A comparison of the antiamnesic effects of erythropoietin derivatives and their mutant forms on the level of S100b protein in the serum of rats with ischemic damage to the prefrontal cortex

Fatima M Shakova1, Irina A Zakharova1, Tatyana I Kalinina2, Vitaly L Yurin2, Galina A Romanova1, Sergey G Morozov1

1 FSBI Institute of General Pathology and Pathophysiology, 8 Baltiyskaya str., 125315 Moscow, Russian Federation
2 State Research Institute of Genetics and Selection of Industrial Microorganisms 1 - 1st Dorozhny proezd, 117545 Moscow, Russian Federation

ABSTRACT

New hybrid proteins based on erythropoietin (EPO), EPO-TR and EPO-Fc, and their mutant forms, MEPO-Fc and MEPO-TR, which lack hematopoietic activity but retain the cytoprotective properties of erythropoietin, were created via genetic engineering. The assessment of the antiamnesic efficiency of the obtained proteins was conducted on the 4th day after bilateral phototrothombosis of the medial prefrontal cortex of rats and it was dependent on the strength of the conditioned passive avoidance reflex before ischemia. The concentration of S100b protein, a glial marker of brain tissue damage in the serum of rats, was assessed using enzyme-linked immunosorbent assay (ELISA) within the same period. A one-time intranasal administration of erythropoietin derivatives EPO-Fc and EPO-TR, as well as their mutated forms, MEPO-Fc and MEPO-TR, at a dose of 50 μg/kg, one hour after ischemic brain cortex injury, was associated with the preservation of the skill that was developed before ischemia. A significant decrease in the level of S100b protein in serum was found when EPO-TR was administered. Administration of the other tested derivatives showed a tendency to decrease the S100b level, which was most pronounced in animals treated with MEPO-TR. Our results confirm the neuroprotective efficacy of these novel proteins as potential drugs for the treatment of experimental focal ischemic brain damage.

Keywords: ischemia, prefrontal cortex, S100b, derivatives and mutated erythropoietin forms, rats

Biotecnología Aplicada 2017;34:2201-2204

Introduction

Stroke has been one of the leading causes of disease and of death for many years [1-3]. Approximately 80% of all strokes are transient ischemic attacks. A search for complex anti-pathogenic therapies to lower the degree of neurodegeneration and to help in restoring disturbed central nervous system functions will significantly reduce the pathologic consequences of stroke. These are current medical issues and important social tasks.

It is known that there are biochemical markers (glial and neuronal specific proteins) that are used for the detection of central nervous system injuries. For instance, the calcium binding protein S100b has been identified as a glial marker of brain tissue damage of different origins, including mechanical trauma and stroke [4]. The use of modern technology allows the quantitative assessment of the concentration of S100b protein in many biological liquids, particularly in serum; its concentration in blood samples consistently correlates with the degree of brain damage. It has been established that, at an early stage of ischemic stroke, microglial cells in the peri-infarct zone

EPO derivatives decrease serum S100b levels in rats.

Therefore, the aim of this work was to compare the effects of EPO derivatives and their mutant forms on the preservation of the passive avoidance reaction and the serum levels of S100b protein in rats with photochemical ischemic damage in the prefrontal cortex.

Materials and methods

Animals and housing conditions

Experiments were performed on 2.5-3 three-month-old outbred male rats (n = 70), weighing 200-220 g each, obtained from the vivarium of the FSBI Institute of General Pathology and Pathophysiology, Russia. The animals were kept in a vivarium at 12-hour light and dark cycles with free access to water and food. Animals were handled following the animal healthcare Directive 2010/63/EU on the protection of animals used for scientific purposes.

EPO derivatives

The erythropoietin EPO-TR, EPO-Fc derivatives and their mutated MEPO-TR and MEPO-Fc variants were previously described [12]. Briefly, EPO-TR variant is a fusion protein containing the TR domain of the glycoprotein MUC1-(TR), which has additional sites for O-glycosylation. EPO-Fc protein fusion consists of EPO with a C-terminal-linked IgG-Fc domain. The MEPO variants contain the R103E substitution in the context of the EPO monomer. Cell lines on the basis of CHO producing proteins were obtained using the Flp/ FRT-dependent site-specific transgene integration in the DG44(FRT+/Dhfr+)+ genome of the recipient CHO cells. The four mutant recombinant proteins were purified from cell supernatants using affinity chromatography on an anti-EPO antibody column [12].

Induction of passive avoidance

The conditioned passive avoidance response (PA) was induced according to a previously described scheme [6]. The latent period (LP), i.e., the time from the start of the test to the moment when the rat passed the hole between illuminated and dark compartments of the chamber, was determined. When the rat re-entered the dark compartment, the door was closed and electrical current was passed through metal rods of the floor (1.3 mA, 50 Hz, 5 s). PA was formed if the LP was higher than or equal to 300 s. Animals with lower LPs were excluded from the experiment. The antiinamncic effect of tested EPO derivatives and mutated forms was evaluated on the 4th day after photochemical induced damage to the prefrontal cortex.

Bilateral focal ischemic infarction was modelled in the rat prefrontal cortex (fields Fr1 and Fr2) by photochemically induced thrombosis [13, 14]. The operation was performed under anaesthesia (chloral hydrate intraperitoneally at 300 μg/kg). After the administration of the photosensitive dye rose Bengal (40 mg/kg intravenously; Sigma, USA), the rat’s head was fixed in a stereotactic frame. The unit used for irradiation consisted of a source of cold light (250 W halogen lamp) and light fibre with an inner diameter of 3 mm. The light fibre was placed at a distance of 1 mm from the cranial surface, 2 mm rostral to bregma, 2 mm lateral to the sagittal suture. Each hemisphere of the brain was exposed to 1000 s of photolysis. This resulted in bilateral focal cerebral ischemic damage to the prefrontal cortex.
irradiated with cold light with a λ = 560 nm for 15 min. The control group was not exposed to any influence other than elaboration PA.

**EPO administration and experimental treatments**

The derivatives of erythropoietin and the mutant forms were administered intranasally once 1 h after the injury at a dose of 50 µg/kg. Animals were randomly distributed among seven groups of 10 rats each and subjected to the following treatments: 1) Intact control rats for ELISA; 2) Control: trained passive avoidance reaction before photothermobilos; 3) Phototherapy + 0.9 % NaCl solution in a volume of 50 µL; 4) Phototherapy + EPO-Fc at 50 µg/kg; 5) Phototherapy + EPO-TR at 50 µg/kg; 6) Phototherapy + MEPO-Fc at 50 µg/kg; 7) Phototherapy + MEPO-TR at 50 µg/kg.

**Determination of S100b protein levels in serum samples**

To assess the level of S100b protein in the serum samples of rats, the “Rat soluble protein-100 B (S-100B) ELISA Kit” (catalogue number: CSB-E08066r; Cusabio Biotech Co., Ltd, USA) was used according to the instructions from the manufacturer. The analysis was the high-sensitivity ELISA (enzyme-linked immunosorbent assay) method, the range of protein identification was 3.12-2000 pg/mL. S100b protein concentration in the samples was assessed by using the working curve made according to the standards of known S100b concentrations [15, 16]. The control group consisted of intact rats.

**Statistical analysis**

Statistical data processing was conducted with Statistics 6.0 software. The values of the latent period in the conditioned passive avoidance reflex and the level of S100b protein contained between the groups were compared by using the Mann-Whitney U test. Differences were considered statistically significant for p < 0.05.

**Results**

The functional state of the central nervous system was assessed by measuring the LP passive avoidance reaction before and after ischemic damage of the cerebral cortex of rats. Before photothermobilos, the LP in all trained animals was 300 s. The passive avoidance reaction was evaluated on the 4th postoperative day. After a single intranasal administration of derivatives of erythropoietin and their mutant forms, the LP passive avoidance reaction on the 4th day after ischemia was reduced to similar levels in animals treated with EPO-Fc, EPO-TR, MEPO-Fc and MEPO-TR (205, 198, 200 and 211 s, respectively) at 50 µg/kg each, a strikingly different behavior when compared to the 68 s value of control animals receiving 50 µL of 0.9 % NaCl. The data showed that treatment with standard doses of the EPO-Fc and EPO-TR derivatives of native EPO and their mutant forms MEPO-TR and MEPO-Fc resulted in a significant preservation of the passive avoidance reaction that had been established prior to ischemia (Figure 1).

A study of the serum content of S100b protein in rats with photochemical ischemic brain damage and the effect of erythropoietin derivatives and mutant forms was carried out on the 4th day after photothermobilos (Figure 2). A two-fold increase was shown in the level of S100b protein in rats with photochemical damage to the prefrontal cortex, in comparison with the intact control animals. The intranasal administration of EPO-TR (50 µg/kg) significantly reduced this index, which confirms the neuroprotective effect of EPO-TR. With the introduction of other studied derivatives, there was a tendency of the level of S100b to decrease, the lowest levels attained in the MEPO-TR group. Thus, a single intranasal administration of EPO-Fc, EPO-TR, MEPO-Fc or MEPO-TR after bilateral photochemical damage to the prefrontal cortex of rat brain produced antiangiogenic effects. The levels of S100b protein in the same animals on the 4th day showed a neuroprotective effect of EPO-TR at a dose of 50 µg/kg (p < 0.05). Treatment with the other studied derivatives was

Figure 1. Effect of intranasal administration of carbamylated erythropoietin derivatives and their mutant variants forms on the conditioned passive avoidance reflex (PA) in rats with bilateral photothermobilos in the prefrontal cortex. Recombinant fusion EPO derivatives EPO-Fc and EPO-TR and their respective R103E mutant variants MEPO-TR and MEPO-Fc, were administered to outbred rats at 50 µg/kg. Animals were evaluated on day 4 after ischemic damage. LP: latent period. Control: passive avoidance (PA) without treatment. NaCl 0.9 % was administered in 50 µL. * Statistically significant differences in LP after treatment as compared to LP of the conditioned PA of all EPO derivatives experimental groups (p < 0.05; Mann-Whitney U test); it was also significant as compared to the LP of the conditioned PA reflex before photothermobilos (p < 0.05; Wilcoxon test).


Figure 2. Influence of intranasal administration of erythropoietin carbamylated derivatives and their mutant variants forms EPO-Fc, EPO-TR and MEPO-TR and MEPO-Fc, respectively, on the amount of S100b protein in the serum of rats with bilateral photothermobilos in the prefrontal cortex. Evaluations were made on day 4 after ischemic damage. * Statistically significant differences in comparison with the group treated with 50 µL of 0.9 % NaCl (Mann-Whitney U test; * p < 0.05; ¶ p < 0.06).
associated with a trend in the decrease of the levels of S100b, particularly with MEPO-TR.

**Discussion**

An evaluation of the therapeutic efficacy of EPO derivatives and mutant proteins was carried out by determining the degree of preservation of the conditioned passive avoidance reflex after photochemical damage of the vessels of the prefrontal cerebral cortex. The level of the S100b protein was measured in the sera of treated and control rats, this marker relevant for evaluating brain tissue damage. In fact, high concentrations of this protein are found in individuals with brain trauma and neurodegenerative and cerebrovascular lesions, including stroke [5].

We have previously shown that a photochemically induced bilateral thrombosis of the blood vessels of the prefrontal areas of the cerebral cortex leads to the formation of an ischemic focus. This lesion captures the entire thickness of the cortex and it is separated from the surrounding intact tissue by a clearly defined border; the damage to the cortex accompanied by a loss of the conditioned reflex of passive avoidance [6, 7]. Previous studies have shown the antiamnesic and neuroprotective effects of the studied erythropoietin derivatives [8, 9]. At the same time, in this study, there was a reliable preservation of passive avoidance that had been established before ischemia and a decrease in serum S100b levels in animals that were treated with EPO-TR. This index tended to decrease when other EPO derivatives were used. Thus, this test of ischemic damage to the brain tissue once again confirmed the neuroprotective efficacy of these drugs for the treatment of experimental focal ischemic brain damage.

Our data provide the evidence that fusion proteins (EPO-TR and EPO-Fc), bearing R103E mutation could have the same properties. Using our preclinical model of focal ischemic damage to the prefrontal cortex of rats, we were able to show significant preservation of skill generated before ischemia, indicating the nootropic and neuroprotective activity of the hybrid proteins. Based on these results, we suggest that our mutant variants of EPO fusion proteins are effective and highly specific in neuroprotection. Further studies should be conducted on animal models to suggest which EPO variant could be used in clinical trials. We plan to obtain more data in our next experiments and verify pharmacological properties of these proteins compared with other neuroprotective molecules.

**Acknowledgements**

This work was supported by the program of the Presidium of the Russian Academy of Sciences “Fundamental Researches for the Development of Biomedical Technologies” for 2014-2016.

**Conflicts of interest statement**

The authors declare that they have no conflicts of interest.