within 30 min after intranasal administration (iN). Moreover, delivery of IGF-1 into the brain activates the IGF-1 signaling pathways (Thorne et al., 2004). There are several reports on successful iN administration of IGF-1 in treatment of various brain injuries. For example, iN administration of IGF-1 has been found to reduce infarct volume and improve neurological function in adult rats following middle cerebral artery occlusion (Liu et al., 2001a; Liu et al., 2001b). Intranasal IGF-1 treatment has also been shown to improve behavior and Purkinje cell pathology in SCA1 mice (Vig et al., 2006). In our previous study, icv injection of IGF-1 was performed before cerebral hypoxia (Lin et al., 2005) due to the low tolerance of rat pups to the icv surgery immediately after severe cerebral HI. Hence, the non-traumatic intranasal administration of IGF-1 provides a feasible approach to achieve the post-HI treatment which is of a greater significance in clinical practice than pre-treatment. To our knowledge, iN-IGF-1 has not yet been reported in the treatment of newborn animals.

Materials and methods

Chemicals

Unless otherwise stated, all chemicals used in this study were purchased from Sigma (St. Louis, MO). Recombinant human IGF-1 (rhIGF-1) was purchased from Cell Sciences (Canton, MA). NeuN (a marker of neurons), NG2 (a marker of oligodendroglial early progenitors) and MBP (a marker of myelination) antibodies were acquired from Chemicon (Temecula, CA). Doublecortin (Dcx, a marker of neuronal progenitors) antibody was from Santa-Cruz (Santa Cruz, CA). BrdU antibody was purchased from Abcam (Cambridge, MA). Antibodies against Akt, phosphorylated Akt (pAkt) or active form of caspase-3 were purchased from Cell Signaling (Danvers, MA).

Preliminary study

To investigate whether rhIGF-1 administered by iN penetrates into the brain, normal P7 SD rat pups were placed on their backs and anesthetized with isoflurane (5% for induction and 1.5% for maintenance). After pups were sedated, 50 µg of rhIGF-1 dissolved in 5 µl PBS containing 0.1% BSA was given into the right naris using a fine tip. The pups were then maintained sedated with isoflurane for 10 min to ensure that they stayed on their backs. All pups woke up within 1-2 min when isoflurane was withdrawn and were returned to their dams. One hour after the first dose, a second dose of rhIGF-1 was infused into the right naris following the same procedure. For pups in the control group, 0.1% BSA was given by iN administration. Pups were decapitated at 30 min, 1 or 2 h after the second dose of rhIGF-1. The brain was separated into six parts: right olfactory bulb (OB), left OB, right frontal brain (FB), left FB, right posterior brain (PB) and left PB. The FB and PB were separated coronally at about the bregma level. Brain tissue was stored at -80°C till further use. Brain concentration of rhIGF-1

was measured using a commercial ELISA kit (R&D systems, Minneapolis, MN). To verify the penetration of iN administered rhIGF-1 into the HI rat brain, the same experiment was conducted in rat pups immediately after the HI insult.

Animal treatment

The Rice-Vannucci model (Rice et al., 1981) was used in the current study. Briefly, P7 SD rat pups were anesthetized with isoflurane (5% for induction and 1.5% for maintenance) and the right common carotid artery was exposed and double ligated with 6-0 silk sutures. The skin was then sutured and the pups were returned to their dams. After 1 h of recovery, rat pups were subjected to a hypoxic exposure (8% oxygen with 92% balanced nitrogen) for 2 h in a glass chamber submerged in a water bath at 37°C. To determine the therapeutic window of iN rhIGF-1, rhIGF-1 was administered at 0 h, 1 h or 2 h after hypoxia following the method described above. Rat pups in the control group received cerebral HI and iN administration of 0.1% BSA. Pups that underwent sham operation and vehicle treatment served as the sham group. Brain injury was evaluated in the P9 and P22 rat brain. The experimental procedure was approved by the Institutional Animal Use and Care Committee at the University of Mississippi Medical Center and was in accordance with the guidelines of the National Institutes of Health on the care and use of animals.

Brain section preparation and Immunohistochemistry

On P9 and P22 (2 and 15 days after HI), rat pups were anesthetized, and transcardiacally perfused with normal saline, followed by 4% PBS-buffered paraformaldehyde. For frozen sectioning, coronal brain sections at 10 µm of thickness were prepared in a cryostat. Sections were used for hematoxylin and eosin (H&E) staining and immunohistochemical staining.

For immunostaining of individual antigen, sections were run at one time to minimize variations. Sections were first incubated with primary antibodies (NeuN, 1:200; MBP, 1:500; Dcx, 1:200; NG2, 1:200) at 4°C overnight, and then incubated with appropriate secondary antibodies after washing. For visualization of MBP, sections were incubated in ABC complex (Vector, Burlingame, CA) with diaminobenzidine tetrahydrochloride (DAB) as a chromogen. For other antigens, sections were incubated with fluorophore-conjugated secondary antibody. The results were examined under a light or fluorescent microscope at appropriate wavelengths. Sections incubated in the absence of primary antibodies served as negative controls.

Detection of cell death

Cell death was detected using terminal deoxynucleotidyl transferase-mediated uridine 5'-triphosphate-biotin nick end labeling (TUNEL) kit (Chemicon). Sections were incubated with terminal deoxynucleotidyl transferase at 37°C for 1 h, followed by incubation with FITC-labeled anti-digoxigenin for 30 min at room temperature. Results were examined with a fluorescence microscope.

Western blotting

To investigate whether the Akt pathway is involved in the neuroprotection of rhIGF-1, Akt and pAkt expression was determined by western blotting after cerebral HI. Brain tissues were rapidly harvested, and hemispheres were split and frozen at 0, 2, 12 and 24 h after rhIGF-1 treatment (post-treatment 1 h group). Each sample was homogenized in ice-cold tissue extraction buffer (Invitrogen, Carlsbad, CA) containing 1% protease inhibitor cocktail. The homogenates were centrifuged at 9200 g for 15 min at 4°C for preparation of cytosolic fractions. Protein concentration of each sample was measured by the Bradford method and adjusted to 4 mg/ml. Following denaturing, samples containing 20 µg of protein were loaded into each well of a NuPAGE precast 8-16% Bis-Tris gel (Invitrogen). After electrophoresis, proteins

were transferred to a nitrocellulose membrane (Invitrogen). Membranes were blocked in NuPAGE blocking buffer (Invitrogen), and then incubated in the primary antibody (β -actin, 1:10000; Akt, 1:5000; pAkt 1:5000; or caspase-3, 1:5000) for 2 h at room temperature. After washing, the membranes were incubated with HRP-conjugated secondary antibodies for 1 h at room temperature and then processed with ECL western blotting detection reagents (GE Healthcare, Piscataway, NJ). The results were documented and analyzed with a ChemiDoc XRS system (Bio-Rad, Hercules, CA). Membranes were stripped with restore plus western blot stripping buffer (Pierce, Rockford, IL) and re-blotted. The band optical density (OD) for Akt, pAkt or caspase3 was normalized with the OD for β -actin.

Cell proliferation study

Cell proliferation was examined by the BrdU uptake method. BrdU is an analog of thymidine. It is incorporated into DNA as a proliferating cell passes through the S-phase of the cell cycle. For BrdU labeling, rat pups were given seven injections of BrdU (100 mg/kg daily from P7 to P13) intraperitoneally. Animals were transcardially perfused on P22 and brain sections were prepared as described above.

For BrdU immunostaining, sections were washed twice in 2XSSC, followed by incubation in a mixture of 50% formamide in 2XSSC at 65°C for 2 h. After washing in 2XSSC, sections were incubated in 2M HCl at 37°C for 30 min, and then washed with five changes of PBS (0.1M, PH7.4). Sheep anti-BrdU antibody (1:200) was applied to the sections and allowed to incubate at 4°C overnight. After washing, sections were incubated with biotin-conjugated secondary antibody at room temperature for 1 h, followed by the incubation with FITC-conjugated avidin for 1 h. To investigate the proliferation profile of neurons and oligodendrocytes, double labeling of BrdU and NeuN, or NG2 was performed.

Immunostaining data analysis

The brain pathological score was evaluated in H&E stained brain sections. The following brain region was examined and given a score from 0-4: cortex, striatum, hippocampus and subcortical white matter track. 0, no pathological changes were found. 1, only scattered pyknotic cells was observed under a high magnification (x 100). 2, pyknotic cells were found under a low magnification (x 25) but the damaged area was less than half of the whole area. 3, pyknotic cells were found in more than half of the whole area. 4, pyknotic cells were found in the whole area. The white matter damage score was evaluated by the extent of white matter rarefaction and necrosis.

Semi-quantification of MBP immunostaining using a computerized video-camera-based image analysis system (Image-Pro) was performed as described previously (Lin et al., 2005). MBP positive staining was primarily localized at the corpus callosum, external capsule and subcortical white matter. Hence, these areas were outlined. Mean optical density (OD) was determined. The area adjacent to the outlined area but without MBP positive staining was considered as the background. Background OD was also measured and subtracted from the signal OD. The corrected OD was used to represent the intensity of MBP positive staining.

For quantification of the density of TUNEL+, Dcx+ or BrdU+ cells, brain sections at the bregma and the middle dorsal hippocampus were used. Two consecutive sections were used at each level. Cells were counted in the cortex, striatum, hippocampus and white matter areas. Positively stained cells were counted in at least three randomly chosen high-power views (100x, 0.0768 mm²) at each brain region. The cell counting results were converted to cells per mm² and the mean value was used to represent one single brain. The pathological scoring and cell counting were done by an investigator blind to the treatment of animals.

Neurobehavioral testing

To determine if IGF-1 treatment improved neurological functions, neurobehavioral performance was tested from P5 to P21. Our previous studies have shown that performance of the neonatal rat in these tests was impaired following HI (Fan et al., 2005). All tests were performed by an investigator who was unaware of the treatment of animals.

Passive avoidance—It gives information about learning and memory capabilities as well as maturation of the inhibitory process (Hermans et al., 1992). On P20, rats were trained in a step-down type of passive avoidance apparatus. The experimental chamber was made of plexiglass. The floor of the chamber was made of parallel 2-mm-caliber stainless steel rods spaced 1 cm apart from each other and connected with an electric shock generator. The safe part was a piece of wood board placed at a corner of the chamber above the metal rods. Each animal was placed initially on the safe platform. When the rat stepped down onto the floor, it was given a foot shock (1 s, 0.5 mA). Although the rats repeatedly stepped up and down, they eventually remained on the board. The number of shocks required to retain an individual animal on the board for 2 min was recorded as a measure of acquisition of passive avoidance. The next day rats were placed on the wood board, the time elapsed before the rat stepped down to the floor was recorded as a measure of memory of the acquired passive avoidance. Following the avoidance test, locomotor activity was determined in an open field test as previously described (Fan et al., 2005).

Pole test—This test was used to assess the maturation of ascending and descending skills (Altman & Sudarshan, 1975). The rat was confronted with a situation in which it had to turn around and climb down a pole. For this purpose, a wooden stick with a cork ball on its top was installed vertically on a platform with a sawdust-filled box at the base serving as protection for the falling pups. The rat was placed directly under the ball at the top of the pole with its head held upwards. The mature pattern of descending consists of turning around on the rod, releasing grip with the forepaws, rotating of the trunk and supporting of the body during this maneuver by the hindpaws, then descend with the head in the leading position. Each pup was given three trials a day. Maturation of skill was assessed by a score defined as following: 0, falling down; 1, descending down with the head up; 2, descending down with the release of grip with the forepaws; 3, descending down with head turning around.

Elevated-plus maze test—This test was used to assess the anxiety behavior (Agmo & Belzung, 1998). The procedure is based on rodent's natural tendency to avoid open space. The plus maze consists of two open arms and two enclosed arms emanating from a common central platform to form a plus shape. The entire apparatus was elevated to a height of 40 cm above the floor. A video camera and illumination lamps were mounted on at the ceiling. The anxiety-related behaviors for each animal were recorded for 5 min by the video system on P19. At the beginning of the test, the rat was placed on the central platform with its head facing an open arm. The results were analyzed by computer software and parameters recorded were the numbers of open or enclosed arm entries, and the time each animal spent in various sections of the maze. The results were expressed as the number of open or enclosed arm entries, and the percentage of time spent in open arms or enclosed arms (time spent in open arms or enclosed arms divided by the sum of time spent in either arm).

Statistics

Data were expressed as the mean $\pm$ SD. Data were compared by t-test, one-way ANOVA or Kruskal-Wallis One Way analysis of Variance on Ranks, followed by multiple comparisons. The significance level was set at $P < 0.05$.

Results

rhIGF-1 diffused into the neonatal brain after iN

rhIGF-1 was not detected in any part of the brain in the vehicle-treated animals from either the normal or HI-treated group. As shown in Fig 1A, high concentration of rhIGF-1 was detected in the right OB at 30 min after a second dose of rhIGF-1 in the normal brain. rhIGF-1 concentration in the right FB and PB was 93 and 49 pg/mg protein respectively, which was about 5-10 % of that in the right OB. Brain concentration of rhIGF-1 was decreased 1 h later (Fig 1A), and was not detected at 2 h (data not shown). rhIGF-1 concentration in the left brain was lower than that in the right brain (Fig 1A&B).

rhIGF-1 concentration in the right OB of the HI-treated group at 30 min or 1 h was similar to that in the normal group. However, the concentration in the right FB and PB was 215 and 110 pg/mg protein respectively at 30 min after the second dose, and was 108 and 60 pg/mg protein respectively 1 h later, which was significantly higher than that in the normal brain (Fig 1A). rhIGF-1 was not detected at 2 h (data not shown). rhIGF-1 concentration in the left brain was also lower than that in the right brain in the HI-treated animals (Fig 1A&B).

iN-rhIGF-1 reduced the HI-induced brain injury in the neonatal rat

Cerebral HI caused severe cell death and tissue loss in the cortex, striatum, hippocampus and white matter area in the ipsilateral, but not contralateral control rat brain. As an example, HI-induced hippocampal injury is shown in Fig 2. No any sign of brain damage was detected in the sham brain on both P9 (Fig 2A&C) and P22. Severe brain damage was observed in the hippocampus of the HI brain (Fig 2B&D), while rhIGF-1 treatment at 0 h (Fig 2E&G) or 1 h (Fig 2F&H) after HI insult remarkably attenuated brain injury. Neuropathological scoring showed that rhIGF-1 treatment at 0 h (Table 1) or 1 h (Table 2) after cerebral HI significantly reduced the injury in the ipsilateral brain as compared to the control rat brain. However, post-treatment at 2 h after cerebral HI failed to offer neuroprotection (Table 3). On P22, similar neuroprotection was observed in the post-treatment 0 and 1 h groups (Table 4).

Immunostaining of NeuN (Fig 3A) further confirmed the HI-induced loss of neurons and abnormal neuronal morphology in the ipsilateral cortex, striatum and hippocampus of the control rat brain on both P 9 (Fig 3A/b) and P 22 (Fig 3A/f). Post-treatment 0 h (Fig 3A/c&g) or 1 h (Fig 3A/d&h) with rhIGF-1 increased the NeuN-positive cells on both P9 and P 22.

Cerebral HI impaired myelination as well. As shown in Fig 3B, the sham rat brain showed normal MBP staining pattern in the subcortical white matter track on P9 (Fig 3B/a) and P22 (Fig 3B/e). Cerebral HI greatly reduced the MBP positive staining in the ipsilateral control rat brain (Fig 3B/b&f), while post-treatment 0 h (Fig 3B/c&g) or 1 h (Fig 3B/d&h) with rhIGF-1 improved the MBP staining on both P9 and P 22. The mean OD data of MBP staining further confirmed that IGF-1 treatment improved myelination in the P9 and P22 rat brain (Table 5).

iN-rhIGF-1 improved neurological functions in the juvenile rat

Passive avoidance—Cerebral HI increased the number of electric foot shocks needed to retain the rat on the safe board at P20. Post-treatment with rhIGF-1 attenuated the HI-induced learning deficits (Fig 4A). No differences in memory of the acquired avoidance and locomotor activity among groups were observed (data not shown).

Pole test—The maturation of skill increased with age. Pups from the sham group started to succeed in the pole test from P17. Cerebral HI significantly delayed the maturation of skills as compared to the sham group, while post-treatment with rhIGF-1 at 0 or 1 h significantly improved the pole skill as compared to the control group (Fig 4B).

Elevated plus-maze test—A higher number of entries into the open arm were observed in the vehicle-treated HI group as compared to the sham group (Fig 4C). Cerebral HI insult also increased the retention in the open arm (Fig 4D). IGF-1 treatment inhibited HI-induced less anxiety-like behavior in the elevated plus-maze test (Fig 4C&D).

iN-rhIGF-1 increased phosphorylation of Akt and inhibited apoptotic cell death

The number of TUNEL-positive cells in the ipsilateral control brain at 2 d following cerebral HI increased significantly as compared to the sham brain (65.4 ± 10.6 vs. $3.8 \pm 1.3/\text{mm}^2$, $P < 0.05$). Post-treatment 0 h ($33.7 \pm 9.2/\text{mm}^2$) or 1 h ($40.5 \pm 7.4/\text{mm}^2$, $P < 0.05$) with rhIGF-1 significantly reduced the number of TUNEL-positive cells in the ipsilateral brain as compared to the control brain.

As shown in Figure 5, Western blotting results showed that ipsilateral caspase 3 expression at 12–24 h following cerebral HI was increased significantly as compared to the sham brain ($P < 0.05$), while rhIGF-1 treatment reduced activation of caspase 3 as compared to the vehicle-treated control group ($P < 0.05$) (Fig 5A&B). pAkt expression in the ipsilateral brain decreased at 2–12 h following cerebral HI as compared to the sham brain ($P < 0.05$), then restored at 24 h later. IGF-1 treatment increased the phosphorylation of Akt as compared to the vehicle-treated control brain (Fig 5A&C). Caspase 3 and pAkt did not change in the contralateral rat brain following cerebral HI or rhIGF-1 treatment (Data not shown). The total Akt level did not change within 24 h after cerebral HI (Fig 5A).

iN-rhIGF-1 increased proliferation of neuronal and oligodendrocyte progenitor cells

Dcx immunostaining was performed in the P9 rat brain. Dcx-positive cells increased significantly in the ipsilateral hemisphere 2 d after cerebral HI (Fig 6B) as compared to the sham brain (Fig 6A) (35.9 ± 2.8 vs. $9.3 \pm 1.2/\text{mm}^2$, $P < 0.05$). IGF-1 treatment further increased the number of Dcx-positive cells in the ipsilateral brain (Fig 6C) of the PT 0h ($51.0 \pm 4.7/\text{mm}^2$) and PT 1h ($48.9 \pm 3.7/\text{mm}^2$) groups ($P < 0.05$ compared to the control brain).

To further investigate the proliferation profile of neuronal and oligodendroglial progenitors, double labeling of BrdU with NeuN or NG2 was performed. BrdU was loaded from P7 to P13 daily and animals were sacrificed on P22. BrdU was found to co-localize with some NeuN (Fig 6D–F) or NG2 (Fig 6G–I)-positive cells. Our results showed that cerebral HI induced proliferation of NeuN-positive and NG2-positive cells. The number of NeuN/BrdU-positive cells was increased in the ipsilateral brain after cerebral HI as compared to the sham brain (9.4 ± 1.2 vs. $2.7 \pm 1.0/\text{mm}^2$, $P < 0.05$). Post-treatment 0 h ($18.6 \pm 2.1/\text{mm}^2$) or 1 h ($17.4 \pm 1.5/\text{mm}^2$) with rhIGF-1 further significantly increased the number of NeuN/BrdU-positive cells as compared to the vehicle-treated control group ($P < 0.05$). Cerebral HI also induced proliferation of NG2-positive cells. The number of NG2/BrdU-positive cells was increased in the ipsilateral brain after cerebral HI as compared to the sham brain (26.4 ± 2.2 vs. $19.7 \pm 1.1/\text{mm}^2$, $P < 0.05$). Post-treatment 0 h ($33.4 \pm 2.8/\text{mm}^2$) or 1 h ($35.2 \pm 2.2/\text{mm}^2$) with rhIGF-1 further increased the number of NG2/BrdU-positive cells as compared to the vehicle-treated control group ($P < 0.05$).

Discussion

The blood-brain barrier presents a major problem in developing treatment for diseases in the central nervous system as it prevents a number of potential therapeutic agents from reaching the brain. IGF-1, a promising treatment for various brain injuries (Guan, 2008), has been shown to provide neuroprotection to newborn rats (Brywe et al 2005; Lin et al., 2005) and fetal sheep (Cao et al., 2003; Guan et al., 2001; Johnston et al., 2001) when administered centrally. However, delivery to the brain remains substantial obstacle to the practical use of potential

therapeutic agents such as IGF-1. Repeated icv. administration is not practical in newborn rodents, which are extensively used in the research of perinatal brain HI injury. Moreover, central administration appears not to be practical for a large number of patients who need critical care with severe brain damage. Therefore, the current study was conducted to investigate whether a non-invasive route of iN-rhIGF-1 delivery can offer neuroprotection in the P7 neonatal rat. Our results showed that rhIGF-1 was successfully delivered into the brain by iN. Furthermore, it reduced the HI-induced brain damage in the neonatal rat and improved neurological function of the juvenile rat. Our findings might provide a potential therapeutic approach to neonatal brain injuries, particularly in the treatment of chronic diseases where repeated dosing is necessary.

In the current study, rhIGF-1 peaked at 30 min after iN in the right OB, FB and PB in both the normal and HI group. The concentration of rhIGF-1 at right PB and FB was 50-100 pg/mg protein, which is approximately 0.7-1.4 nM. In a similar study (Thorne et al., 2004), when 40 µg IGF-1 was given by iN to normal adult rats, brain IGF-1 concentration peaked 30 min later. IGF-1 concentration in the brain ranged from 0.3-1.4 nM (Thorne et al., 2004), which is comparable to our results. Our results also showed that after cerebral HI, rhIGF-1 concentration in the right PB and FB was 110-215 pg/mg protein (1.5-3 nM) 30 min after a second iN, which was significantly higher than that in the brain without the HI. This finding indicates that a HI insult enhanced the movement of rhIGF-1 into the brain. The mechanism of this process is unknown. However, others have reported that a cerebral HI insult enhances the movement of exogenous IGF-1 into the cerebrum through white matter tracts and perivascular spaces (Guan et al., 1996a). Our results demonstrated that iN is a safe and effective way to deliver rhIGF-1 to the brain in newborn animals as well. Given that an IGF-1 concentration as low as 0.1-10 nM (Cheng & Mattson, 1992; LeRoith et al., 1993) can elicit most protective and survival promoting effects, the rhIGF-1 concentration in this study should be sufficient for pharmacological effects being achieved in multiple brain regions. The mechanism of the protein transport from nasal cavity to the brain is not known at present. Several potential pathways have been proposed: (1) adsorptive or receptor-mediated endocytosis into olfactory sensory neurons followed by intracellular transport to the OB, (2) non-specific fluid phase endocytosis into olfactory sensory neurons followed by intracellular transport to OB and (3) extracellular diffusion into the olfactory submucosa along open intracellular clefts in the olfactory epithelium with subsequent diffusion into OB (Thorne et al., 1995).

Our study showed that iN-rhIGF-1 at 0 or 1 h after cerebral HI reduced the brain injury compared to the vehicle-treated control group. The neuroprotective effect of IGF-1 in neonatal cerebral ischemia has been reported in several other studies (Brywe et al., 2005; Cao et al., 2003; Guan et al. 2000&2001; Wood et al., 2007). However, some of them only administered IGF-1 immediately after cerebral HI (Brywe et al., 2005; Wood et al., 2007), which has relatively lower clinical significance. Here we found that the therapeutic window of iN-IGF-1 was 1 h after cerebral HI insult and that post-treatment at 2 h after HI failed to offer neuroprotection. Others have showed that the therapeutic window of IGF-1 was 2 h after reperfusion (Guan et al., 1996b; Guan et al., 2000). Some factors might contribute to the difference in the therapeutic window: animal age, animal model, the method of IGF-1 administration and the cerebral temperature during recovery. A lower brain temperature could extend the therapeutic window to 6 h after cerebral HI (Guan et al., 2000). In addition to morphological parameters, our neurobehavioral study further demonstrated the neuroprotective effect of iN-rhIGF-1. Performance of juvenile rats following neonatal HI in the pole, passive avoidance and plus maze tests reflects the impairment to their motor skill, learning ability and anxiety level. Our results showed that iN-rhIGF-1 improved the neurological function of juvenile rats. This is consistent with our previous results, which showed that icv-administered IGF-1 improved neurobehavioral performance of juvenile rats after a neonatal HI insult (Lin et al., 2005).

IGF-1 plays an important role in cell survival and prevention of apoptotic cell death (Crowder & Freeman, 1998; Fukunaga & Kawano 2003; Feldman et al, 1997; Russel et al., 1998). Survival-promoting effects are proposed to be elicited by activation of intracellular signaling cascades such as phosphatidylinositol-3 kinase (PI3K) pathways (Feldman et al, 1997; Russel et al., 1998). Activation of PI3K leads to phosphorylation and activation of Akt (pAkt), which can inhibit the pro-apoptotic agents such as Bad, caspase 9, caspase 3, Forkhead transcription factors and NF- κ B (Fukunaga & Kawano, 2003). Therefore, we examined the pAkt level after cerebral HI. Our results showed that pAkt level was depleted at 2 h after HI, but started to recover at 12 h after HI, and returned to the sham level 24 h after HI. IGF-1 treatment reversed the initial depletion of pAkt. Caspase 3 activity was increased significantly at 12-24 h after cerebral HI as compared to the sham group, while IGF-1 treatment reduced the caspase-3 level markedly. Moreover, cerebral HI increased the number of TUNEL-positive cells, while IGF-1 treatment reduced the number of TUNEL-positive cells by 40-50% in the ipsilateral hemisphere 2 d after cerebral HI as compared to the vehicle-treated brain. Changes in Akt phosphorylation have been reported after cerebral ischemia in different animal models. pAkt was found to show a temporal increase during reperfusion or permanent ischemia (Ouyang et al., 1999; Tomimatsu et al., 2001). In contrast, results from the current study and studies by other investigators (Brywe et al., 2005; Yoshimoto et al., 2001) indicate there is a temporal decrease in pAkt after cerebral ischemia. This early reduction in pAkt after HI might indicate a trophic factor withdrawal response, which is in line with a decrease in IGF-1 mRNA after HI in the neonatal rats (Clawson et al., 1999). In the present study, IGF-1 treatment reversed the HI-induced reduction of pAkt level, which might in turn trigger the anti-apoptotic cascades and inhibit the activation of caspase 3. The enhanced Akt phosphorylation has also been reported in the neonatal rat with intracerebral administration of IGF-1 following HI (Brywe et al., 2005).

There is much evidence supporting a key role of IGF-1 in proliferation and maturation of neurons and oligodendrocytes both in vivo and in vitro (D'Ercole et al., 1996&2008). Exogenous IGF-1 has been found to stimulate proliferation of neuronal (Dempsey et al., 2003) or oligodendroglial progenitors (Cao et al., 2003; Lin et al., 2005; Wood et al., 2007) after cerebral ischemia. Inhibiting IGF-1 activity by intracerebroventricular infusion of IGF-1 antibody significantly prevents the ischemia-induced neural progenitor proliferation (Yan et al., 2006). In the present study, we found that cerebral HI increased proliferation of Dcx, NeuN and NG2+ cells in the ipsilateral hemisphere, which reflects the self-repair ability of the young brain. The post-HI neural cell proliferation has also been reported in several other studies (Miles & Kernie, 2008; Felling et al., 2006; Sung et al., 2007; Yang & Levison, 2007). Our results showed that IGF-1 treatment further increased the number of Dcx+ cells on P9 and increased the NeuN/BrdU+, NG2/BrdU+ cells on P22 by 100% and 40-50%, respectively. These results indicate that in addition to its anti-apoptotic effect, rhIGF-1 acts as a mitogenic agent as well in the current model. Since the BrdU was injected at one week after cerebral HI (P7-P13) and the rats were sacrificed on P22, our results demonstrated that the proliferating neurons and oligodendrocytes survived for at least one week, which is encouraging for possible treatment to enhance brain self-repair. Recently, it has been found that the PI3K/Akt signaling pathway also plays an important role in neural cell proliferation and differentiation (Otaegi et al., 2006; Peng et al., 2004; Sung et al., 2007). Furthermore, in vitro studies have demonstrated that the IGF-1-induced neural progenitor cell proliferation and differentiation requires PI3K/Akt (Cui & Almazan, 2007; Kalluri et al., 2007; Peltier et al., 2007). Here we found in vivo that IGF-1 treatment increased the pAkt level in the early post-HI stage and enhanced neural cell proliferation 2 weeks following HI. Therefore, the PI3K/Akt signal transduction pathway might not only participate in inhibition of apoptotic cell death, but also in enhancing proliferation of neural progenitor cells after cerebral HI.

In summary, rhIGF-1 administered by iN administration was successfully delivered into the brain of P7 neonatal rats. iN-rhIGF-1 not only attenuated the HI-induced brain injury, but also

improved the neurological function in juvenile rats. Inhibition of neural cell apoptosis and enhancing proliferation of neural progenitor cells might contribute to its neuroprotective effects.

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Fig 1.

Brain concentration of rhIGF-1 in the right (A) and left (B) hemisphere after two doses of iN administration. Each group contained 10 animals. rhIGF-1 in right FB and PB peaked at 30 min after the second dose of IGF-1 in both the normal and HI-treated groups and began to decrease 1 h later (A). rhIGF-1 in left hemisphere was lower than that in the right (A&B). rhIGF-1 concentration in the FB and PB of the HI-treated brain was significantly higher than that of the normal brain (A&B). * $P < 0.05$ as compared to the same part of the normal brain at the same time point.

Fig 2. H&E staining of rat brain sections 2 d following cerebral HI. No pathological changes were found in the sham group (A&C). The vehicle-treated HI group showed severe tissue damage and cell death in the hippocampus area (B&D). Post-treatment 0 h (E&G) or 1 h (F&H) with rhIGF-1 reduced the HI-induced damage. Scale bar: 500 μ m in A, B, E and F; 50 μ m in C, D, G and H.

Fig 3.

Panel A, NeuN immunostaining in the P9 and P22 rat brain. Neonatal HI was performed on P7 as described in the text. Normal pattern of NeuN immunostaining was observed in the cortex of the P9 (a) and P22 (e) sham brain. NeuN-positive cells were reduced markedly following cerebral HI (b&f). Post-treatment 0 h (c&g) or 1 h (d& h) with rhIGF-1 partially reversed the HI-induced loss of NeuN staining. Scale bar: a-d, 50 μ m; e-h, 200 μ m. Panel B, MBP immunostaining in the P9 and P22 rat brain. Neonatal HI was performed on P7 as described in the text. Normal pattern of MBP staining was observed in brain sections at the bregma level of the P9 (a) and P22 (e) rat brain. Cerebral HI greatly reduced MBP positive staining in the ipsilateral control rat brain (b&f), while post-treatment 0 h (c&g) or 1 h (d&h) with rhIGF-1 improved the loss of MBP staining in both the P9 and P 22 rat brain. Scale bar: a-d, 50 μ m; e-h, 500 μ m.

Fig 4.

Neurobehavioral tests after cerebral HI. A. Acquisition of passive avoidance in P20 rats. The number of electric shocks shown in the figure represents the learning ability of rats to acquire avoidance. Cerebral HI increased the number of electric shocks required in the HI control rats, while iN-rhIGF-1 significantly reduced the number of electric shocks required as compared to the control group. B. Maturation of skills determined in the pole test. Performance in the pole test was determined from P5 to P21 and the grade scale was defined as described in the text. Pole skill matures with age. Cerebral HI severely harmed development of the pole skill, while iN-rhIGF-1 improved the pole skill as compared to the control group. C&D. The elevated-plus maze test was conducted on P19 as described in the text. C represents the number of open arm or close arm entries. D represents the percentage of time spent in open arms or closed arms. A higher number of entries into the open arm were observed in the vehicle-treated HI group as compared to the sham group (C). Cerebral HI insult also increased the retention in the open arm (D). IGF-1 treatment inhibited HI-induced less anxiety-like behavior in the elevated plus-maze test (C&D). * $P < 0.05$ compared to the sham group. # $P < 0.05$ compared to the HI control group.

Fig 5.

Effects of rhIGF-1 on phosphorylation of Akt and caspase3 activation following cerebral HI. Representative western blotting results of caspase 3, Akt and pAkt are shown in A. Quantification of the OD data showed that activated caspase3 level was increase at 2-24 h after cerebral HI and IGF-1 (post-treatment 1 hr) significantly reduced activation of caspase3 (B). Akt expression did not change within 24 h following cerebral HI. pAKt level decreased significantly at 2 and 12 h after HI, while IGF-1 treatment reversed the reduction of pAkt (C). * P<0.05 compared to the sham group. # P<0.05 compared to the HI control group at the same time point.

Fig 6.

Effects of rhIGF-1 on neural cell proliferation. Cerebral HI increased the number of Dcx+ cells (B) in the ipsilateral hemisphere as compared to the sham brain (A). IGF-1 treatment further increased the number of Dcx+ cells on P9 (C). Double labeling showed some NeuN+ cells (E) were co-localized with BrdU+ cells (D) in the ipsilateral hippocampal area (F) on P22. Some NG2+ cells (H) were also co-localized with BrdU+ cells (G) in the ipsilateral cortex (I). Scale bar: A-C, G-I, 50 μ m. D-F, 25 μ m. Arrows in F and I represent the double-labeled cells.

Table 1
Neuropathological scoring 2 d after cerebral HI (PT0h)

	Control (n=11)	IGF-0h (n=11)	Decrease %	P
Cortex	2.8±0.4	1.5±0.3	46%	0.021
Striatum	2.6±0.3	1.6±0.3	38%	0.041
Hypocampus	2.6±0.4	1.4±0.3	46%	0.031
White matter	2.7±0.4	1.1±0.3	59%	0.003
Total	10.8±1.5	5.5±1.2	49%	0.013

Neuropathological scoring is evaluated as described in Methods. rhIGF-1 treatment immediately after cerebral HI (PT 0h) significantly reduced the neuropathological score as compared to the control group.

Table 2
Neuropathological scoring 2 d after cerebral HI (PT1h)

	Control (n=11)	IGF-1h (n=11)	Decrease %	P
Cortex	2.3±0.3	0.9±0.2	61%	0.002
Striatum	2.4±0.3	1.3±0.2	46%	0.004
Hypocampus	3.1±0.3	1.4±0.3	55%	0.001
White matter	2.1±0.5	0.9±0.3	57%	0.025
Total	9.8±1.8	4.3±0.8	56%	0.001

Neuropathological scoring is evaluated as described in Methods. rhIGF-1 treatment 1 h after cerebral HI (PT1h) significantly reduced the neuropathological score as compared to the control group.

Table 3

Neuropathological scoring 2d after cerebral HI (PT2h) Control (n=9) IGF-2h (n=10) Decrease % P

	Control (n=9)	IGF-2h (n=10)	Decrease %	P
Cortex	2.3±0.4	1.9±0.5	17%	0.531
Striatum	1.9±0.4	1.7±0.5	11%	0.748
Hypocampus	2.9±0.4	2.0±0.5	31%	0.182
White matter	2.2±0.5	1.6±0.5	27%	0.411
Total	9.3±1.6	7.2±1.9	23%	0.412

Neuropathological scoring is evaluated as described in Methods. rhIGF-1 treatment 2 h after cerebral HI (PT2h) did not significantly change the neuropathological score as compared to the control group.

Table 4

Neuropathological scoring 15 d after cerebral HI

	Control (n=5)	IGF-1, PT0h (n=6)	Decrease %	IGF-1, PT1h (n=6)	Decrease %
Cortex	2.6±0.6	1.0±0.4 [*]	62%	1.0±0.4 [*]	62%
Striatum	2.2±0.4	0.8±0.3 [*]	64%	0.7±0.3 [*]	68%
Hypocampus	3.0±0.5	1.5±0.5 [*]	50%	1.0±0.5 [*]	67%
Total	7.8±1.3	3.3±1.1 [*]	58%	2.7±1.0 [*]	65%

Neuropathological scoring is evaluated as described in Methods. rhIGF-1 treatment immediately (PT0h) or 1 h (PT1h) after cerebral HI significantly reduced the neuropathological score.

^{*} P<0.05 as compared to the control group.

Table 5
Mean optical density of MBP staining following cerebral HI

	P9	P22
Sham	0.0472±0.007 (n=11)	0.170±0.021 (n=5)
Control	0.009±0.004* (n=11)	0.080±0.050* (n=5)
IGF-PT0h	0.033±0.014*# (n=11)	0.124±0.015*# (n=6)
IGF-PT1h	0.028±0.010*# (n=11)	0.130±0.023*# (n=6)

Mean optical density of MBP staining was determined as described in Methods. Cerebral HI decreased the MBP staining in both the P9 and P22 rat brain, while IGF-1 treatment at 0 or 1 h after HI partially reversed the HI-induced hypomyelination.

* P<0.05 as compared to the sham group.

P<0.05 as compared to the HI control group.