Liposome-Encapsulated Hemoglobin Ameliorates Impairment of Fear Memory and Hippocampal Dysfunction After Cerebral Ischemia in Rats

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Abstract. Liposome-encapsulated hemoglobin (LEH) has been developed as a blood substitute. In spite of its size (1/30 – 1/40 of erythrocytes), LEH has an oxygen-carrying capacity comparable to erythrocytes. Thus, LEH is expected to carry oxygen into vital organs via collateral routes during ischemia induced by vascular embolism. In the present study, we examined the therapeutic effects of LEH on behavioral impairments in rats after four-vessel occlusion (4VO) for 30 min. In the open-field test, locomotor activity in 4VO rats did not alter 7 days after ischemia. However, in the contextual fear conditioning (CFC) test, the freezing rate was significantly decreased in 4VO rats, although no behavioral changes in the Y-maze test and elevated plus-maze test were observed. Phosphorylation of the cyclic AMP response element-binding protein (CREB) in the hippocampal CA1 region after the CFC test was attenuated. These 4VO-induced impairments were significantly alleviated by the administration of LEH (5 ml/kg, i.v.) during occlusion. Moreover, LEH did not alter hippocampal blood flow and tissue oxygen pressure during 4VO, but it did suppress hyperoxia after ischemia–reperfusion. These findings suggest that LEH, an artificial oxygen carrier, could be a novel therapeutic agent for brain dysfunction after acute cerebral ischemia.

Keywords: artificial oxygen carrier, liposome-encapsulated hemoglobin, hippocampus, ischemia, contextual fear conditioning

Introduction

Artificial oxygen carriers have been developed as an alternative to blood for transfusions, which have many advantages over blood transfusions such as utility regardless of blood type and a lack of viral contamination (1, 2). Liposome-encapsulated hemoglobin (LEH) is a novel artificial oxygen carrier that has been developed for clinical applications to enable long-term storage and subsequent transfusion. The liposomal surface is modified by polyethylene glycol to improve biocompatibility and to avoid aggregation of particles. The LEH particles (mean diameter: 230 nm) are extremely small compared to erythrocytes, approximately 1/30 the size (3 – 5). LEH is expected to carry oxygen into vital organs via collateral routes during ischemia because its size may allow O₂ delivery beyond the plasma obstruction to areas where erythrocytes seldom reach.

Several studies have demonstrated that LEH has beneficial effects on the sequelae of brain ischemia and hemorrhage. LEH reduced the size of cerebral infarction in rats subjected to photochemically induced thrombosis (PIT) of the middle cerebral artery (MCA) (6, 7) and also in primates treated with MCA occlusion (MCAO) (8). Transfusion of LEH rescued lethal progressive hemodilution and improved hemodilution-induced metabolic acidosis in rats (9). However, the efficacy of LEH administration on cognitive impairments after brain ischemia–reperfusion is still unclear.

At the present time, whether LEH actually supplies oxygen to the infarct area is still controversial. LEH elevates the plasma oxygen content in rats (6). However,
by using positron emission tomography, an LEH analog was observed in the cerebral cortex but not in the ischemic core of the rats with MCAO caused by PIT (10). Thus, both confirmation that LEH delivers oxygen to the infarction area and determination of the mechanisms that support the protective effects of LEH against brain ischemia are required.

Acellular hemoglobin has been reported to act as nitric oxide (NO) scavengers and to have constrictive effects on peripheral vessels (11, 12). The LEH used in the present study has been reported to have no NO scavenging action with lethal progressive hemodilution in model rats (9) and is not believed to have pressor effects. However, the effects of LEH on hemodynamics remain controversial.

The hippocampus is believed to be responsible for the formation of episodic memory (13, 14). It has been also proposed that the hippocampus and associated structures form the neuronal basis underlying depression and anxiety (15 – 17). In the hippocampus, the Cornu ammonis 1 (CA1) area in particular is vulnerable to hypoxia induced by cerebral ischemia (18). Thus, it can be predicted that reduction of hypoxia during hypoperfusion is essential to avoid the sequelae caused by stroke after brain ischemia, such as dementia, depression, and anxiety.

The four-vessel occlusion (4VO) rat model, a model for brain ischemia, was devised by Pulsinelli and Brierley (19). Bilateral vertebral arteries of the rat were permanently occluded by pinching with bipolar coagulator tweezers under pentobarbital anesthesia (40 mg/kg, i.p.). Seven days after surgery, the rats were reperfused by releasing the clip after 30 min. Rats in the control sham-operation group underwent the same treatments except for the occlusion of the bilateral vertebral arteries. During this manipulation, each animal’s rectal temperature was maintained at 37 ± 0.5°C with an electric heating pad.

A transient cerebral ischemia model (4VO) was prepared by bilaterally occluding the vertebral arteries and common carotid arteries according to the method of Pulsinelli and Brierley (19). Bilateral vertebral arteries of rats were permanently occluded by pinching with bipolar coagulator tweezers under pentobarbital anesthesia (40 mg/kg, i.p.). Seven days after surgery, the rats were reperfused by releasing the clip after 30 min. Rats in the control sham-operation group underwent the same treatments except for the occlusion of the bilateral vertebral arteries and common carotid arteries. During this manipulation, each animal’s rectal temperature was maintained at 37 ± 0.5°C with an electric heating pad.

**Behavioral experiments**

Seven days after the ischemia–reperfusion, behavioral experiments were started. The Y-maze test, open field test, elevated plus-maze (EPM) test, and contextual fear conditioning (CFC) test were conducted over 5 days, in that order. One behavioral test was conducted per day.

**Y-maze test:** Spontaneous alternation behavior in the Y-maze was assessed (26). Tests were started 7 days after occlusion of the common carotid arteries. The Y-maze used in the present study consisted of three arms. Entry into the arm was defined as when the hind paws of the...
rats were completely within the arm. Spontaneous alternation was counted when rats entered all three arms in overlapping triplet sets. The percentage of alternation was calculated as (successive triplet sets / total number of arm entries − 2) × 100. Tests were performed for 8 min.

Open field test: The open field apparatus was a square chamber (90-cm length × 90-cm width × 40-cm height). To evaluate the locomotor activity, the traveled distance was recorded and automatically analyzed by the LimeLight2 software package (Actimetrics, Inc., Wilmette, IL, USA), and the total number of crossings, the times animals cross the lines dividing 9 × 9 squares in the open field, in 30 min was also recorded. Tests were started 8 days after occlusion of the common carotid arteries.

EPM test: Anxiety-like behavior was evaluated with the EPM test (27, 28) and was assessed as described in our previous reports (29 – 31). The plus-maze, consisting of two open arms (50-cm length × 10-cm width) and two enclosed arms (50-cm length × 10-cm width × 40-cm height), was elevated 50 cm above the floor. The arms extended from a central platform (10 × 10 cm). The illumination of the room was kept at 20 lx during the tests. The behavior of each rat over the course of 10 min was automatically analyzed by the software package LimeLight2. The time spent in the open and enclosed arms and the number of open and enclosed arm entries were recorded. Tests were started 9 days after occlusion of the common carotid arteries.

CFC test: The CFC test was used to evaluate fear-related learning and memory (32) and was assessed as described in our previous reports (29, 30). To quantify an aspect of the fear response in the chamber, electrical foot shocks were administered five times (2 s, 0.3 mA, 30-s intervals) 24 h before the tests. The tests were conducted 11 days after occlusion of the common carotid arteries. Rats were re-exposed to the chamber without foot shocks for 5 min, and freezing behavior was defined as a lack of movement, except for respiration, accompanied by an arched back and retraction of the ears (33). The freezing behavior was analyzed automatically by the FreezeFrame software package (Actimetrics, Inc.).

Histochemical experiments

Rats were anesthetized before 15 min of perfusion by intraperitoneal injection of pentobarbital (40 mg/kg) and then perfused with saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer 1 h after finishing the CFC test. The brains were sectioned at a 30-μm thickness. Based on the atlas of Paxinos and Watson (34), the section located 3.14-mm posterior from the bregma was selected. Histochemical experiments were performed on free-floating coronal sections.

Phosphorylated cyclic AMP response element-binding protein (pCREB) immunostaining: The brain sections were treated with 30% H2O2, methanol and H2O (1:5:4) for 30 min. After a 1-h incubation in 0.01 M phosphate–buffered saline and normal goat serum, the sections were incubated overnight in 0.01 M phosphate–buffered saline containing 0.1% Triton X-100 and rabbit anti-pCREB antibody (Upstate Biotechnology, Waltham, MA, USA; 1:1000 dilution). The sections were then incubated for 1 h in 0.01 M phosphate–buffered saline containing 0.1% Triton X-100 and biotinylated goat anti-rabbit IgG (Vector Labs, Burlingame, CA, USA) followed by a 20-min incubation in 0.01 M phosphate–buffered saline and avidin–biotinylated horseradish peroxidase complex (Vectastain Elite ABC Kit, Vector Labs). The reaction product was visualized by transferring the sections to a 50 mM Tris-HCl buffer (pH 7.6) containing 0.05% diaminobenzidine, 0.6% nickel ammonium sulfate, and 0.01% H2O2. Immunoreactivity to pCREB was evaluated with the MCID densitometric video image analysis system (Imaging Research, Inc., St. Catharines, ON, Canada), according to the method of Bilang-Bleuel et al. (35) and Izumi et al. (36).

The unit areas of the hippocampal CA1 (700 × 200 μm), CA3 (200 × 500 μm), dentate gyrus (500 × 500 μm), somatosensory cortex II – IV (1000 × 1000 μm), and basolateral nucleus of the amygdala (400 × 400 μm) were digitally recorded by a CCD camera (CCD-IRIS; Sony, Tokyo) connected to a photomicroscope (BX50; Olympus, Tokyo). The gray area of pCREB positive cells was assessed by automated selection of those cells within the unit areas that satisfied the following criteria: 1) the gray value of the cell nucleus is higher than the threshold value (threshold gray value = 50% higher than the background gray value) and 2) nucleus diameter is bigger than 4 μm (to exclude cell debris and artifacts). The measurements were repeated 4 times on the right and left side of 2 brain sections and averaged.

Nissl staining: Additional brain sections were stained with toluidine blue for verification and comparison with previously established patterns of post-ischemic neuronal cell loss (24).

Measurements of hippocampal blood flow (HBF), hippocampal oxygen pressure, and peripheral hemodynamic parameters

The vertebral arteries of rats were permanently occluded by pinching with bipolar coagulator tweezers under pentobarbital anesthesia (40 mg/kg, i.p.) 7 days before the experiment. The rats were anesthetized with halothane (5% induction, 2% surgery, and 1% maintenance) in 30% O2 and 70% N2. The trachea was cannulated for mechanical ventilation (3 ml/stroke, 60 strokes/
The bilateral common carotid arteries were gently separated from the carotid sheath and vagal nerves. A 4-0 nylon thread was hitched to each artery. A catheter was inserted in the left femoral artery and connected to a pressure transducer for continuous recording of arterial blood pressure (BP) and heart rate (HR) (Nihon Kohden, Tokyo). The head was immobilized with a stereotaxic frame, and the cranial bone and the dura were carefully removed over the hippocampus.

A Laser-Doppler probe with a diameter of 0.5 mm was inserted into one side of the hippocampus (coordinate: 3.5-mm posterior to the bregma, 2.0-mm lateral to the bregma, and 2.5-mm ventral from the cortical surface) to record HBF at a 2.0 – 3.0-mm depth from the cortical surface. HBF was continuously monitored using Laser-Doppler Flowmetry (Omegaflow FLO-C1; Omegawave, Tokyo). The signals were sampled once a second. The tissue oxygen pressure (pO2) and temperature on another side of the hippocampus were determined using a combined oxygen/temperature probe (coordinate: 3.5-mm posterior to the bregma, 2.0-mm lateral to the bregma, and 3.0-mm ventral from the cortical surface) and Tissue Oxygenation / temperature monitor (OxyLab pO2; Oxford Optronix Limited, Oxford, UK) over 10 s at intervals of 50 s.

After the baselines of all parameters were stabilized, the bilateral common carotid arteries were occluded for 30 min by pulling on the hitched nylon threads. These measurements were continued until 60 min after reperfusion. The parameters were recorded with the PowerLab Data Acquisition System/Chart v.5.5.6 (ADInstruments Pty., Ltd., Castle Hill, NSW, Australia) and averaged every 5 min. During this experiment, each animal’s rectal temperature was maintained at 37 ± 0.5°C with an electric heating pad.

**Measurements of systemic arterial pressure and HR in intact rats**

Intact rats were anesthetized with halothane (5% induction, 2% surgery, and 1% maintenance) in 30% O2 and 70% N2. The trachea was cannulated for mechanical ventilation. A catheter was inserted in the left femoral artery and connected to a pressure transducer for continuous recording of arterial BP and HR. The head was immobilized with a stereotaxic frame. After baselines of all parameters were stabilized, LEH or saline (5 ml/kg) was injected via a tail vein. The two parameters were recorded 90 min after LEH or saline administration.

**Statistics**

Values in the texts, tables, and figures are each the mean ± S.E.M. and n indicates the number of rats. Multiple group comparisons in the behavioral and histochemical experiments were performed using one-way analysis of variance (ANOVA) and the LSD post hoc test. Differences were considered significant at P < 0.05. Values in hemodynamic parameters are expressed as a percentage of the baseline before bilateral common carotid artery occlusion or LEH administration. Area-under-the-curve (AUC) (% min × 10^3) was calculated. Statistical analysis of AUC data was performed using a two-tailed Student’s paired t-test. Differences were considered significant at P < 0.05.

**Results**

**Decreased freezing time in the CFC test on 4VO rats is improved by LEH**

In the open-field test, the locomotor activity in 4VO rats did not change compared to that of the sham-operated control (Fig. 1b). However, in the CFC test, the freezing rate was significantly decreased to 40.3 ± 5.8% in 4VO rats compared to 64.6 ± 3.4% in the control group (Fig. 1d), although no behavioral changes in the Y-maze test (Fig. 1a) or EPM test (Fig. 1c) were observed. The decreased freezing rate induced by 4VO was significantly alleviated by administration of LEH (5 ml/kg, i.v.) during occlusion to 57.2 ± 4.1% (Fig. 1d).

**Cell densities are not altered by 4VO**

In the Nissl staining, a significant reduction of cell densities was not observed in any regions of hippocampus of the 4VO group compared to the sham-operation group at 11 days after ischemia–reperfusion. Moreover, cell densities were not altered by administration of LEH (Fig. 2).

**LEH improves the decreased phosphorylation of CREB after the CFC test by 4VO in the hippocampal CA1 region**

As shown in Fig. 2, post-ischemic neuronal cell loss in the hippocampal area, a vulnerable region of ischemic injury in the brain, was not observed using 4VO rats or LEH administered to 4VO rats. However, phosphorylation of CREB 1 h after the CFC test was significantly reduced in the hippocampal CA1 region by 4VO treatment (2,465 ± 630 μm²) compared to the sham-operated control (12,024 ± 2,135 μm²). Moreover, administration of LEH improved this reduced pCREB in the hippocampal CA1 region of 4VO rats (7,901 ± 909 μm²) (Figs. 3, 4).

**The hyperoxia occurring after ischemia–reperfusion in the hippocampus is eliminated by administration of LEH**

Bilateral common carotid artery occlusion after per-
permanent occlusion of bilateral vertebral arteries decreased pO₂ to approximately 30% of baseline levels during the occlusion in both the saline- and LEH-administered groups. However, transient hyperoxia was observed just after the reperfusion and then pO₂ increased continuously in the saline-treated group. These responses disappeared, and pO₂ levels gradually recovered in the LEH-treated group (Fig. 5a).

HBF was reduced to approximately 60% of baseline levels during the occlusion in both saline- and LEH-administered groups. After the reperfusion, there was no difference in these two groups. In the LEH-treated group, however, HBF tended to decrease 30–60 min after reperfusion, but it was not statistically significant (P = 0.095) (Fig. 5b).

During bilateral common carotid artery occlusion, systemic arterial BP and HR were not changed by LEH administration. However, after the reperfusion, systemic arterial BP was increased to approximately 120% of baseline levels by LEH administration (P = 0.046) (Fig. 5c). Likewise, HR was slightly increased 30–60 min after reperfusion, but it was not statistically significant (P = 0.106) (Fig. 5d).

**LEH has no apparent effect on systemic arterial pressure and HR in intact rats**

Systemic arterial BP was not affected by administration of LEH in intact rats (Fig. 6a). However, HR was slightly increased by LEH administration, but it was not statistically significant (P = 0.108) (Fig. 6b).

**Discussion**

In the present study, we determined the following as-
Fig. 2. Effects of liposome-encapsulated hemoglobin (LEH) on cellular structure in Nissl staining 11 days after ischemia–reperfusion. Rats were perfused 1 h after finishing the contextual fear conditioning test. The brains were sectioned at a 30-μm thickness, which were stained with toluidine blue. In the Nissl staining, a significant reduction of cell densities was not observed in any regions of the hippocampus of the 4VO group compared to the sham-operation group at 11 days after ischemia–reperfusion. a), b) Sham: sham-operated rats without 4VO; c), d) Saline: 4VO rats administered saline; e), f) LEH: 4VO rats administered LEH. a), c), e) Low-power field of the hippocampus; b), d), f) high-power field of the hippocampal CA1 region.

Fig. 3. Effects of liposome-encapsulated hemoglobin (LEH) on cyclic AMP response element-binding protein (CREB) phosphorylation in immunohistochemical staining. Rats were perfused 1 h after finishing the contextual fear conditioning (CFC) test. The brains were sectioned at a 30-μm thickness, which were immunostained for phosphorylated CREB. Phosphorylation of CREB 1 h after the CFC test was significantly reduced in the hippocampal CA1 region by 4VO treatment compared to the sham-operated control. Moreover, administration of LEH improved this reduced pCREB in the hippocampal CA1 region of 4VO rats. a), b) Sham: sham-operated rats without 4VO; c), d) Saline: 4VO rats administered saline; e), f) LEH: 4VO rats administered LEH. a), c), e) Low-power field of hippocampus; b), d), f) high-power field of hippocampal CA1 region.
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pects of LEH effects on cerebral ischemia: 1) the beneficial effects of LEH on impaired cognitive performance and CREB phosphorylation in the hippocampal CA1 region induced by transient ischemia and 2) the suppressant effects of LEH on hyperoxidation after reperfusion. The latter implies that novel mechanisms underlie the cerebroprotective actions of LEH.

LEH ameliorated the reduction of freezing behavior in the CFC test caused by 4VO (Fig. 1d). The CFC test is a universal behavioral paradigm for studying the neuronal basis of learning and memory. Freezing behavior as a measure of fear expression has been used as an index of learning and memory (37, 38). The decreased freezing rate induced by 4VO in the CFC test may reflect impairment of long-term memory.

We observed no alteration of locomotor activity caused by 4VO in the open field test (Fig. 1b). Previous studies have shown that at the first day after operation, the ischemia-induced increase in locomotor activity is most prominent. It decreases over the following two days, and at 5 – 7 days after operation there is no difference between the controls and ischemic animals (39, 40). In this study, as we conducted the open field test 8 days after occlusion, the present result regarding locomotor activity conforms to these previous reports. Thus, it is unlikely that locomotor activity was altered by 4VO in the other behavioral tests.

In the present Y-maze test, spontaneous alternation behavior was unchanged by 4VO (Fig. 1a). Spontaneous alternation behavior reflects the operation of short-term memory (41, 42). Our results from the Y-maze test suggest that 4VO does not impair short-term memory.

In clinical studies, it has been reported that acute stroke provokes not only cognitive deficits but also anxiety disorder in approximately 20% – 30% of stroke survivors as neuropsychiatric complications (43, 44). Our results

Fig. 4. Quantitative analyses of phosphorylated cyclic AMP response element-binding protein (pCREB) in immunohistochemical staining; effects of liposome-encapsulated hemoglobin (LEH). Immunoreactivity to pCREB was evaluated with the densitometric video image analysis system. The unit areas of the hippocampal CA1, CA3, dentate gyrus, somatosensory cortex, and basolateral nucleus of the amygdala were digitally recorded by a CCD camera connected to a photomicroscope. The gray area of pCREB-positive cells was assessed by automated selection of those cells within the unit areas. The measurements were repeated 4 times on the right and left side of 2 brain sections and averaged. a) Measurement fields of phosphorylated CREB in the brain map, b) hippocampal CA1, c) hippocampal dentate gyrus, d) hippocampal CA3, e) somatosensory cortex, f) basal amygdala. Each column shows data on the Sham (sham-operated rats without 4VO: white), Saline (4VO rats administered saline: black), and LEH (4VO rats administered LEH: stripe) groups. Data represent the mean ± S.E.M. The number in parenthesis indicates the number of animals in each group. *P < 0.05 vs. the sham-operated rats, †P < 0.05 vs. the saline-treated rats.
of the EPM test, however, demonstrated that 4VO did not change the levels of anxiety-like behavior in rats (Fig. 1c). Bantsiele (2009) also reported no change in the EPM test after ischemia–reperfusion with 4VO (23). Our preliminary data showed that milder ischemia than that caused by 30 min of 4VO evoked anxiety-like behavior in the EPM test (T. Yamaguchi and N. Hamadate, unpublished). Thus, whether anxiety disorder occurs might

**Fig. 5.** Effects of liposome-encapsulated hemoglobin (LEH) on hippocampal pO2, hippocampal blood flow, systemic blood pressure, and heart rate in 4VO rats. In the rats subjected to permanent vertebral artery occlusion 7 days before the experiment under halothane anesthesia and mechanical ventilation, arterial blood pressure and heart rate were continuously recorded via the left femoral artery. Hippocampal blood flow was continuously monitored using Laser-Doppler Flowmetry, and hippocampal oxygen pressure (pO2) in another side of the hippocampus was determined using a Tissue Oxygenation monitor. a) Hippocampal pO2, b) hippocampal blood flow, c) systemic blood pressure, d) heart rate. Each graph shows data from the Saline (4VO rats administered saline: filled circle) and LEH (4VO rats administered LEH: open circle) groups. Data represent the mean ± S.E.M. The number in parenthesis indicates the number of animals in each group. *P < 0.05 vs. the saline-treated rats at the same time. NS: not significant.

**Fig. 6.** Effects of liposome-encapsulated hemoglobin (LEH) on systemic blood pressure and heart rate in intact rats. With intact rats anesthetized with halothane, arterial blood pressure and heart rate were continuously recorded via left femoral artery. After baselines of all parameters were stabilized, LEH or saline was injected via a tail vein. Two parameters were recorded 90 min after LEH or saline administration: a) systemic blood pressure and b) heart rate. Each graph shows data from the Saline (Intact rats administered saline: filled circle) and LEH (Intact rats administered LEH: open circle) groups. Data represent the mean ± S.E.M. The number in parenthesis indicates the number of animals in each group.
depend on the degree of ischemia. Further studies are required to clarify this issue.

The reduction of freezing behavior induced in the CFC test by 4VO (Fig. 1d) implies impairment of long-term memory unrelated to innate fear (Fig. 1c). Moreover, the reduction of freezing behavior induced by 4VO was suppressed by administration of LEH during ischemia (Fig. 1d) without causing a behavioral change in the other tests (Fig. 1: a – c), indicating that impairment of long-term memory after ischemia–reperfusion could be improved by LEH administration without adverse effects. LEH administration offers the possibility of a new therapeutic approach to disorders induced by ischemia–reperfusion such as stroke.

Our observations suggest that the reduction of freezing behavior by 4VO (Fig. 1d) can occur without accompanying neuronal cell death (Fig. 2). Although 4VO treatment has been reported to delay neuronal cell death in the hippocampal CA1 region in a previous study (24), obvious neuronal cell death was not induced by 4VO in the present study (Fig. 2). Discrepancy of the results can be attributed to the differences of experimental conditions. For example, the neuroprotective effects could be attributed to the volume expansion with intravenous saline (5 ml/kg) during ischemia in the present study. In our recent study, we also reported that obvious neuronal cell death did not occur in intravenous saline-injected 4VO rats, using the staining method with neutral red, a marker of irreversible damage after ischemia (45). Furthermore, we also confirmed the same evidence using the 2,3-triphenyltetrazolium chloride (TTC) staining, a marker of intact cellular metabolism (data not shown). These histological findings indicated that microscopic changes, such as neuronal cell death, were not evident in the 4VO rat hippocampus. Furthermore, our results from the Nissl staining (Fig. 2) and Y-maze test (Fig. 1a) agree with other recent studies correlating neuronal cell death in the hippocampal CA1 region in a previous study (24), indicating that impairment of long-term memory after ischemia–reperfusion could be improved by LEH administration without adverse effects. LEH administration offers the possibility of a new therapeutic approach to disorders induced by ischemia–reperfusion such as stroke.

CREB phosphorylation was impaired (Figs. 3, 4) in addition to cognitive performance in the CFC test (Fig. 1d). The transcription factor CREB is activated by phosphorylation at serine 133 by CREB kinases such as protein kinase A and calcium/calmodulin-dependent protein kinase (CaMK) IV (48, 49). This phosphorylation is an essential step in the activation of CREB-mediated transcription (50 – 53), and Stanciu et al. showed a biphasic pattern of CREB activation at 0 – 1 and 3 – 6 h triggered by the CFC test (54). Therefore, the phosphorylation of CREB after CFC test has been used to identify brain regions activated in gene expression–dependent memory processes (55, 56). Moreover, recent studies have indicated that retrieval of fear memory initiates two processes, consolidation and extinction. These two processes appeared to depend on new gene expression following CREB phosphorylation (57), and protein synthesis in the hippocampus is necessary for the memory consolidation process (58). The suppressed CREB phosphorylation caused by 4VO seen after the CFC test implies that the hippocampal circuit function required for memory process is impaired. Reduction of freezing behavior (Fig. 1d) may result from impairment of the memory acquisition and/or consolidation phase, although it is still not certain which phase was impaired. Synaptic plasticity such as LTP also indicates memory formation. LTP in the hippocampal CA1 region is induced by massive glutamate release from excitatory input and subsequent activation of NMDA receptors. After flowing calcium influx, CaMK is activated in the post synapse. Transiently activated CaMK in the nucleus of pyramidal cell phosphorylates CREB. Brain-derived neurotrophic factor (BDNF) expression resulting from CREB phosphorylation indicates long-term memory. Resulting damage in these signalings may be detected as decrease of freezing late in the CFC test. In the present study, LEH showed protective effects on this impairment of CREB phosphorylation induced by transient ischemia in the hippocampal CA1 area (Fig. 4b). These results suggest that LEH has cerebroprotective actions that may combat the impairment of the hippocampal circuit function that is induced by ischemia–reperfusion.

Unexpectedly, administration of LEH did not increase the pO2 in the hippocampus during ischemia in the 4VO rats compared with saline-injected rats (Fig. 5a). However, a previous study using MCAO model rats showed that LEH carries oxygen into the other ischemic regions (59). This discrepancy might be due to the differences in the types of models between them. From these results, it is indicated that LEH could produce cerebroprotective effects via another mechanism in addition to oxygen supplementation. Namely, there are two possible mechanisms for the ameliorating effects of LEH on the hippocampal dysfunction by ischemia–reperfusion. 1) The hyperoxia observed in saline-injected rats after ischemia–reperfusion (Fig. 5a) could be avoided in LEH-injected rats by the differences of oxygen-dissociation profiles between red blood cell and LEH (3, 6). 2) Reactive oxygen species (ROS) generated by ischemia–reperfusion that contribute to neuronal cell dysfunction (60 – 62) could be scavenged by LEH (63). Although further studies are required to clarify this issue, the ROS-scavenging effects and/or the suppression of hyperoxia by LEH may be possible mechanisms that explain the cerebroprotective effects of LEH against the sequelae of brain ischemia in the present study.

The pegylated LEH has been reported to have no pres-
sor effects with hemodilution model rats (9), but in this study slight differences were observed in the pressor responses after ischemia–reperfusion by injection of LEH (Fig. 5c). The hemodilution model rats might not be exposed to hypoxia differently from 4VO rats because the blood was continuously withdrawn from the femoral artery with a simultaneous transfusion of LEH in the hemodilution model rats (9). Therefore, our present findings that systemic arterial pressure was increased after LEH injection in 4VO rats raised the suspicion that LEH may have no pressor effects (Fig. 5c). Thus, we tested the effects of LEH administration on the hemodynamics of intact rats. However, in intact rats the pressor response was not observed during 90 min after the injection of LEH (Fig. 6: a, b). Although LEH might be a weak vasoressor under extraordinary circumstances such as hypoxia, we speculate that LEH would not cause any serious problems in cardiovascular function.

In conclusion, our present findings demonstrate that LEH has cerebroprotective effects against transient cerebral ischemia. Furthermore, our research indicates that LEH might be a promising candidate not only as an artificial blood cell substitute for transfusion but also as a therapeutic agent for acute ischemic stroke.

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