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Intranasal recombinant human erythropoietin protects rats against focal cerebral ischemia

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Abstract

Erythropoietin (EPO) is a hematopoietic growth factor with tissue-protective properties, and can protect animals from cerebral ischemic injury. However, the central nervous effects of EPO as a glycoprotein is limited by the potential complication resulted from its erythropoietic activity and the problem of the penetration through blood–brain barrier (BBB). To avoid these limitations, in this study we administered recombinant human EPO (rhEPO) intranasally (i.n.) to evaluate its neuroprotective effect in the rats with focal cerebral ischemia induced by middle cerebral artery occlusion (MCAO). We found that rhEPO i.n. at doses of 4.8, 12 and 24 U (administered 10 min after MCAO and 1 h after reperfusion) reduced infarct volume, brain swelling and cell damage in the ischemic hemispheres, and improved behavioral dysfunction 24 h after cerebral ischemia. Intraperitoneal rhEPO (5000 U/kg) also showed the protective effect, but the heat-inactivated rhEPO did not show any effect. Thus, intranasal administration of relatively small doses of rhEPO protects rats from acute injury after focal cerebral ischemia, suggesting that intranasal rhEPO may be a more effective and safer administration route for treatments of ischemic or other brain diseases.

Keywords: Recombinant human erythropoietin; Intranasal delivery; Cerebral ischemia; Neuroprotection

Erythropoietin (EPO) is a hematopoietic growth factor (or a cytokine) with tissue-protective properties [5,8,13]. It has been reported that EPO possesses neuroprotective effects on hypoxic/ischemic cerebral damages [2,4,9,15,23] and experimental subarachnoid hemorrhage [1,7]. In a clinical trial in 40 patients, EPO improved earlier in the stroke scores and had significantly better functional outcomes at the termination of the study of 30 days [8]. However, the potential complication of EPO therapy is the harmful increases in the red cell mass and reactive platelets resulted from its hematopoietic activity, which may hamper its clinical use. For example, in the mice overexpressing EPO, the hematocrit levels were dramatically increased and the infarct volumes were enlarged after cerebral ischemia [24]. To resolve this problem, one desirable way is the development of modified molecules of EPO that retain the beneficial tissue-protective actions but not stimulate the bone marrow. A non-erythropoietic variation of EPO, asialoerythropoietin (asialoEPO), has been reported to not increase the hematocrit of mice and rats, but exhibit a broad spectrum of neuroprotective activities, as demonstrated in models of cerebral ischemia, spinal cord compression, and sciatic nerve crush [6]. Another possible way may be the alternative administration routes. Currently, the administrations of EPO used in the animal models of cerebral ischemia are systemic (intraperitoneal or subcutaneous) and intracerebroventricular (i.c.v.) injections [13]. Systemic injections have the problem of the penetration through blood–brain barrier (BBB), and cannot avoid the undesirable systemic effects on other tissues, while i.c.v. injection can cause brain damages and infections, and is not suitable for clinical use. It has been reported that EPO can across BBB to protect experimental brain injury, but the dose (5000 U/kg) is much larger than that needed

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for erythropoiesis [4]. Here, we consider whether intranasal administration of small doses of EPO is a simple route to penetrate BBB and provides a neuroprotective effect on cerebral ischemia.

Intranasal administration of therapeutic agents is capable of directly transporting into the central nervous system (CNS), especially for agents with poor bioavailability and for the delivery of biosensitive and high-molecular weight compounds, such as proteins and peptides. Intranasal delivery of several peptides/proteins to the brain has been reported in human [3] and animals [14,20,21]. Moreover, intranasal administration of insulin-like growth factor-1 (IGF-1) has been shown to attenuate focal cerebral ischemic injury [10,11]. This suggests that intranasal administration of a neuroprotective peptide or protein may be a simple and non-invasive method to treat cerebral ischemia. However, whether intranasal administration of EPO is effective on acute injury in cerebral ischemia is still unknown. Therefore, in the present study we examined whether intranasal administration of EPO has the therapeutic benefit in focal cerebral ischemia in rats.

Male Sprague–Dawley rats weighting 250–350 g were purchased from the Experimental Animal Center of Zhejiang Academy of Medical Sciences (Certificate No. SCX20030001). Rats were housed under a controlled temperature, 12-h light/12-h dark cycle and allowed free access to food and water. All experiments were carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals.

Rats were anesthetized with an intraperitoneal injection of chloral hydrate (400 mg/kg). Polyethylene tube was inserted into the right femoral artery for continuously monitoring blood pressure using a computer-assisted system (MedLab-U/4cs, Nanjing MedEase Inc., Nanjing, China). Before operation and 30 min after reperfusion, the arterial blood pH, p_{AO_2}, p_{ACO_2} and hemoglobin (Hb) were monitored (Blood gas analyzer ABL700, Denmark), blood glucose was measured (One Touch^{TM} Basic Complete Blood Glucose Monitoring System, Lifescan Inc., USA), and rectal temperature was maintained at $37 \pm 0.5\,^{\circ}C$ by means of heating blanket and lamp during the surgery.

Focal cerebral ischemia was induced by the suture occlusion method as previously described by Zea Longa et al. [12]. Briefly, a nylon suture (diameter 0.26 mm) was inserted 18–20 mm to occlude the origins of the anterior cerebral artery, the middle cerebral artery, and the posterior communication artery, and withdrawn for reperfusion after 1 h of occlusion. Sham-operation animals were treated in the same manner except that the common carotid arteries were not clamped and the suture was not inserted. Recombinant human EPO (rhEPO) or saline was administered 10 min after MCAO and 1 h after reperfusion. Injection of rhEPO (0.5 ml) was purchased from Kirin Kunpeng (China) Bio-Pharmaceutical Co. Ltd. (Shanghai, China) that contained 3000 U rhEPO and 5 mg gelatin (pH 5.4–6.4) in one vial.

Intranasal administration of rhEPO was based on the method as previously described by van den Berg et al. [22]. The rats were fixed in the stereotaxic frame and lying in the supine-70° angle position, a polyvinyl chloride tube (i.d. 0.5 mm, o.d. 1.0 mm) attached to a 1 ml syringe was inserted into the left and right nostrils, respectively, for ≈2 cm. A volume of 20 μl (0.6, 2.4, 6 and 12 U rhEPO/20 μl sterile saline for one nostril) was delivered by gently pushing the plunger of the syringe into two nasal cavities, respectively. After delivery, the tube was removed and the rats were keeping at this position for 30 min to avoid rhEPO loss. To exclude the non-specific effect, the inactivated rhEPO (by heating at 56 °C for 30 min) was intranasally administered as a control. To compare to the effect of systemic administration, rhEPO (5000 U/kg) was intraperitoneally injected at the same time points.

Twenty-four hours after reperfusion, the neurological scores were determined by a modified described by Zea Longa et al. [12]: 0, no deficit; 1, failure to extend left forepaw fully; 2, circling to the left; 3, failing to the left; 4, no spontaneous walking with a depressed level of consciousness. The inclined plane test was performed to assess balance and coordination, based on modifications of a method described by Yonemori et al. [27]. Rats were placed on a board (25 cm × 15 cm) covered by copper wire mesh (0.2 mm) 24 h after MCAO. Once they stayed stably the board was inclined from horizontal to vertical. The holding angle at which the animal fell from the board was recorded. The test was repeated for three times and the average holding angle was used.

After evaluation of neurological scores and inclined plane test, the rats were re-anesthetized with chloral hydrate and decapitated. The brains were quickly removed and coronally dissected into 2-mm thick slices. The slices were stained with 2% 2,3,5-triphenyltetrazolium chloride (TTC) at 37 °C for 30 min. All the slices with the caudal face upward were recorded with a digital camera (C-1400, Olympus, Japan); then the image data were transferred to a computer and analyzed using an image analyzer (AnalyPower 1.0, Zhejiang University, Hangzhou, China). The areas of both hemispheres and the infarct areas were calculated. The left (non-ischemic) stained areas (AL) and right (ischemic) stained areas (AR) were measured. AL minus AR was defined as the infarct area of this slice. Infarct area times the thickness of the slice (2 mm) was the infarct volume. Total infarct volume was the sum of the infarct volumes from all of the slices. Brain edema was evaluated by the ratio of ischemic/non-ischemic hemispheres.

Serial sections (8 μm) were prepared by cryostat (Leica CM 1900) from the indicated slices, fixed in 10% buffered formalin for 10 min, and then stained with hematoxylin and eosin. The apparently survival neuron-appearing cells in hippocampal CA1 region, temporo-parietal cortex III and IV layers (3.8–4.0 mm caudal to bregma), and striatum (0.4–0.2 mm rostral from bregma) were counted under a light microscope.