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European Journal of Pharmacology 566 (2007) 83–87
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Neuroprotective effects of the novel polyethylene glycol-hemoglobin conjugate SB1 on experimental cerebral thromboembolism in rats

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Received 22 December 2006; received in revised form 25 February 2007; accepted 28 February 2007
Available online 12 March 2007

Abstract

SunBio1 (SB1) is a novel polyethylene glycol-bovine hemoglobin conjugate. It is a small molecule that shows high oxygen-delivery capacity, and exhibits extended plasma half-life compared to hemoglobin alone, thus reducing renal toxicity. The aim of the present study was to evaluate potential neuroprotective effects of SB1 using a rat middle cerebral artery occlusion model. The middle cerebral artery of male Sprague–Dawley rats was occluded with a thrombotic blood clot and SB1 was administered via intra-arterial infusion 5 min after the operation. Brain tissue was harvested after 2 h, and cerebral infarct volumes were calculated from coronal sections stained with 2,3,5-triphenyltetrazolium chloride. Three to 6 days after the procedure, sub-groups of animals were subjected to an open field test and the Morris water maze to assess locomotor activity and learning/memory function. Thrombotic blood clots induced extensive brain infarction and edema; however, these were significantly reduced in SB1 treated animals. In addition, SB1 treatment increased locomotor activity in open field tests, and improved the learning/memory deficits caused by the thromboembolism. These results suggest that SB1 has neuroprotective effects against ischemic brain injury caused by thromboembolism.

Keywords: Hemoglobin; Polyethylene glycol conjugate; Stroke; Neuroprotection

1. Introduction

Stroke is a major cause of death and a leading cause of adult long-term disability worldwide (Bonita et al., 2004). Recently, the number of stroke patients has significantly increased due to prolonged human lifespan. This has prompted enormous efforts to develop effective treatments that minimize brain lesions and neurological deficits (Koroshetz and Moskowitz, 1996).

The formation of blood clots is a common cause of stroke. Patients are often treated by surgical removal of the clot, thrombolytic medication, or a combination of both treatments. Recent approaches in stroke therapy focus on the combinatorial use of thrombolytic and neuroprotective agents. Neuroprotective agents are used to prevent oxidative stress caused by either reperfusion surgery, or the restoration of blood flow following infusion of thrombolytic drugs (Elger et al., 2006; Haga et al., 2003; Lekieffre et al., 1997). Thus, administration of free radical scavengers, calcium channel blockers, calpain inhibitors, and inducing hypothermia, have been shown to exert neuroprotective effects in different models of cerebral ischemia (Elger et al., 2006; Haga et al., 2003; Lekieffre et al., 1997; Maksimovich et al., 2006; Markgraf et al., 1988). The efficacy of such treatments however, is relatively low, due to limited penetration of the central nervous system and/or differences between animal models and human cases (Hara et al., 2000).

Hemoglobin (Hb) and its variants are considered to be alternative therapeutic candidates in the early stage of stroke treatment due to their high oxygen-carrying capacity. Hb

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doi:10.1016/j.ejphar.2007.02.061

modifications such as crosslinking, polymerization and conjugation, have been proposed to improve oxygen carrying efficiency while decreasing the toxicity of native Hb (Chang, 2004). Diaspirin cross-linked Hb, and polyethylene glycol (PEG)-conjugated Hb, have been extensively evaluated for their beneficial effects on ischemic brain injury. Diaspirin cross-linked Hb has a stable structure and efficient oxygenation properties, which show neuroprotective effects via hemodilution (Cole et al., 1993; D'Agnillo and Alayash, 2000). PEG-conjugated Hb, has been tested as a blood replacement fluid. This conjugate also has good tissue oxygenation capacity compared to unmodified Hb, and exhibits minimal effects on renal function (Conover et al., 1997, 1999).

SunBio1 (SB1) is a novel PEG-conjugated bovine Hb compound that has a molecular weight of 104.5 kD with a size of 30–50 nm (Fig. 1). The pharmacokinetic study of SB1 showed improved oxygen carrying capacity compared to other PEG-conjugated Hb compounds that have been developed, and the plasma half-life of SB1 is 9.6 h in rodents (Lee et al., 2006) and 15–30 h in beagle dogs (Kwon et al., 2004). Since SB1 has high oxygen carrying capacity and increased plasma half-life, which reduce side-effects such as renal toxicity and hypertension, it is expected to be a potential therapeutic candidate for ischemic brain injury. The aim of this study is to evaluate neuroprotective effects of SB1 using a rat middle cerebral artery occlusion model.

2. Materials and methods

2.1. Animals

Twelve week-old male Sprague–Dawley rats (350–400 g) (Daehan Laboratory Animal Center, Korea) were housed in the Laboratory Animal Research Center at Chungbuk National University. The animals ($n=10$ per group) were maintained at a constant temperature of 21±1 °C, 12-h light/dark cycle and fed with standard rodent chow. All experimental procedures were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85–23, revised, 1996), and the protocol was approved by the Institutional Animal Care and Use Committee of Laboratory Animal Research Center, Chungbuk National University, Korea.

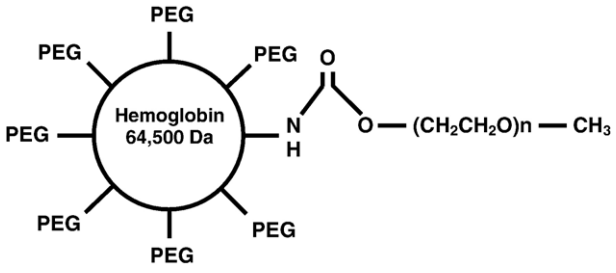

Fig. 1. Chemical structure of polyethylene glycol (PEG)-hemoglobin SunBio1 (SB1). The total molecular weight of SB1 is 104.5 kDa including hemoglobin and conjugated PEG. The expected size of the molecule is approximately 30–50 nm.

2.2. Experimental thromboembolism

Animals were anesthetized with 1.5% enflurane, and the right middle cerebral artery was obstructed with an artificial thrombus, as previously described (Zhang et al., 1997). Briefly, the bifurcation of the right common carotid artery was exposed, then the branches of external carotid artery were divided and pulled down to assist polyethylene tube insertion into the internal carotid artery.

The artificial thrombus was prepared by mixing 4 parts of fresh rat blood and 1 part of thrombin (60 U/ml in 0.05 M phosphate-buffered saline) in a polyethylene tube (0.28 mm in internal diameter). The clot was released into the internal carotid artery by injecting 0.1 ml of saline from a Hamilton syringe connected to the tube. The clot was left within the internal carotid artery until the animals were killed. During surgery, the rectal temperature of the animals was maintained in the range 36.5–37.5 °C using a heating pad.

2.3. SB1 treatment

SB1 is a PEG-conjugated hemoglobin that is composed of 5.6% bovine hemoglobin and 4.0% PEG in isotonic saline (SunBio Inc., Korea, Fig. 1). The SB1 was prepared as a 96 mg/ml solution for infusion. Five minutes after surgical occlusion of the middle cerebral artery, animals were treated with SB1. Two different doses of SB1 (240 or 480 mg/kg) or saline (vehicle) were injected via femoral arterial catheter using an infusion pump. Prior to, and after the SB1 injection, blood was withdrawn from each animal and hematocrit values were determined.

2.4. Image analysis and histopathology

In order to determine the areal size of infarction, brains were removed 2 h after artificial thromboembolism. The brains were briefly washed in cold saline and then 2 mm coronal sections were prepared using a brain matrix. The sections were stained with 2,3,5-triphenyltetrazolium chloride at 37 °C for 60 min (Beech et al., 2001).

Digital images of stained sections were obtained using a flatbed scanner (Hewlett-Packard, USA) within 1 h after staining. The sections were placed directly on the scanning screen, and images were taken from the rostral tip to the caudal end using the caudal surface from each section. Image analysis was performed using Photoshop software (Adobe Systems Inc, San Jose, CA) as previously described (Beech et al., 2001). The infarction size was measured by manually outlining the margins of infarct area using the Lasso tool, and subsequently the pixel-based area was calculated. The infarct volume was presented as mm^3, and represents the estimated volume resulting from the summation of surface areas multiplied by the thickness of each section.

Brain edema may affect the accuracy of infarct size estimation within a period of 6 h to 3 days (Lin et al., 1993). Based on the compensation of brain swelling in the ischemic hemisphere, the infarct size in each rat was corrected by sizing the left and right hemispheres and applying the following

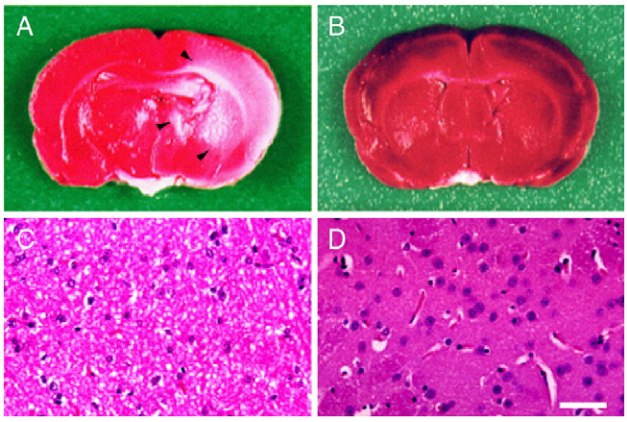

Fig. 2. Representative images of coronal brain sections stained with 2,3,5-triphenyltetrazolium chloride (A and B) and hematoxylin–eosin stained photomicrographs (C and D). Artificial thromboembolism induced cerebral infarction is outlined by arrows (A). SunBio1 (SB1, 480 mg/kg) treatment prevented cerebral infarction (B). Neural cells in the infarction area exhibited spongiform degeneration (C) while minimal neurodegeneration was observed in SB1-treated animals (D). Scale bar = 50 μm.

formula: corrected infarct size = the size of left hemisphere − (the size of ipsilateral hemisphere − measured infarct size) (Belayev et al., 1996). The rate of brain swelling was measured as follows: % edema = (the volume of ipsilateral hemisphere − the volume of contralateral hemisphere)/the volume of left hemisphere × 100.

Cytoarchitectural changes were also examined to ensure the presence of neurodegeneration induced by the thromboembolism, as well as to examine potential neuroprotective effects of SB1. Brains from thromboembolism-, and high-dose SB1-treated groups were fixed in 10% neutral buffered formalin, processed using standard techniques, and then embedded in paraffin wax. Subsequently, 6 μm sections were cut and stained with hematoxylin–eosin then examined under a light microscope.

2.5. Behavioral analyses

Following a 3 day post-surgical recovery period, locomotor activity was evaluated to assess behavioral changes in treated animals. Prior to the test, animals were adapted in a dark cage for 30 min, and then transferred to a 60 × 60 × 40 cm open box. The number of rearing and leaning behaviors, as well as total traveling distance, were recorded for 60 min using a video tracking system (HVS Image, United Kingdom) (Shen et al., 2005).

To evaluate the functional integrity of memory acquisition, animals were subjected to Morris water maze performances 3 days following experimental thromboembolism (Grauer and Kapon, 1993). Each animal was given 4 trials a day at 30-min intervals, from each quadrant of a 27 °C water pool that was rendered opaque with milk powder. Mean latency (s) and distance (cm) to find a hidden platform were recorded.

2.6. Statistical analysis

The results are presented as means ± S.E.M. The significance of differences of all results was analyzed by one-way analysis of variance (ANOVA) followed by a Dunnett's *t*-test correction.

3. Results

3.1. Neuroprotective effect of SB1

Artificial thromboembolism (TE group) induced by middle cerebral artery occlusion for 2 h in rats led to an extensive infarction in the ipsilateral (occluded side) hemisphere including the cortex, hippocampus and thalamus (Fig. 2A), whereas relatively mild to minimal infarction was observed in high-dose SB1-treated rats (SB1 group, Fig. 2B). Animals in the TE group showed degeneration and loss of neural cells, and spongiform change (malacia) of neuropils (Fig. 2C), but only a few degenerating neurons were observed in the high-dose SB1 group (Fig. 2D).

Image analysis of the infarct volume revealed significant differences between the TE group, and both low- and high-dose SB1 groups (Table 1). The absolute and corrected infarct volumes in the TE group were 159.5 ± 12.3 mm^3 and 145.1 ± 9.2 mm^3, respectively, whereas no infarction was observed in normal and sham-operated animals. The volume of the ipsilateral hemisphere in the TE group was increased by 2.3% compared to the contralateral hemisphere, due to edema. SB1 treatment showed dose-dependent neuroprotective effects. In the SB1 group treated with 240 mg/kg, the infarction volume was decreased to 16% when compared to the TE group. The SB1 group treated with 480 mg/kg showed a marked reduction in infarction volume as the volume was decreased to 1.4% of TE group, showing effective prevention of neural injuries caused by thromboembolic stroke.

The hematocrit values from low- and high-dose SB1 treatments exhibited an approximately 6% decrease in both groups. The hematocrit values before and after low-dose SB1

Table 1
Absolute and corrected infarct volumes and percent changes in edema

Treatment	Absolute infarct volume (mm^3)	Corrected infarct volume (mm^3)	Edema (%)
Normal	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Sham operation	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Thromboembolism	159.5 ± 12.3 [a]	145.1 ± 9.2[a]	2.3 ± 0.4[a]
+SB1 (240 mg/kg)	25.5 ± 5.8 [b]	19.9 ± 4.6[b]	2.0 ± 0.3
+SB1 (480 mg/kg)	2.3 ± 0.7[b]	1.9 ± 0.5[b]	0.3 ± 0.1[b]

[a] Significantly different from normal or sham operation ($P < 0.05$).
[b] Significantly different from thromboembolism ($P < 0.05$).

Table 2
Rearing/leaning and traveling distance in open field test

Treatment	Rearing/leaning (counts/h)	Traveling distance (cm/h)
Normal	31.8 ± 1.1	2,609.3 ± 94.8
Sham operation	28.4 ± 0.5	2,682.7 ± 66.9
Thromboembolism	14.6 ± 1.9 [a]	1,730.4 ± 113.0[a]
+SB1 (240 mg/kg)	20.0 ± 1.6	2,522.5 ± 77.1 [b]
+SB1 (480 mg/kg)	25.8 ± 1.3[b]	2,678.4 ± 92.8[b]

[a] Significantly different from normal or sham operation ($P < 0.05$).
[b] Significantly different from thromboembolism ($P < 0.05$).

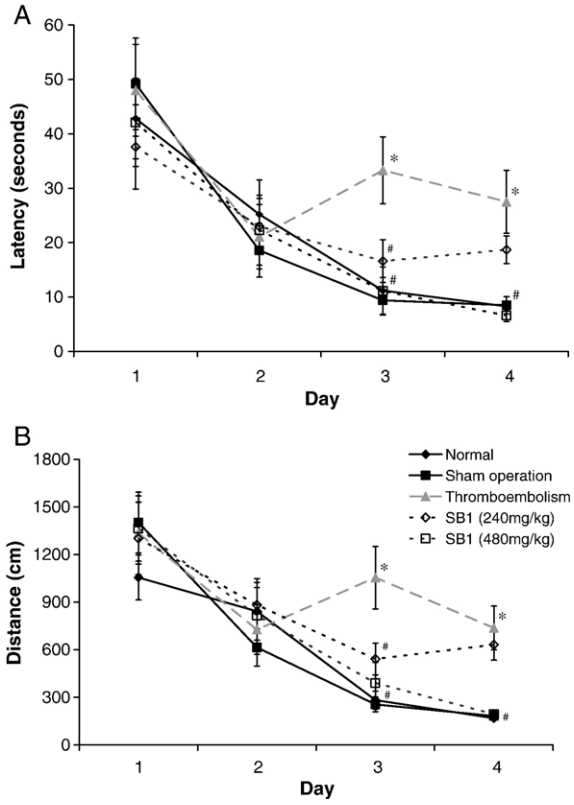

Fig. 3. Latency (A) and distance (B) to find a hidden platform in the Morris water maze test. Low- (240 mg/kg) and high-dose (480 mg/kg) SunBio1 (SB1) treatment improved both latency and distance to find the hidden platform in animals with thromboembolism. *Significantly different from normal or sham operation ($P<0.05$). #Significantly different from thromboembolism ($P<0.05$).

treatment were 54.0±1.0 and 51.0±0.6 respectively, whereas those for high-dose SB1 treatment were 52.7±0.9 and 49.7±0.3.

3.2. Behavioral analyses

Artificial thromboembolism resulted in significant motor deficits characterized by decreased rearing/leaning and traveling distance (Table 2). In the open field test, animals in the TE group showed approximately 50% less rearing/leaning activity when compared to normal and sham operated groups. The total traveling distance in the TE group was also decreased by about 35% compared to normal and sham operated groups. When animals were treated with SB1, however, the rearing/leaning activity was improved by 37% (240 mg/kg) and 77% (480 mg/kg) compared to the TE group. The total traveling distance was also improved with SB1 treatment by 47% (240 mg/kg) and 55% (480 mg/kg) compared to the TE group.

Memory acquisition was significantly delayed when the animals with artificial thromboembolism were subjected to Morris water maze performances after the 3-day recovery period (Fig. 3). Differences in latency and distance to find the hidden platform were observed in the third day trial, as SB1 treated animals found the platform significantly faster than animals in the TE group. In particular, the high-dose SB1-treated group showed an almost identical pattern of learning and memory acquisition as was observed in normal and sham operated animals.

4. Discussion

This study demonstrates that SB1 has neuroprotective effects against ischemic brain injury caused by middle cerebral artery occlusion in rats. SB1 administration also prevents behavioral deficits including motor function and memory acquisition.

A number of focal ischemia models have been developed in mice, rats and gerbils (Carmichael, 2005). Each model provides a unique opportunity to evaluate a number of potential therapeutic agents. One of the models described by Zhang et al. (1997) employed a technique using infusion of an artificial thrombin clot to produce consistent focal infarction in the territory of the middle cerebral artery. We found that this model is particularly useful for several reasons. The first major advantage of this model is the close resemblance of its cerebral circulation to that of a human. Moreover, the model reproducibly generates a large infarct which closely simulates the clinical situation of ischemic stroke, using a minimally invasive technique. Finally, the rat provides an optimal model to evaluate deficits in behavioral function, such as locomotive activity and learning/memory impairment (Hunter et al., 1998).

Currently, one of the major therapeutic strategies for human stroke is thrombolytic agent administration (Caplan, 2004; Traynelis and Lipton, 2001). Thrombolytic therapies are known to decrease neuronal damage and improve recovery of function after an acute ischemic stroke. It is conceivable that thrombolytic therapies effectively restore blood flow and improve the clinical outcome for stroke patients. However, the window of efficacy with such therapy is unfortunately shorter than 3 h, and the treatment is often associated with a serious risk of systemic and intracerebral hemorrhage, which may increase death rates (Barsan et al., 1993). Moreover, an abrupt restoration of blood flow following reperfusion surgery or infusion of thrombolytic agents may exacerbate the tissue injuries due to oxidative stress (Elger et al., 2006; Haga et al., 2003; Lekieffre et al., 1997).

In this context, hemoglobin and its variants have been studied as an alternative treatment for ischemic brain injury. Since isolated hemoglobin exhibits renal toxicity and has low oxygen affinity, several modifications have been proposed to overcome the limitation of hemoglobin treatment (Chang, 2004). SB1 is a novel PEG-conjugated bovine hemoglobin that has been recently developed. Several modifications result in SB1 having a high affinity for oxygen and low toxicity. It was found that SB1 possesses 8–12 mm Hg of P_{50} (oxygen pressure of blood at 50% oxygen saturation of Hb), thus facilitating the delivery of oxygen to ischemic cells more effectively than previously developed PEG-conjugates with 20–32 mm Hg of P_{50} (unpublished results). In addition, the extended plasma half-life of SB1 may reduce side-effects caused by PEG-Hb (Kwon et al., 2004).

Our results show that SB1 effectively reduced ischemic brain injury by decreasing infarction volume and preventing accompanying behavioral deficits. The beneficial effects were dose-dependent, as high-dose SB1 treatment showed the greatest prevention against ischemic brain injury and behavioral deficits. Although the exact mechanism of the neuroprotective effects of SB1 remains to be clarified, it seems that its small size

and low P_{50} play important roles. The estimated size of SB1 is approximately 30–50 nm, therefore SB1 may be able to pass through the narrow openings between vessel wall and thrombus, thus supplying hypoxic cells with oxygen. Moreover, once spontaneous blood clot lysis takes place, SB1 can easily penetrate gaps within the infracted area and supply oxygen. Alternatively, with its high oxygen-carrying capacity, SB1 may supply oxygen via collateral blood vessels, and prevent hypoxic brain injuries within affected areas.

Previous reports have shown that hemodilution is an effective way to protect neuronal cells from the ischemic insult (Cole et al., 1993, 1997). In our experiment, we found that there is about a 6% decrease in hematocrit value in both low- and high-dose SB1 treated groups. Hemodilution may have played a role in protecting ischemic injury in our model; however, the effect may have been minimal because the magnitude of hemodilution is far less than in previous reports showing neuroprotective effects at 10–30% hemodilution.

In conclusion, these results show that SB1 is a potential therapeutic agent that can be used in early stages of stroke to prevent ischemic brain injury. Further studies are warranted to evaluate the neuroprotective effects and mechanism of action of SB1 in different models of ischemia.

Acknowledgement

This work was supported in part by the Korea Research Foundation Grant (Basic Research Promotion Fund KRF-2005-005-J15002).

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