RESEARCH

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

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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 photothrombosis 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

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RESUMEN

Comparación del efecto antiamnésico de derivados de la eritropoyetina y sus especies mutadas sobre los niveles de la proteína S100b en suero de ratas con daño isquémico en la corteza prefrontal. Con anterioridad se obtuvieron mediante ingeniería genética cuatro nuevas especies híbridas de la eritropoyetina (EPO), llamadas EPO-TR y EPO-Fc (fusionadas por el extremo C-terminal al dominio TR de la glicoproteína humana MUC1 y al dominio FC de una molécula de IgG, respectivamente), y sus variantes mutadas MEPO-TR y MEPO-Fc. Estas moléculas carecen de actividad hematopoiética pero retienen las propiedades citoprotectoras de la eritropoyetina. En este estudio se evaluó su eficiencia antiamnésica en ratas, a los cuatro días de haberles inducido protrombósis bilateral de a corteza media prefrontal, dicha evaluación siendo dependiente de la severidad del reflejo de evasión pasiva condicionado previo a la isquemia. Se determinaron los niveles de la proteína S100b mediante un ensayo de inmunoadsorción (ELISA) en dicho periodo, por ser esta molécula un marcador en células de la glia del daño tisular cerebral. Una sola administración de cualquiera de las cuatro nuevas proteínas derivadas de la EPO en una dosis de 50 μg/kg, una hora después del daño isquémico en la corteza cerebral, se asoció con la preservación por los animales de las habilidades desarrolladas antes de la isquemia. Se observó un incremento significativo en los niveles de proteína S100b en suero cuando se administró la variante EPO-TR. Las otras tres proteínas indujeron una tendencia a la disminución de los niveles de S100b, más pronunciada en los animales tratados con la variante MEPO. Nuestros resultados confirman la eficacia neuroprotectora de estas cuatro nuevas variantes de EPO, como candidatos terapéuticos potenciales para el tratamiento del daño isquémico focal experimental del cerebro.

Palabras clave: isquemia, corteza prefrontal, eritropoyetina, variantes de EPO, mutaciones de EPO, ratas

*I*ntroduction

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

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express the S100b family proteins and actively proliferate, whereas the proteins are expressed for no longer than 3 days after a stroke. These findings indicate that a permanent microglia population becomes activated early in brain tissue in response to ischemia. The correlation has been demonstrated between the general protein fraction, the size of the ischemic zone, and the clinical outcome of stroke [5].

One of the experimental models that fully reproduce this clinical picture of focal brain ischemia is the photochemical thrombosis of cortical vessels. This model allows the quantitative assessment of neuroprotective and antiamnesic effects of substances used for the pharmacological correction of cerebral ischemic pathology [6-9]. A preclinical trial of carbamylated forms of new hybrid proteins based on EPO was conducted earlier on the focal ischemic stroke model [8, 9]. It has been shown that this chemical modification of both EPO and its derivatives (EPO-TR and EPO-Fc) leads to the loss of erythropoietin activity but maintains the neuroprotective action [10]. To activate cytoprotection, unlike erythropoiesis, significantly higher doses of EPO are needed. This can also induce side effects such as elevated haematocrit and platelet activation, ultimately causing thrombosis. However, there are still no mutated EPO forms able to display a cytoprotective function and stimulating erythropoiesis downwards.

There has been recently shown that certain mutant variants of EPO, such as the one carrying the arginine at position 103 replaced by glutamic acid (R103E), lose erythropoietic function but, otherwise, retain a high cytoprotective efficacy [11]. Mutant EPO molecules that carry the substitution (R103E) were produced via genetic engineering techniques in monomeric form of EPO-TR and in a dimer form of the recombinant protein, in which the immunoglobulin Fc-fragment was formed due to the dimerization of two Fc-fragments. CHO-producing mutant proteins were established with plasmids that carried mutant EPO genes, and the Flp / FRT site-specific transgenes were incorporated into the genome of Flp-In / CHO cells. Erythropoietic activity of the resulting mutant hybrid protein was evaluated in *in vitro* tests, in which the capacity of purified mutant proteins to initiate the proliferation of UT-7epo cells was estimated in comparison to the standard EPO preparation.

The erythropoietin activity of the obtained mutated hybrid proteins was analysed *in vitro*, in which the capacity of eliminated mutated proteins to initiate the proliferation of UT-7eop cells sensitive to EPO was examined in comparison with the standard EPO. The resulting data showed that EPO ability to interact with the receptor and to initiate UT-7epo cell proliferation was reduced more than 1000 times in a mutant bearing the R103E substitution in the context of the EPO monomer. In the case of dimer molecules with Fc, the molecule had a 100 times lower capacity to induce proliferation. Thus, the mutant EPO-TR and EPO-Fc erythropoietin molecules exhibited greatly reduced erythropoietic activity. Advantageously, the preparation and purification of these proteins require no further chemical modification, which makes the process more reproducible and time-saving for biotechnological production.

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-h light regimen 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 fusion protein 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 antiamnesic 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 stereotaxic 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

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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 photothrombosis; 3) Photothrombosis + 0.9 % NaCl solution in a volume of 50 μL; 4) Photothrombosis + EPO-Fc at 50 μg/kg; 5) Photothrombosis + EPO-TR at 50 μg/kg; 6) Photothrombosis + MEPO-Fc at 50 μg/kg; 7) Photothrombosis + 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-100B (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 3.12-200.00 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 Statistica 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 photothrombosis, 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 photothrombosis (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 antiamnesic 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

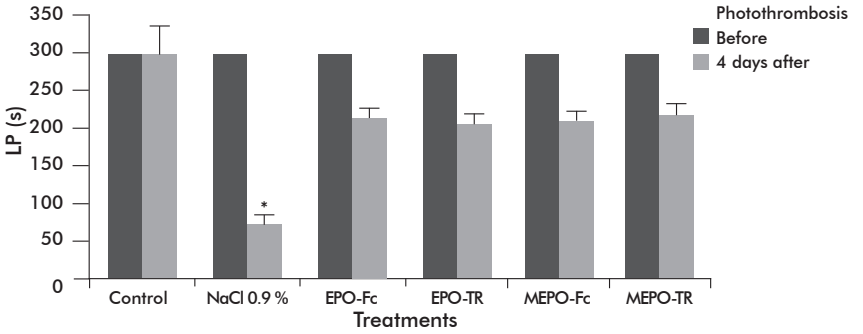

Figure 1. Effect of intranasal administration of carbamylated erythropoietin derivatives and their mutant variants forms on the conditioned passive avoidance reflex (PA) in rats with bilateral photothrombosis in the prefrontal cortex. Recombinant fusion EPO derivatives EPO-Fc and EPO-TR and their respective 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 photothrombosis ($p < 0.05$; Wilcoxon test).

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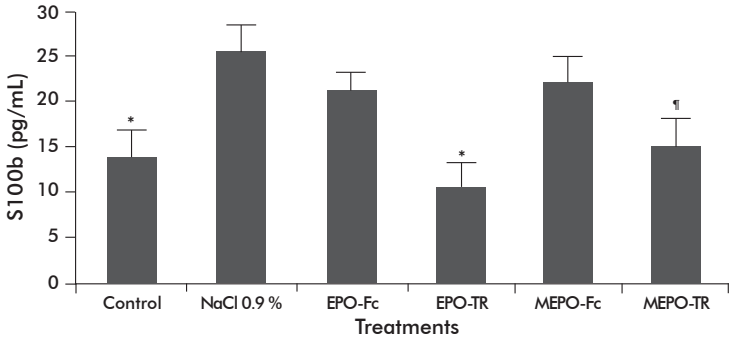

Figure 2. Influence of intranasal administration of erythropoietin carbamylated derivatives and their mutant variants forms EPO-Fc, EPO-TR and MEPO-TR and MEPO-Fc, respectively, on the amount of S100b protein in the serum of rats with bilateral photothrombosis in the prefrontal cortex. Evaluations were 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.

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Conflicts of interest statement

The authors declare that they have no conflicts of interest.

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