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Whole body hypothermia broadens the therapeutic window of intranasally administered IGF-1 in a neonatal rat model of cerebral hypoxia-ischemia

Shuying Lin, **Philip G. Rhodes**, and **Zhengwei Cai***
Department of Pediatrics, Division of Newborn Medicine, University of Mississippi Medical Center, Jackson, MS 39216

Abstract

To investigate whether whole body hypothermia after neonatal cerebral hypoxia-ischemia (HI) could broaden the therapeutic window of intranasal treatment of IGF-1 (iN-IGF-1), postnatal day 7 rat pups were subjected to right common carotid artery ligation, followed by 8% oxygen inhalation for 2 h. After HI, one group of pups were returned to their dams and kept at room temperature (24.5±0.2°C). A second group of pups were subjected to whole body hypothermia in a cool environment (21.5±0.3°C) for 2 or 4 h before being returned to their dams. Two doses of 50 μg recombinant human IGF-1 were administered intranasally at a 1 h interval starting at 0, 2 or 4 h after hypothermia. Hypothermia decreased the rectal temperature of pups by 4.5°C as compared to those kept at room temperature. While hypothermia or iN-IGF-1 administered 2 h after HI alone did not provide neuroprotection, the combined treatment of hypothermia with iN-IGF-1 significantly protected the neonatal rat brain from HI injury. Hypothermia treatment extended the therapeutic window of IGF-1 to 6 h after HI. The extended IGF-1 therapeutic window by hypothermia was associated with decreases in infiltration of polymorphonuclear leukocytes and activation of microglia/macrophages and with attenuation of NF-κB activation in the ipsilateral hemisphere following HI.

Keywords

hypothermia; IGF-1; intranasal administration; neonatal hypoxia-ischemia; polymorphonuclear cell infiltration; NF-κB

1. Introduction

Insulin-like growth factor-1 (IGF-1) is a broad neurotrophic factor widely distributed in the central nervous system (Baskin et al., 1988). Exogenous IGF-1 given soon after brain injury has been shown to offer neuroprotection in both the adult (Schäbitz et al., 2001) and newborn animals (Brywe et al., 2005; 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 cerebral hypoxia-ischemia (Lin et al., 2005). However, the icv injection might not

*Corresponding author Phone number: 601-984-2786, Fax: 601-815-3666, zcai@umc.edu.

be practical in humans, as it requires surgery with potential risks of infection and may cause other complications. Therefore, we developed a non-invasive IGF-1 delivery method through intranasal (iN) administration of IGF-1. In our previous study (Lin et al., 2009), we found that intranasally administered IGF-1 penetrated into the neonatal rat brain successfully. iN-IGF-1 not only attenuated pathological changes induced by cerebral hypoxia-ischemia (HI), but also improved neurological dysfunctions in the juvenile rats following HI (Lin et al., 2009). However, the therapeutic window of iN-IGF-1 is relatively narrow. In our neonatal cerebral HI model, iN-IGF-1 showed neuroprotection only when administered within 1 h following cerebral HI (Lin et al., 2009). To be clinically useful, a broad window of therapeutic opportunity is desirable.

In recent years, selective head cooling combined with mild whole body cooling or whole body cooling alone have been shown to be effective neuroprotective strategies for the prevention of neonatal hypoxic-ischemic encephalopathy in human full term newborn infants (Azzopardi et al., 2009; Shankaran et al., 2005). In fact, experimental studies in both adult and newborn animal models have found that hypothermia, when applied with minimal delay after the insult and for an appropriate duration and degree, significantly reduces HI-induced neuronal death (Agnew et al., 2003; Tooley et al., 2003; Gunn et al., 1997). Some possible mechanisms are involved in the neuroprotective effects of hypothermia. For example, hypothermia has been shown to inhibit neuronal apoptosis (Zhao et al., 2005), reduce brain metabolic rate (Williams et al., 1997), block excitatory neurotransmitters (Baker et al., 1991) and reduce the products of oxidative stress following cerebral HI (Brooks et al., 2002). IGF-1 has also been shown to be a potent anti-apoptotic agent (Guan 2008) and a potent mitogen following brain injury (Wiltrout et al., 2007). In our previous study, we demonstrated that IGF-1 treatment activated the pAkt pathway and inhibited the activation of caspase-3 following cerebral HI. Moreover, it promoted the proliferation of neural progenitor cells during the tissue repair phase in a neonatal HI model (Lin et al., 2009). Hence, hypothermia shares some similar neuroprotective mechanisms with IGF-1, but they also act on other mechanisms. Therefore, in the current study, we investigated whether whole body hypothermia in combination with iN-IGF-1 could offer extra neuroprotection, or broaden the therapeutic window of IGF-1 in a neonatal rat model of cerebral HI. Furthermore, we investigated how brief hypothermia immediately after cerebral HI extended the therapeutic window of IGF-1.

2. Results

2.1. Hypothermia for up to 4 h was safe in the neonatal rat

The mean rectal temperature was 33.5±0.3°C in rats before the hypoxia treatment and it was raised to 35.0±0.2°C immediately after hypoxia. In the normothermic group, the rectal temperature of rat pups was maintained at 33.4±0.2°C from 30 min after HI throughout the entire experimental duration. In the hypothermic group, the rectal temperature of rat pups was decreased to 29.5±0.4°C at 30 min after HI, and maintained at 28.9±0.3°C from 1 h after HI throughout the entire hypothermia duration. Therefore, the hypothermic treatment decreased the rectal temperature by 4.5°C as compared to the normothermic group ($P<0.05$). Once hypothermia was terminated, the rat pups were returned to their dams. The rectal temperature of the hypothermic rat pups was elevated to 33.1±0.3°C within 30 min after the termination of hypothermic treatment ($P>0.05$, as compared to that of the normothermic group), and remained at the normal level throughout the whole experiment.

The average body weight of P7 rat pups before HI was 13.5±0.5 g. As shown in Fig 1, the rat pups in the sham group gained 6% of body weight in 6 h. However, all rat pups that were subjected to cerebral HI had a significant decrease in body weight within 6 h after HI ($P<0.05$) as compared to the sham group. Rat pups began to gain weight at 24 h after HI. But

the weight gain in the HI rat pups was significantly lower than that of the sham rats at 24 and 48 h following HI ($P<0.05$). Following HI, rat pups in hypothermic (for 4 h) and normothermic groups did not show significant difference in body weight gain at 0–24 h after HI ($P>0.05$). However, the rat pups in the hypothermia (HT)+IGF-1 group (IGF-1 was given at 0 h after hypothermia for 4 h) gained more weight at 48 h after HI as compared to the other 3 HI groups ($P<0.05$).

The blood glucose level in the sham rats was 105±4 mg/dl. As shown in Fig 2, the blood glucose level decreased significantly immediately after hypoxia treatment. During the recovery stage, the blood glucose level stayed normal after 2 or 4 h of the hypothermic treatment. However, when hypothermia was extended to 6 h, the blood glucose level dropped significantly (62±8 mg/dl, $P<0.05$ as compared to the sham group). These data suggested that hypothermia for up to 4 h did not cause hypoglycemia or adversely affected the body weight gain and it was safe to the neonatal rat. Therefore, the duration of hypothermia was set at 2 or 4 h in our experiments.

2.2. Hypothermia for 2–4 h broadened the therapeutic window of IGF-1

Representative H&E stained brain sections at the ipsilateral hippocampus are shown in Fig 3. No pathological changes were found in the sham rat brain (Fig 3A). Cerebral HI caused severe tissue damage at the cortex, striatum, hippocampus (Fig 3B) and white matter areas. IGF-1 treatment following 4 h of normothermia (Fig 3C) or hypothermia for 4 h alone (Fig 3D) did not attenuate the brain damage, while the combination of hypothermia and IGF-1 treatments decreased the extent of brain injury (Fig 3E). As shown in Table 1A, hypothermia for 2 h combined with immediate IGF-1 treatment (IGF 0h) decreased the total pathological score by 40% as compared to the group that was treated with normothermia and vehicle ($P<0.05$). Similarly, hypothermia for 4 h combined with immediate IGF-1 treatment (IGF 0h, Table 1B) or IGF-1 treatment at 2 h later (IGF 2h, Table 1C) also reduced the pathological scores by 43% and 34%, respectively, as compared to the group that was treated with normothermia (NT)+BSA ($P<0.05$). Hypothermia or IGF-1 treatment alone did not offer neuroprotection ($P>0.05$) in all treatment regimens (Tables 1A, B & C). However, IGF-1 did not offer neuroprotection when administered at 4 h following the hypothermic exposure (HT4h+IGF4h regimen) ($P>0.05$ as compared to the NT+BSA group, data not shown).

2.3. Hypothermia treatment reduced infiltration of polymorphonuclear(PMN) cells and activation of microglia/macrophages in the infarct area following HI

CD43 antibody was used to detect infiltrated PMN cells. As shown in Fig 4, only a few CD43-positive cells (5.3±1.1 cells/mm^2) were seen in the P7 sham rat brain (Fig 4A). Cerebral HI induced an accumulation of CD43+ cells (28.9±2.4 cells/mm^2) in the cortex, striatum and hippocampus of the ipsilateral hemisphere as early as 4 h following HI (Fig 4B), whereas the hypothermic treatment reduced the PMN cell infiltration (18.9±2.6 cells mm^2, $P<0.05$ as compared to the normothermic group, Fig 4C). PMN cell infiltration was more evident at 48 h following HI. The combination of hypothermia and IGF-1 treatment significantly reduced PMN cell infiltration (36.3±5.8 cells mm^2, Fig 4F) as compared to the normothermia and vehicle-treated group (58.0±7.3 cells mm^2, $P<0.05$, Fig 4E).

The hypothermia and IGF-1 treatment also attenuated activation of microglia/macrophages in the rat brain following HI. Only a few ED1-postitive cells (6.8±1.5 cells/mm^2) were observed in the sham rat brain (Fig 4G). At 4 h following cerebral HI, microglia/macrophages were activated, as indicated by the increased number of ED1-positive cells in the ipsilateral hemisphere of the normothermic group, especially at the cingulum area (23.6±2.8 cells/mm^2, Fig 4H), while the hypothermic treatment significantly decreased the

number of activated of microglia/macrophages (15.6±2.0 cells/mm^2, Fig 4I). More microglia/macrophages were activated in the infarct areas including the cortex, striatum, internal capsule and external capsule at 48 h following cerebral HI in the normothermia and vehicle-treated group (68.4±6.5 cells/mm^2, Fig 4K), while hypothermia+IGF-1 treatment reduced the number of ED1+ cells to 42.9±5.8 cells/mm^2 ($P<0.05$, Fig 4L).

2.4. Hypothermia inhibited the HI-induced activation of Nuclear Factor-KappaB (NF-κB)

In the current study, because 4h was the longest hypothermia duration, which did not alter the blood glucose level significantly in rat pups while provided neuroprotection in combination with the IGF-1 treatment, NF-κB activation was measured in the HT4h+IGF0h regimen. As shown in Fig 5, cerebral HI induced activation of NF-κB at 2 h after HI and the elevation of NF-κB activation lasted for 24 h following HI. Hypothermic treatment reduced the elevation of NF-κB level at 2–4 h following cerebral HI as compared to the normothermic groups. At 12 h following HI, activation of NF-κB in the HT+BSA group was increased to a level similar to that for the NT group with or without IGF-1 treatment. However, activation of NF-kB in the HT+IGF-1 group still remained at a lower level at 12 h after HI as compared to other HI groups ($P<0.05$). At 24 h activation of NF-κB began to decrease in the normothermic groups as well as in the HT+BSA group, but was still higher in all groups as compared to the sham group. No significant difference was detected among the four HI groups ($P>0.05$).

2.5. Hypothermia in combination of IGF-1 increased phosphorylation of Akt and inhibited activation of caspase-3

Expression of Akt and pAKt, as well as activation of caspase-3 in the rat brain were determined by western blotting in the HT4h+IGF0h regimen. As shown in Figure 6, western blotting results demonstrated that the total Akt level did not change within 24 h after cerebral HI. Cerebral HI caused a reduction in the pAKt level in the ipsilateral brain at 4 h following cerebral HI. Hypothermia or IGF-1 treatment did not alter the pAKt level as compared to that in the NT+BSA-treated rat brain, while a combination of hypothermia and IGF-1 treatment increased the phosphorylation of Akt (Fig 6B) at 12 h following cerebral HI ($P<0.05$ as compared to the NT+BSA-treated rat brain). Cerebral HI also induced activation of caspase-3 in the ipsilateral rat brain at 12–24 h following HI. Hypothermia or IGF-1 treatment alone did not change the activated caspase-3 level as compared to the NT+BSA-treated rat brain, while combination of hypothermia and IGF-1 treatment greatly reduced the activation of caspase-3 at 12 and 24 h following cerebral HI (Fig 6C, $P<0.05$ as compared to the NT+BSA-treated rat brain).

3. Discussion

Our previous study demonstrated that iN-rhIGF-1 protected the neonatal rat brain from HI-induced brain injury (Lin et al., 2009). However, IGF-1 offered neuroprotection only when administered within 1 h following HI insult (Lin et al., 2009). Our current study showed that brief whole body hypothermic treatment immediately after HI insult for 4 h extended the therapeutic window of IGF-1 to 6 h following HI (HT4h+IGF2h). Although selective head cooling combined with mild whole body cooling has been shown protection against neonatal HI brain injury, it requires specific facilities. As shown in the present study, the extended therapeutic window of IGF-1 with brief whole body hypothermia might be of a greater clinical significance.

Due to the small size of the rat pup, it was technically difficult to monitor the cerebral temperature directly. Since the rectal temperature is closely related to cerebral temperature (Sahuquillo and Vilalta, 2007), we measured the rectal temperature of pups and used it as an

indication of cerebral temperature. In the current study, hypothermia reduced rectal temperature of rat pups by 4.5°C as compared to the normothermia group. This hypothermia was mild to moderate. The extent of hypothermia is comparable to a similar study conducted in neonatal rats (Jatana et al., 2006).

In the current study, in order to lower the body temperature, rat pups in the hypothermia group were separated from their dams for up to 4 h. As shown in Fig 2, the blood glucose level decreased significantly immediately after the severe hypoxia. Following HI, the blood glucose level in the normothermia group recovered within 2 h and remained at the normal level. In the hypothermic group, rat pups were separated from their dams and were deprived of maternal feeding. Although these rat pups could maintain their blood glucose levels during the first 4 h following HI, probably through mobilization of their glycogen storage, they eventually could not maintain their blood glucose level at 6 h after HI. Lack of feeding in the hypothermia group appears to be the most likely cause for the significant drop in the blood glucose level at 6 h. It is technically difficult to provide feeding to rat pups during hypothermia. Based on these findings, the hypothermia duration in the current study was set at 2 or 4 h following cerebral HI. As a matter of fact, body weight loss in the hypothermia group during the first 4 h after HI was not greater than that in the normothermia group (Fig. 1, NT+BSA vs HT+BSA), and rat pups in the HT+IGF-1 group gained more weight at 48 h following cerebral HI than those in other HI groups, which might be due to the attenuated brain injury. Therefore, whole body hypothermia for a short duration (up to 4 h) was safe in our current animal model.

Consistent with our previous finding (Lin et al., 2009), delayed iN-IGF-1 treatment (2–6 h following HI insult) under normothermic condition did not provide neuroprotection in the current study. Also, hypothermia for 2–4 h alone did not decrease the pathological score in the HI rat brain as compared to the normothermia and vehicle-treated group either. Other researchers also found that post-insult hypothermia offers neuroprotection only when the duration is as long as 24–72 h (Ohmura et al., 2005; Tomimatsu et al., 2001), while short-term hypothermia during brain recovery appears not to be neuroprotective (Guan et al., 2000; Jatana et al., 2006; Lasarzik et al., 2009).

Our results also showed that the combined treatment of hypothermia with iN-IGF-1 reduced total pathological score in the rat brain by 34–43%. In our previous study, iN-IGF-1 treatment at 0 or 1 h after the HI insult reduced the total pathological score by 49–56%. Therefore, the combination of hypothermia and IGF-1 treatments did not offer extra neuroprotection, but only extended the therapeutic window of iN-IGF-1. This finding indicates that both the timing of iN-IGF-1 treatment and the core temperature during recovery are important for the successful treatment of perinatal HI. It was found in a study with adult rats that the cerebral temperature during recovery closely modulated the window of opportunity for neuronal rescue with IGF-1 (Guan et al, 2000). When rats were recovered from a HI insult in a warm environment, IGF-1 offered neuroprotection only when it was administered 2 h after HI insult. However, when rats were recovered in a cool environment, the therapeutic window of IGF-1 treatment was extended to 6 h post insult (Guan et al, 2000). Results from the present study with neonatal rats are consistent with the reported adult rat data.

In the current study we found that the post-insult hypothermia for 2–4 h did not reduce the HI-induced brain injury. However, it greatly broadened the therapeutic window of iN-IGF-1 treatment. To explore the possible mechanisms that might contribute to this finding, we investigated whether transient hypothermia immediately following cerebral HI would cause any pathophysiological changes in the neonatal rat brain. First, we found that the hypothermic treatment for 4 h reduced the PMN infiltration and activation of microglia/macrophages at 4

h after HI as compared to the normothermic treatment. Among all blood cell types, PMNs are the first to infiltrate the brain after stroke in human and animal stroke models (Lindsberg and Grau, 2003; Ritter et al., 2000). After experimental stroke there is an adhesion of PMNs to venules within 1 h in a rat model (Ritter et al., 2000) and within the first 2 h in a pig model (Gidday et al., 1997). PMNs are capable of expressing inflammatory factors such as formyl peptide receptor-1 and neutrophil cytosolic factor-4, which can activate the brain inflammatory response and cause brain damage (Tang et al., 2006). Macrophage/microglia is another important type of inflammatory cells in the brain. Macrophage/microglia are recruited and activated in the central nervous system as early as 2 h after cerebral HI (Garden and Möller, 2006). They produce several pro-inflammatory factors such as interleukin 1β, tumor necrosis factor-α and inducible nitric oxide synthase, which may play key roles in the pathophysiology of perinatal brain damage (Fan et al., 2010; Pang et al., 2007). Consistent with our findings, other studies also show that hypothermic treatment attenuates the PMN infiltration (Prandini et al., 2005) and macrophage/microglia activation following cerebral ischemia (Webster et al., 2009). Therefore, in the current study, hypothermic treatment might attenuate HI-induced inflammatory responses and delay the onset of secondary neuronal death, thus providing a wider window for iN-IGF-1 to exert its anti-apoptotic effects.

Secondly, we also found in the present study that hypothermia delayed and reduced NF-κB activation in the rat brain following HI (Fig 5). Many other researchers also reported that hypothermia attenuated NF-kB activation and brain inflammation in focal (Han et al., 2003) and global cerebral ischemia (Webster et al., 2009). NF-κB is a major transcription factor involved in brain inflammation following various brain insults. It consists of a family of proteins that contain the Rel-homology domain, including p50, p65, p52, c-rel, and Rel-B. NF-κB is normally sequestered in the cytoplasm where it is bound to a family of inhibitory proteins known as the inhibitor of NF-κB (IκB). Inflammatory stimuli activate a family of upstream kinases which phosphorylate IκB leading to its degradation and the liberation of NF-κB to enter the nucleus and induce the downstream gene expression (Rothwarf et al., 1999). Activation of NF-kB increases the production of proinflammatory cytokines, chemokines and adhesion molecules, which together can promote apoptosis and neuronal cell death (O'Neill and Kaltschmidt, 1997). Emerging evidence has demonstrated a role for NF-κB activation in HI-induced cerebral damage in adult (Scheider et al., 1999) and neonatal (Lin et al., 2006;van den Tweel et al., 2006) models of cerebral ischemia. In the current study, we showed that cerebral HI induced activation of NF-κB in the ipsilateral hemisphere starting at as early as 2 h following HI and lasting for 24 h. Hypothermic treatment decreased the elevation of NF-κB activation at 2–4 h following cerebral HI as compared to the normothermic groups and the additional IGF-1 treatment at 4 h following HI further maintained a low level of NF-κB activation as compared to other HI groups at 12 h following HI. A recent study has shown that while early inhibition of NF-κB activation at 0–3 h after cerebral HI provided strong neuroprotection, late inhibition of NF-kB activation (18–21 h after HI) aggravated brain injury in a similar neonatal rat model of cerebral HI (Nijboer et al., 2008). Consistent with their findings, the early inhibition of NF-κB activation after cerebral HI (2–4 h) in the current study appears to be of great significance in neuroprotection seen in our model. It is uncertain in the current study whether decreased NF-kB activation at 12 h in the hypothermia+IGF-1 group also contributes to the observed neuroprotection or it might prevent endogenous neuroprotection and compromise the protection of IGF-1 as does by the deteriorate late inhibition (18–21 h) of NF-kB (Nijboer et al., 2008). Since brain injury in the hypothermia+IGF-1 group was significantly improved as compared to that in either the normothermia+IGF-1 group or the hypothermia+BSA group, where NF-kB activation at 12 h was increased, it is possible that decreased NF-kB activation at 12 h after HI in the current study also contributes to the observed neuroprotection. There are also investigators who showed that post-insult hypothermia augmented activation of NF-

κB (Arai et al., 2008;Fairchild et al., 2004). The different effects of hypothermia on NF-κB activation might be caused by the difference in animal models, duration of hypothermia and the targeted brain temperature. Further investigations are needed to elucidate the detailed role of NF-κB at various times after the HI insult and its down-stream signaling pathways in the neuroprotection of hypothermia and IGF-1.

Thirdly, we also investigated whether the pAkt signaling pathway was involved in the neuroprotection of the combined treatment of hypothermia with IGF-1. Phosphoinostitide-3-kinase (PI3K)/Akt survival signal pathways have been found to play critical roles in neuronal survival after cerebral ischemia (Brywe et al., 2005; Zhao et al., 2005). Several studies have demonstrated that hypothermia inhibits the dysfunction of the Akt pathway after cerebral ischemia. For example, in an adult focal brain ischemia model, hypothermia blocked the ischemia-induced pAkt changes and maintained pAkt activity (Zhao et al., 2005). In a neonatal HI model, hypothermia was found to enhance pAkt activity at 24 h after HI and blocked cytochrome C release from the mitochondria and caspase activation (Zhu et al., 2006). However, there are other reports that showed reduced pAkt levels following hypothermia treatment in a neonatal HI model (Tomimatsu et al., 2001). In the current study, hypothermia alone did not prevent the HI-induced decrease in pAkt level in this severe model of neonatal HI. This contradiction might be caused by differences in animal models, severity of the HI and duration of hypothermia. Our previous work demonstrated that pAKt levels were decreased significantly at 2 h after HI, while iN-IGF-1 administered at 1 h after cerebral HI maintained pAkt level at 2 and 12 h following HI and significantly inhibited activation of caspase-3 (Lin et al., 2009). Our current study showed that IGF-1 given at 4 h following cerebral HI in the normothermic condition did not have an effect on the decreased phosphorylation of Akt, while the combination of hypothermia and IGF-1 increased phosphorylation of Akt at 12 h after HI and reduced activation of caspase-3 (Fig 6). These data indicated that in our current animal model, the neuroprotective effect and therapeutic window of IGF-1 were temperature dependent.

In summary, whole body hypothermia for up to 4 h broadened the therapeutic window of iN-IGF-1 to 6 h after the insult in a neonatal rat model of cerebral HI. Hypothermia treatment reduced infiltration of PMNs and activation of macrophage/microglia in the infarct area following HI. It also inhibited activation of NF-κB at the early stage of brain recovery. These effects might delay the HI-induced pathophysiological changes in the neonatal rat brain and thus provide a wider therapeutic window for IGF-1 treatment.

4. Experimental Procedure

4.1. 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). Antibodies against α-tubulin, Akt, phosphorylated Akt (pAkt) or active form of caspase-3 were purchased from Cell Signaling (Danvers, MA). Antibodies against ED1 (marker of activated microglia/macrophages) and CD43 (marker of PMN cells) were purchased from Millipore (Temecula, CA).

4.2. Animal model

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. Pups that underwent sham operation and normoxic inhalation served as the sham group. All experiments were performed following an institutionally approved protocol in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

4.3. Whole body hypothermia

Rat pups were randomly assigned to the hypothermia or normothermia group immediately after HI exposure. Pups in the normothermia group were returned to their dams after HI and kept in cages at room temperature (24.5±0.2°C). In order to keep the body temperature at a low level, rat pups in the hypothermia group were separated from their dams and placed in a cage at 21.5±0.3°C. To test the safety of hypothermia, the duration of hypothermia was set at 2 h, 4 h or 6 h. Rectal temperature was monitored at the following time points: before hypoxia, immediately after hypoxia and every 30 min during hypothermia using a digital thermometer (Fisher Scientific, Houston, TX). Body weight was measured before HI, every 2 h during hypothermia, 24 and 48 h after HI. Percentage changes in body weight (the body weight before the HI treatment on P7 was used as the baseline level) were calculated at different time points. Blood glucose levels were monitored every 2 h during hypothermia using a blood glucose meter (Bayer, Pittsburgh, PA). To monitor the blood glucose level, the tail of rat pups was topically anesthetized with sensorcaine solution first. About 1 mm of tail was then cut and the blood sample was collected for the measurement.

4.4. Intranasal administration of IGF-1

Intranasal administration of IGF-1 was performed as described before (Lin et al., 2009). Briefly, 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. IGF-1 or vehicle (0.1% BSA in PBS) administration was started at 0 h, 2 h or 4 h (IGF0, 2, 4 h) following hypothermia or normothermia exposure for 2 or 4 h. Therefore, this treatment regime represented a post-treatment of IGF starting from 2–8 hr after HI. Pups in the sham group went through the same anesthesia procedure and were infused with vehicle and kept under normothermia.

4.5. Experimental group

Our previous study demonstrated that IGF-1 offered neuroprotection only when administered at 1 h following cerebral HI (Lin et al., 2009). To determine whether hypothermia treatment broadens the therapeutic window of IGF-1, whole body hypothermia was started immediately after cerebral HI. The duration of hypothermia was set at 2 or 4 h. IGF-1 post- treatment was started at 0, 2, or 4 h following hypothermia. Therefore, we had the following experimental schemes:

1. Hypothermia (or Normothermia) for 2 h and followed by immediate IGF (or BSA) treatment, HT (NT) 2h+IGF (BSA) 0h;
2. Hypothermia (or Normothermia) for 4 h and followed by immediate IGF (or BSA) treatment, HT (NT) 4h+IGF (BSA) 0h;
3. Hypothermia (or Normothermia) for 4 h and with IGF (or BSA) treatment 2h later, HT (NT) 4h+IGF (BSA) 2h, and
4. Hypothermia (or Normothermia) for 4 h and with IGF (or BSA) treatment 4h later, HT (NT) 4h+IGF (BSA) 4h.

Equal numbers of male and female rat pups were included in each experimental group.

4.6. Brain section preparation and Immunohistochemistry

At 4 h and 2 d following cerebral 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.

Since the maximum duration of hypothermia in the current study was 4 h, we tried to determine what pathophysiological changes the brief post-insult hypothermia would cause, and how these changes might contribute to the broadened therapeutic window of IGF-1. Therefore, immunostaining of ED1 and CD43 was performed right after the termination of 4 h hypothermia (before IGF-1 treatment), and at 2 d following cerebral HI in the HT4h +IGF0h regimen.

For immunostaining of individual antigens, sections were processed simultaneously to minimize variations. Sections were first incubated with primary antibodies (CD43, 1:200; ED1: 1:200) at 4°C overnight, and next day, incubated with appropriate fluorophore-conjugated secondary antibodies after washing. The results were examined under a fluorescent microscope at appropriate wavelengths. Sections incubated in the absence of primary antibodies served as negative controls.

4.7. NF-κB measurement

NF-κB activation was measured in the HT4h+IGF0h regimen. For measurement of NF-κB activation in the brain, brain samples from the ipsilateral hemisphere were acquired at 0, 2, 4, 12 and 24 h following cerebral HI. Nuclear protein was extracted using a commercial kit (Panomics, Fremont, CA). Activation of NF-κB in the rat brain was determined using an ELISA kit (Panomics), following the manufacture's instruction. This kit specifically detects the p50 member of NF-κB.

4.8. Western blotting

To investigate whether the Akt pathway is involved in the neuroprotection of HT+IGF-1, Akt, pAKt and caspase-3 expression was determined by western blotting after cerebral HI in the HT4h+IGF0h regimen. Brain tissues were rapidly harvested at 0, 4, 12 and 24 h after IGF-1 treatment (IGF0 h group) and hemispheres were split and stored at −80°C until use. 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 with the primary antibody (α-tubulin, 1:10000; Akt, 1:5000; pAkt 1:5000; or active form of caspase-3, 1:5000) at room temperature for 2 h. After washing, membranes were incubated with HRP-conjugated secondary antibodies for 1 h and then processed with ECL western blotting detection reagents (GE Healthcare, Piscataway, NJ). 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 α-tubulin.

4.9. Histological data analysis

The brain pathological score in the ipsilateral hemisphere was evaluated in H&E stained brain sections as described in our previous study (Lin et al., 2009). 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 were observed under a high magnification (× 100); 2, pyknotic cells were found under a low magnification (× 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; and 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 as described previously (Lin et al., 2009).

For quantification of the density of ED1 or CD43-positive cells, sections at the bregma and the middle dorsal hippocampus were used. Two consecutive sections were used at each level. Positively stained cells were counted in at least three randomly chosen high-power views (100×, 0.0768 mm^2) at the following ipsilateral brain regions: the cingulum, the external capsule, the internal capsule, the cortex and the striatum. The cell counting results were converted to cells per mm^2 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.

4.10. Statistics

Data were expressed as the mean±SEM. All data were first tested for distribution. Normally distributed data were analyzed by t-test, one-way or two-way ANOVA (for data of NF-kB activation and western blot), followed by multiple comparisons using Turkey's post hoc test. Non-normally distributed data were compared with Rank-Sum test or Kruskal-Wallis One Way analysis of Variance on Ranks. The significance level was set at $P<0.05$.

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References

Agnew DM, Koehler RC, Guerguerian AM, Shaffner DH, Traystman RJ, Martin LJ, Ichord RN. Hypothermia for 24 hours after asphyxic cardiac arrest in piglets provides striatal neuroprotection that is sustained 10 days after rewarming. Pediatr Res. 2003; 4:253–62. [PubMed: 12736390]

Arai T, Kaneko H, Ohnishi H, Matsui E, Fukao T, Kawamoto N, Kasahara K, Kondo N. Hypothermia augments NF-kappaB activity and the production of IL-12 and IFN-gamma. Allergol Int. 2008; 57:331–8. [PubMed: 18690006]

Azzopardi DV, Strohm B, Edwards AD, Dyet L, Halliday HL, Juszczak E, Kapellou O, Levene M, Marlow N, Porter E, Thoresen M, Whitelaw A, Brocklehurst P. TOBY Study Group. Moderate hypothermia to treat perinatal asphyxial encephalopathy. N Engl J Med. 2009; 361:1349–58. [PubMed: 19797281]

Baker AJ, Zornow MH, Grafe MR, Scheller MS, Skilling SR, Smullin DH, Larson AA. Hypothermia prevents ischemia-induced increases in hippocampal glycine concentrations in rabbits. Stroke. 1991; 22:666–73. [PubMed: 2028499]

Baskin DG, Wilcox BJ, Figlewicz DP, Dorsa DM. Insulin and insulin-like growth factors in the CNS. Trends Neurosci. 1988; 11:107–11. [PubMed: 2465607]

Brooks KJ, Hargreaves I, Bhakoo K, Sellwood M, O'Brien F, Noone M, Sakata Y, Cady E, Wylezinska M, Thornton J, Ordidge R, Nguyen Q, Clemence M, Wyatt J, Bates TE. Delayed hypothermia prevents decreases in N-acetylaspartate and reduced glutathione in the cerebral cortex of the neonatal pig following transient hypoxia-ischaemia. Neurochem Res. 2002; 27:1599–604. [PubMed: 12515311]

Brywe KG, Mallard C, Gustavsson M, Hedtjärn M, Leverin AL, Wang X, Blomgren K, Isgaard J, Hagberg H. IGF-I neuroprotection in the immature brain after hypoxia-ischemia, involvement of Akt and GSK3beta? Eur J Neurosci. 2005; 21:1489–502. [PubMed: 15845077]

Fairchild KD, Singh IS, Patel S, Drysdale BE, Viscardi RM, Hester L, Lazusky HM, Hasday JD. Hypothermia prolongs activation of NF-kappaB and augments generation of inflammatory cytokines. Am J Physiol Cell Physiol. 2004; 287:C422–31. [PubMed: 15070815]

Fan LW, Tien LT, Zheng B, Pang Y, Rhodes PG, Cai Z. Interleukin-1beta-induced brain injury and neurobehavioral dysfunctions in juvenile rats can be attenuated by alpha-phenyl-n-tert-butyl-nitrone. Neuroscience. 2010; 168:240–52. [PubMed: 20346393]

Garden GA, Möller T. Microglia biology in health and disease. J Neuroimmune Pharmacol. 2006; 1:127–37. [PubMed: 18040779]

Gidday JM, Park TS, Gonzales ER, Beetsch JW. CD18-dependent leukocyte adherence and vascular injury in pig cerebral circulation after ischemia. Am J Physiol. 1997; 272:H2622–9. [PubMed: 9227539]

Guan J. Insulin-like growth factor-1 and its derivatives: potential pharmaceutical application for ischemic brain injury. Recent Pat CNS Drug Discov. 2008; 3:112–27. [PubMed: 18537771]

Guan J, Gunn AJ, Sirimanne ES, Tuffin J, Gunning MI, Clark R, Gluckman PD. The window of opportunity for neuronal rescue with insulin-like growth factor-1 after hypoxia-ischemia in rats is critically modulated by cerebral temperature during recovery. J Cereb Blood Flow Metab. 2000; 20:513–9. [PubMed: 10724116]

Gunn AJ, Gunn TR, de Haan HH, Williams CE, Gluckman PD. Dramatic neuronal rescue with prolonged selective head cooling after ischemia in fetal lambs. J Clin Invest. 1997; 99:248–56. [PubMed: 9005993]

Han HS, Karabiyikoglu M, Kelly S, Sobel RA, Yenari MA. Mild hypothermia inhibits nuclear factor-kappaB translocation in experimental stroke. J Cereb Blood Flow Metab. 2003; 23:589–98. [PubMed: 12771574]

Jatana M, Singh I, Singh AK, Jenkins D. Combination of systemic hypothermia and N-acetylcysteine attenuates hypoxic-ischemic brain injury in neonatal rats. Pediatr Res. 2006; 59:684–9. [PubMed: 16627882]

Lasarzik I, Winkelheide U, Thal SC, Benz N, Lörscher M, Jahn-Eimermacher A, Werner C, Engelhard K. Mild hypothermia has no long-term impact on postischemic neurogenesis in rats. Anesth Analg. 2009; 109:1632–9. [PubMed: 19843801]

Lin S, Cox HJ, Rhodes PG, Cai Z. Neuroprotection of alpha-phenyl-n-tert-butyl-nitrone on the neonatal white matter is associated with anti-inflammation. Neurosci Lett. 2006; 405:52–6. [PubMed: 16876321]

Lin S, Fan LW, Pang Y, Rhodes PG, Mitchell HJ, Cai Z. IGF-1 protects oligodendrocyte progenitor cells and improves neurological functions following cerebral hypoxia-ischemia in the neonatal rat. Brain Res. 2005; 1063:15–26. [PubMed: 16259966]

Lin S, Fan LW, Rhodes PG, Cai Z. Intranasal administration of IGF-1 attenuates hypoxic-ischemic brain injury in neonatal rats. Exp Neurol. 2009; 217:361–70. [PubMed: 19332057]

Lindsberg PJ, Grau AJ. Inflammation and infections as risk factors for ischemic stroke. Stroke. 2003; 34:2518–32. [PubMed: 14500942]

Nijboer CH, Heijnen CJ, Groenendaal F, May MJ, van Bel F, Kavelaars A. A dual role of the NF-kappaB pathway in neonatal hypoxic-ischemic brain damage. Stroke. 2008; 39:2578–86. [PubMed: 18420947]

Ohmura A, Nakajima W, Ishida A, Yasuoka N, Kawamura M, Miura S, Takada G. Prolonged hypothermia protects neonatal rat brain against hypoxic-ischemia by reducing both apoptosis and necrosis. Brain Dev. 2005; 27:517–26. [PubMed: 15899566]

O'Neill LA, Kaltschmidt C. NF-kappa B: a crucial transcription factor for glial and neuronal cell function. Trends Neurosci. 1997; 20:252–8. [PubMed: 9185306]

Pang Y, Zheng B, Fan LW, Rhodes PG, Cai Z. IGF-1 protects oligodendrocyte progenitors against TNFalpha-induced damage by activation of PI3K/Akt and interruption of the mitochondrial apoptotic pathway. Glia. 2007; 55:1099–107. [PubMed: 17577243]

Prandini MN, Neves Filho A, Lapa AJ, Stavale JN. Mild hypothermia reduces polymorphonuclear leukocytes infiltration in induced brain inflammation. Arq Neuropsiquiatr. 2005; 63:779–84. [PubMed: 16258656]

Rice JE 3rd, Vannucci RC, Brierley JB. The influence of immaturity on hypoxic - ischemic brain damage in the rat. Ann Neurol. 1981; 9:131–41. [PubMed: 7235629]

Ritter LS, Orozco JA, Coull BM, McDonagh PF, Rosenblum WI. Leukocyte accumulation and hemodynamic changes in the cerebral microcirculation during early reperfusion after stroke. Stroke. 2000; 31:1153–61. [PubMed: 10797180]

Rothwarf DM, Karin M. The NF-kappa B activation pathway: a paradigm in information transfer from membrane to nucleus. Sci STKE. 1999; 5:RE1. [PubMed: 11865184]

Sahuquillo J, Vilalta A. Cooling the injured brain: how does moderate hypothermia influence the pathophysiology of traumatic brain injury. Curr Pharm Des. 2007; 13:2310–22. [PubMed: 17692002]

Schäbitz WR, Hoffmann TT, Heiland S, Kollmar R, Bardutzky J, Sommer C, Schwab S. Delayed neuroprotective effect of insulin-like growth factor-i after experimental transient focal cerebral ischemia monitored with mri. Stroke. 2001; 32:1226–33. [PubMed: 11340238]

Schneider A, Martin-Villalba A, Weih F, Vogel J, Wirth T, Schwaninger M. NF-kappaB is activated and promotes cell death in focal cerebral ischemia. Nat Med. 1999; 5:554–9. [PubMed: 10229233]

Shankaran S, Laptook AR, Ehrenkranz RA, Tyson JE, McDonald SA, Donovan EF, Fanaroff AA, Poole WK, Wright LL, Higgins RD, Finer NN, Carlo WA, Duara S, Oh W, cotton CM, Stevenson DK, Stoll BJ, Lemons JA, Guillet R, Jobe AH. National Institute of Child Health and Human Development Neonatal Research Network. Whole-body hypothermia for neonates with hypoxic-ischemic encephalopathy. N Engl J Med. 2005; 353:1574–84. [PubMed: 16221780]

Tang Y, Xu H, Du X, Lit L, Walker W, Lu A, Ran R, Gregg JP, Reilly M, Pancioli A, Khoury JC, Sauerbeck LR, Carrozzella JA, Spilker J, Clark J, Wagner KR, Jauch EC, Chang DJ, Verro P, Broderick JP, Sharp FR. Gene expression in blood changes rapidly in neutrophils and monocytes after ischemic stroke in humans: a microarray study. J Cereb Blood Flow Metab. 2006; 26:1089–102. [PubMed: 16395289]

Tomimatsu T, Fukuda H, Endo M, Watanabe N, Mu J, Kohzuki M, Fujii E, Kanzaki T, Murata Y. Effects of hypothermia on neonatal hypoxic-ischemic brain injury in the rat: phosphorylation of Akt, activation of caspase-3-like protease. Neurosci Lett. 2001; 312:21–4. [PubMed: 11578836]

Tooley JR, Satas S, Porter H, Silver IA, Thoresen M. Head cooling with mild systemic hypothermia in anesthetized piglets is neuroprotective. Ann Neurol. 2003; 53:65–72. [PubMed: 12509849]

van den Tweel ER, Kavelaars A, Lombardi MS, Groenendaal F, May M, Heijnen CJ, van Bel F. Selective inhibition of nuclear factor-kappaB activation after hypoxia/ischemia in neonatal rats is not neuroprotective. Pediatr Res. 2006; 59:232–6. [PubMed: 16439584]

Webster CM, Kelly S, Koike MA, Chock VY, Giffard RG, Yenari MA. Inflammation and NFkappaB activation is decreased by hypothermia following global cerebral ischemia. Neurobiol Dis. 2009; 33:301–12. [PubMed: 19063968]

Williams GD, Dardzinski BJ, Buckalew AR, Smith MB. Modest hypothermia preserves cerebral energy metabolism during hypoxia-ischemia and correlates with brain damage: a 31P nuclear magnetic resonance study in unanesthetized neonatal rats. Pediatr Res. 1997; 42:700–8. [PubMed: 9357946]

Wiltrout C, Lang B, Yan Y, Dempsey RJ, Vemuganti R. Repairing brain after stroke: a review on post-ischemic neurogenesis. Neurochem Int. 2007; 50:1028–41. [PubMed: 17531349]

Zhao H, Shimohata T, Wang JQ, Sun G, Schaal DW, Sapolsky RM, Steinberg GK. Akt contributes to neuroprotection by hypothermia against cerebral ischemia in rats. J Neurosci. 2005; 25:9794–806. [PubMed: 16237183]

Zhu C, Wang X, Xu F, Qiu L, Cheng X, Simbruner G, Blomgren K. Intraischemic mild hypothermia prevents neuronal cell death and tissue loss after neonatal cerebral hypoxia-ischemia. Eur J Neurosci. 2006; 23:387–93. [PubMed: 16420446]

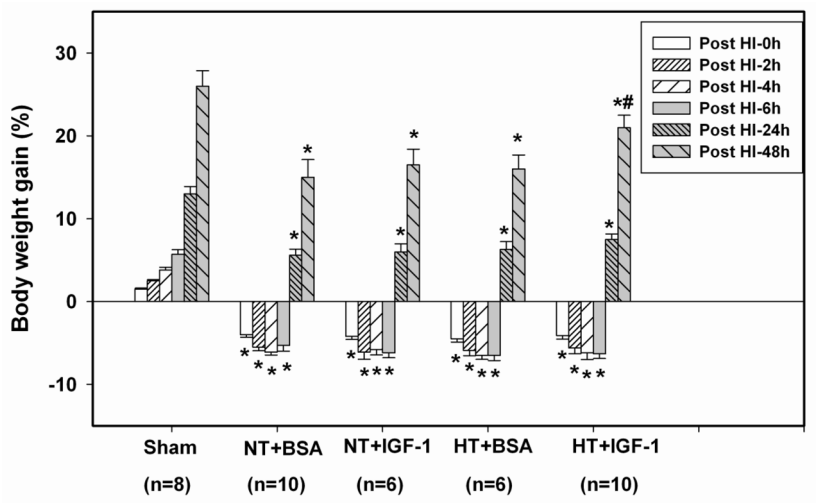


Figure 1.

Body weight changes following cerebral HI. Rat pups were treated with hypothermia (HT) or normothermia (NT) for 4 h, and IGF-1 or vehicle (BSA) was administered at 4 h after the HI. Y axis represents the percentage changes in body weight as compared to the mean weight of the P7 rat before cerebral HI. The number of rat pups in each group is presented in the parenthesis. The rat pups in the sham group gained 6% of body weight in 6 h. All rat pups that were subjected to cerebral HI, however, had a decrease in body weight within 6 h after HI. Rat pups began to gain weight at 24 h after HI. The weight gain in the HI rat pups was significantly lower than that in the sham group at 24 and 48 h following HI ($P<0.05$). Rat pups in the hypothermic and normothermic groups did not show significant difference in body weight gain at 0–24 h after HI ($P>0.05$). However, the rat pups in the HT+IGF-1 group gained more weight at 48 h after HI as compared to the other 3 HI groups ($P<0.05$). *$P<0.05$ vs the sham group at the same time point after the HI insult. #$P<0.05$ vs other three HI groups at the same time point.

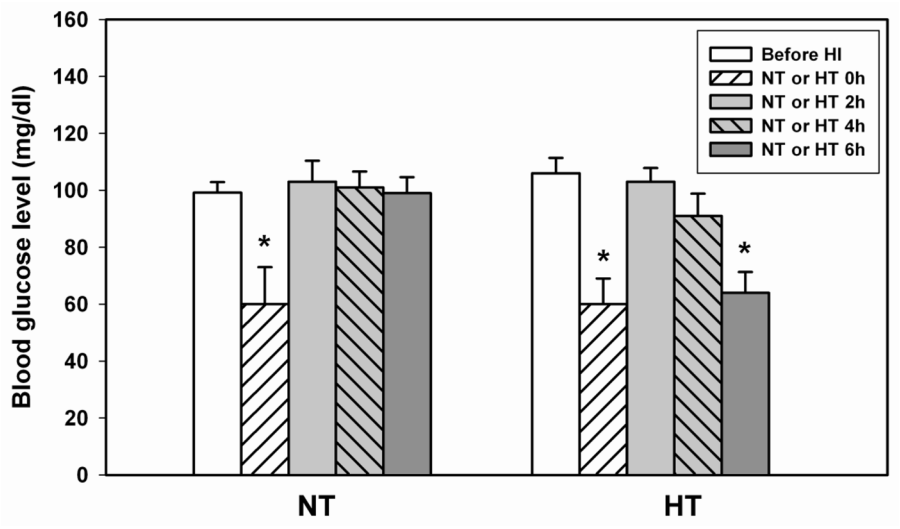


Figure 2.

Blood glucose levels following cerebral HI under the normothermic or the hypothermic condition for various durations. Each group was consisted of 6 animals. The blood glucose level was decreased significantly immediately after hypoxia treatment as compared to that in the sham group ($P<0.05$). During the recovery stage, blood glucose was maintained at a normal level at 2 or 4 h of hypothermia treatment. However, if the duration of hypothermia was extended to 6h, blood glucose level dropped significantly ($P<0.05$). *$P<0.05$ vs the level before HI.

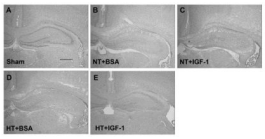
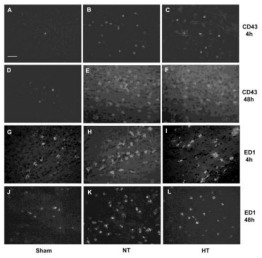


Figure 3.

Representative images of H&E stained rat brain sections at the hippocampal area 2 d after cerebral HI. The rat pups were subjected to a hypothermic exposure for 4 h, followed by an immediate IGF treatment (HT4h+IGF0h). No pathological changes were found in the sham group (A). Cerebral HI caused severe tissue damage in the ipsilateral cortex, striatum, hippocampus and white matter areas of the normothermia and vehicle-treated group (B). IGF-1 treatment (C) or hypothermic treatment (D) alone did not attenuate brain injury, while the combination of hypothermia and IGF-1 treatment (E) reduced the HI-induced brain damage. Scale bar: 500 μm.



Figur 4.

Immunostaining of CD43 and ED1 in the rat brain at 4 and 48 h after cerebral HI. Immediately after HI, rats were exposed to either a hypothermia (HT) or a normothermia (NT) condition for 4 h. For the 48 h data, the rat pups in the HT group received iN-IGF-1 treatment immediately after the termination of hypothermia. Brain samples were collected after the termination of hypothermia (4 h after HI) or 48 h after HI. Representative images of CD43 immunostaining were taken at the ipsilateral cortical area (A–F), while images of ED1 immunostaining were taken at the ipsilateral cingulum area (G–L). Only a few CD43+ cells (A, D) or ED1+ cells (G, J) were observed in the sham brain at 4 and 48 h after HI. Cerebral HI caused the infiltration of PMN cells (B) and activation of macrophages/microglia (H) at 4 h after cerebral HI. Hypothermic treatment immediately after HI reduced PMN infiltration (C) and macrophage/migroglia activation (I) in the ipsilateral brain. The recruitment of PMN cells (E) and macrophage/microglia (K) was even more evident at 48 h after HI. Hypothermia combined with IGF-1 inhibited the recruitment of PMN cells (F) and macrophage/microglia (L). Scale bar: 50 μm.

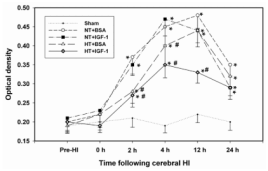


Figure 5.

NF-κB levels in the ipsilateral rat brain following cerebral HI. Each experimental group had 5 animals. After HI, rats were exposed to a hypothermia (HT) or a normothermia (NT) condition for 4 h and then immediately treated with either IGF-1 or vehicle. Cerebral HI caused activation of NF-κB at 2 h after HI and the activation lasted for 24 h. Hypothermic treatment decreased the NF-κB level at 2–4 h following cerebral HI as compared to the normothermic groups. However at 12 h following HI, the NF-κB level in the HT+BSA group was increased, while the level in the HT+IGF-1 group still remained at a lower level as compared to other 3 HI groups ($P<0.05$). NF-κB level at 24 h was still higher in all HI groups as compared to the sham group. But no significant difference was detected among the four HI groups. Results are expressed as the Mean±SD of 5 animals for each group. *$P<0.05$ vs to the sham group at the same time point. # $P<0.05$ vs the NT+BSA group at the same time point.

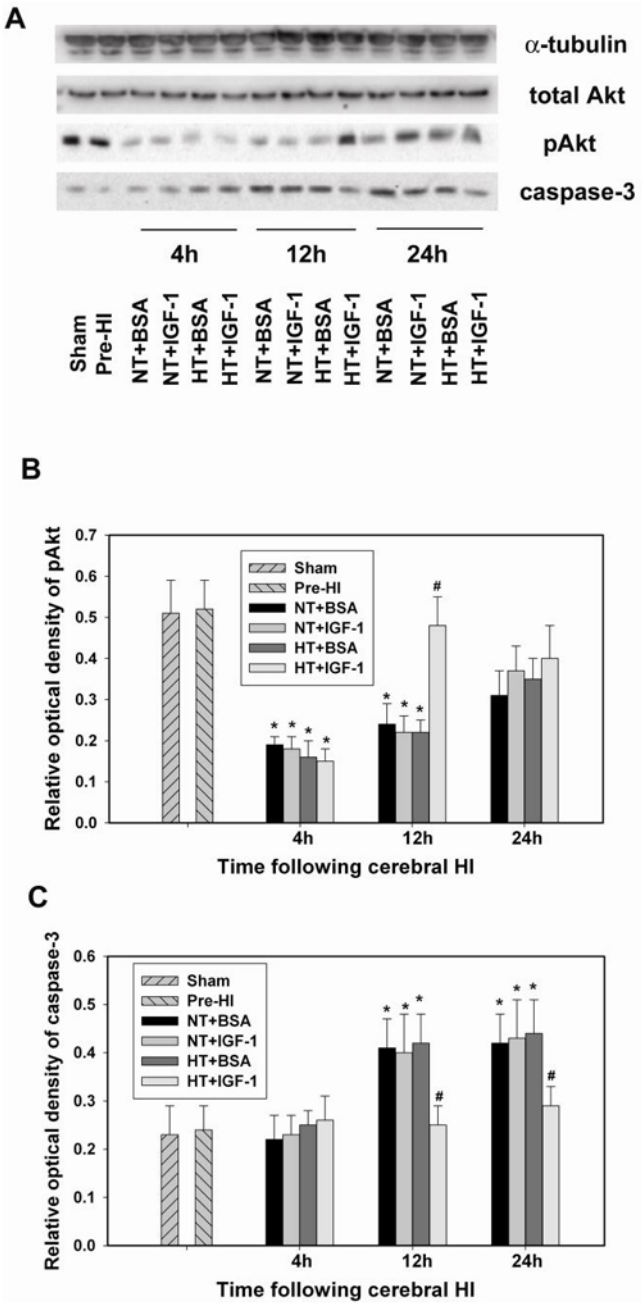


Figure 6.

Effects of HT and IGF-1 treatment on phosphorylation of Akt and activation of caspase-3 following cerebral HI. Each experimental group had 5 animals. After HI, rats were exposed to a hypothermia (HT) or a normothermia (NT) condition for 4 h and then immediately treated with either IGF-1 or vehicle. Representative western blotting results of activated caspase-3, Akt and pAkt are shown in A. Quantification of the OD data showed that the total Akt expression did not change within 24 h following cerebral HI. pAKt level decreased significantly at 12 h after HI, while the combination of hypothermia and IGF-1 treatment reversed the reduction of pAkt (B). Activated caspase-3 level was increased at 12–24 h after cerebral HI and the combination of hypothermia and IGF-1 significantly reduced activation of caspase-3 (C). Each bar represents the Mean±SD of 5 animals. * P<0.05 vs the sham

group. # P<0.05 vs the HI rat pups subjected to the NT+ BSA treatment at the same time point.

Table 1

Neuropathological scores at 2d after cerebral HI under various experimental regimens

	NT+BSA	HT+BSA	NT+IGF-1	HT+IGF-1
A. Regimen HT2h+IGF0h	(n=9)	(n=9)	(n=9)	(n=9)
Cortex	2.9±0.5	2.5±0.4	2.3±0.5	1.7±0.5*
Striatum	2.7±0.3	2.5±0.3	2.2±0.4	1.6±0.4*
Hippocampus	2.7±0.5	2.2±0.4	2.1±0.5	1.5±0.4*
White matter	2.6±0.5	2.3±0.3	2.2±0.4	1.8±0.4*
Total	10.9±1.5	9.5±1.3	8.8±1.2	6.6±1.4*
B. Regimen HT4h+IGF0h	(n=10)	(n=6)	(n=6)	(n=10)
Cortex	2.9±0.4	2.8±0.4	3.0±0.5	1.6± 0.4*
Striatum	2.9±0.4	2.6±0.4	2.7±0.5	1.5±0.5*
Hippocampus	2.8±0.3	2.8±0.5	2.5±0.5	1.7±0.3*
White matter	2.6±0.3	2.4±0.5	2.7±0.4	1.6±0.4*
Total	11.2±1.4	10.6±1.2	10.9±1.5	6.4±1.4*
C. Regimen HT4h+IGF2h	(n=11)	(n=8)	(n=8)	(n=12)
Cortex	3.0±0.4	2.8±0.5	2.7±0.3	1.8±0.3*
Striatum	2.7±0.3	2.9±0.4	2.8±0.4	1.7±0.4*
Hippocampus	2.8±0.4	2.9±0.4	2.7±0.5	1.9±0.3*
White matter	2.7±0.5	2.5±0.3	2.6±0.3	2.0±0.5*
Total	11.2±1.3	11.1±1.3	10.8±1.2	7.4±1.4*

Following cerebral HI, neonatal rats were subjected to hypothermia (HT) or normothermia (NT) for 2 or 4h and received IGF-1 or vehicle (BSA) treatment at 0 or 2 h after HT or NT as indicated in the table. Brain injury was scored in brain sections 2 d after cerebral HI as described in methods. Regardless of the duration of hypothermia or the time of IGF-1 treatment in this experiment, hypothermia or IGF-1 alone did not change the pathological score at 2 d after cerebral HI. Hypothermia for 2 or 4h combined with iN-IGF-1 treatment at either 0 or 2h after hypothermia reduced the neuropathological scoring.

* $P<0.05$ as compared to the NT+BSA group in the same experimental regimen. Data were expressed as mean±SD of the number of animals indicated in the parenthesis.