



Research Paper

Neuroprotective effect of a new variant of Epo nonhematopoietic against oxidative stress



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ARTICLE INFO

Keywords:

Erythropoietin
Erythropoietin receptor
Neuroprotection
Oxidative stress

ABSTRACT

Human erythropoietin is mainly recognized for its hematopoietic function; however, by binding to its receptor (EpoR), it can activate different signaling pathways as STAT, PI3K, MAPK and RAS to increase cellular differentiation or provide neuroprotective effects, among others. A recombinant human erythropoietin variant with low glycosylation and without hematopoietic effect (EpoL) was purified from skimmed goat milk. Recombinant human erythropoietin (Epo) was obtained from CHO cell line and used as control to compare EpoL effects. Neuroprotection studies were performed in PC12 cells and rat hippocampal slices. Cells were pretreated during 1 h with EpoL or Epo and exposed to oxidative agents (H₂O₂ or FCCP); cell viability was assayed at the end of the experiment by the MTT method. Hippocampal slices were exposed to 15 min of oxygen and glucose deprivation (OGD) and the neuroprotective drugs EpoL or Epo were incubated for 2 h post-OGD in re-oxygenated medium. Cell cultures stressed with oxidative agents, and pretreated with EpoL, showed neuroprotective effects of 30% at a concentration 10 times lower than that of Epo. Moreover, similar differences were observed in OGD *ex vivo* assays. Neuroprotection elicited by EpoL was lost when an antibody against EpoR was present, indicating that its effect is EpoR-dependent. In conclusion, our results suggest that EpoL has a more potent neuroprotective profile than Epo against oxidative stress, mediated by activation of EpoR, thus EpoL represents an important target to develop a potential biopharmaceutical to treat different central nervous system pathologies related to oxidative stress such as stroke or neurodegenerative diseases.

1. Introduction

Erythropoietin (Epo) is a glycoprotein hormone belonging to the superfamily of cytokines type I, and is mainly responsible for proliferation, differentiation and maturation of erythroid cells, in both embryonic and adult stages [1–3]. This glyco-hormone is synthesized mostly in the kidney by peritubular capillary interstitial cells in response to changes in tissue oxygen tension, but it is also produced by non-hematopoietic tissues such as liver, retina and brain [1,4–6]. Epo has an extensive N- and O-glycosylation pattern, which regulates its pharmacokinetic and pharmacodynamics properties controlling the interaction to its receptor (EpoR), and modulating its biological activity [7–12].

The interaction of Epo/EpoR activates signaling cascades that control apoptosis to decrease cell death rate in the bone marrow at the final

stages of the development of erythroid progenitor cells, especially in the erythroid colony forming unit (CFU-E) [1]. This activation induces cell proliferation and maturation from normoblasts to reticulocytes, which eliminate the nucleus and are released from the bone marrow into the circulation, where final differentiation to erythrocytes occurs [1,2,11,13].

The Epo non-canonical receptor expressed in non-erythroid tissues is structurally different from classical EpoR because it is composed by one monomer of canonical receptor and a second monomer of region “β-common to immunoglobulin, receptor” also known as CD131 (NonHem-EpoR) [14–17]. Interestingly, the erythropoietin produced in retina and brain differs from the variant produced by the peritubular cells in the kidney in the extent of sialic acid substitutions, the former is almost completely devoid of sialic acid substituents, and this variant

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<http://dx.doi.org/10.1016/j.redox.2017.09.010>

Received 31 July 2017; Received in revised form 5 September 2017; Accepted 18 September 2017

Available online 21 September 2017

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has been called neuro-EPO [18–20]. This Epo variant binding to NonHem-EpoR in non-hematopoietic tissues triggers the activation of a complex molecular network related to proliferation and apoptosis control [21,22]. Neuro-Epo also activates PI3K/AKT, a key step in the transcription of crucial anti-apoptotic proteins, such as BCL2 family; and the inhibition of apoptosis, together with other important pathways leading to cell proliferation and survival events [22–25]. Also, the neuro-EPO binding to EpoR modulates the activity of ionic channels responsible for cell volume maintenance [26] and has shown to increase neurogenesis in stroke-affected areas [27–29]. Erythropoietin's pleiotropic effects, which are confined to non-hematopoietic tissues such as brain [30–32], heart [33] and retina [34], protect against damage caused by ischemia/reperfusion events, inflammation and some neurodegenerative diseases [1,7,22].

Here we have studied the neuroprotective effect of a new variant of recombinant Epo expressed in the mammary gland tissue of non-transgenic animals, called EpoL [35], which was previously characterized with a different glycosylation pattern with a low sialylated bi-antennary structures [36]. Low sialic acid content of EpoL resembles neuro-Epo found in the central nervous system and retina [20,37–39]. Here, we confirm that the EpoL variant did not show hematopoietic activity and afforded protection in *in vitro* neuronal models and slices of hippocampus against oxidative stress through activation of its receptor and intracellular signaling pathways that includes JAK/STAT and up-regulation of BCL2 gene.

2. Materials and methods

2.1. *In silico* modelation of glycosylation and molecular docking

The models of Epo glycosylated were generated using the crystallographic structure of human Epo (PDB code: 1EER), where mutations K24N, K38N and K83N were created to restore the glycosylation sites in Epo using Modeller 9.15 [40]. The oligosaccharides tetra- and di-antennary for the Epo wild type (Epo) and L-glycosylation (EpoL), respectively, were created by SWEET2 (<http://www.glycosciences.de/modeling/sweet2/doc/index.php>). The N-linked glycosylation process for each Epo variant was performed using Maestro (Schrödinger, LLC, New York, NY, 2016), and the resulting models were minimized energetically in a conjugate gradient (Polak-Ribiere) utilizing MacroModel (Schrödinger, LLC, New York, NY, 2016). The NonHem-EpoR was created conserving one chain from the EpoR complemented with the β common receptor chain (PDB code: 2GYS). The complexes Epo-EpoR and Epo-NonHem-EpoR were generated by a rigid docking using ZDock [41], optimized in MacroModel, and finally analyzed energetically with Prime (Schrödinger, LLC, New York, NY, 2016). The interaction surface analysis was performed using InterSurfPro (<http://curie.utmb.edu/usercomplex.html>). Images and molecular surfaces were created by PyMol (version 1.5, DeLano Scientific LLC).

2.2. Epo expression and purification

Recombinant human erythropoietin was obtained from genetically transformed Chinese ovarian hamster cell line (CHO; ATCC CCL-61, USA). The supernatant culture was collected, centrifuged at 390 g for 10 min and purified by Blue-Sepharose and chelating affinity chromatography column. Epo samples were quantified by commercial human EPO ELISA kit (R & D, USA) and stored at -80°C .

2.3. Goat mammary gland adenoviral mediated Epo expression

Three nulliparous Saanen goats (*Capra hircus*) of around 1,8 years old were used during the first month of natural lactation. The expression of recombinant human erythropoietin was carried out by the recombinant adenoviral infusion method described elsewhere [35]. Milk collection started 48 h after adenoviral vector inoculation and was

extended for 12 days.

2.4. EpoL purification from milk

The recombinant human Epo was purified from skimmed goat milk corresponding to the milking days 2–6 in the mammary glands infused with the AdhEPO vector, as was previously described [35]. Milk was diluted five-fold in 100 mM EDTA containing 150 mM NaCl and the pH of samples was adjusted to 4.0 by adding hydrochloric acid while stirring at 4°C . The insoluble fraction was removed by centrifugation and the supernatant was precipitated with 30% ammonium sulfate for 1 h at 4°C . The supernatant was dialyzed overnight at 4°C using 20 mM Tris-HCl, 1% Tween 20 at pH 7.4 and loaded into a Blue-Sepharose and chelating affinity chromatography column. EpoL was quantified by commercial human EPO ELISA kit (R & D, USA) and stored at -80°C .

2.5. *In vivo* hematopoietic activity

Time-course of hematopoietic activity was assessed by three injections of 88 $\mu\text{g}/\text{kg}$ of the recombinant glycoproteins (Epo and EpoL) in C57BL/6 normocytic mice of seven weeks-old and weighing approximately 31 g each, according to the method previously described [42]. After injections, blood samples (60 μL approx) were collected via retro-orbital route on days 0, 5, 10, 15 and 20, and the increase was measured as percentage of total hematocrit. The hematopoietic activity was expressed as the differential increment in mouse hematocrit compared to negative control group, and data were analyzed by two-way ANOVA and Sidak's test.

2.6. PC-12 cell line

(ATCC CRL-1721, USA): Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Hyclone, USA) with 5% fetal bovine serum, 5% horse serum, 100 U/mL penicillin, 100 g/mL streptomycin, and 2 mM L-glutamine and were incubated under standard conditions (37°C , 5% CO_2).

2.7. Epo pre-treatments assay

PC-12 cells were pretreated with Epo or EpoL for 1 h and then stressed with carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP, a decoupling agent of the respiratory chain of electrons, which induces oxidative stress) (60 μM) or H_2O_2 (80 μM) as a model of oxidative stress for 45 min, using the same medium with Epo or EpoL. After that, the percentage of live cells was quantified.

2.8. Cell viability assays

Cell cultures were seeded at a density of 90,000 cells /well and used 24 h after plating. After exposing the cells to each experimental condition they were incubated with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (1 mg/mL) for 30 min, and, thereafter, precipitated MTT was dissolved using isopropanol cooled for 15 min. The tetrazolium ring of MTT can be cleaved by active dehydrogenases in order to produce a precipitated formazan compounds. Absorbance was measured in a multiplate reader (NovoStar, LabTech BMG, Germany) at two wavelengths: 560 nm and 620 nm, and the difference was quantified using NovoStar Software for the different experimental conditions.

2.9. Western blot

Protein lysates from PC-12 cells were separated by 10% acrylamide gel electrophoresis (100 V, 100 mins) and transferred to nitrocellulose membrane, which were later blocked with 5% milk in Tris saline buffer and 0,1% Tween 20 (TBS). The primary antibodies used were: anti-

EpoR (mouse, 1:500, Santa Cruz Biotechnology); anti-BCL-2 (mouse, 1:200, Sigma-Aldrich); and anti- α tubulin (mouse, 1:3000, Santa Cruz Biotechnology). Anti-mouse-HRP (1:5000, Santa Cruz Biotechnology) was used as secondary antibody, and the immunoreactive signals were visualized and quantified by a fluorescence detection system Odyssey FC (LI-COR, USA).

2.10. Real time PCR

Total RNA was purified using TRIZOL (Sigma, USA), and the reaction was performed with the commercial kit KAPASYBR FAST qPCR (KapaBiosystems, USA) and the equipment for Stratagene MX3000P (ThermoFisher, USA) real-time PCR. The qPCR was performed using RNA as template, the primers were ordered from Integrated DNA Technologies (Coralville, USA): BCL2 (Forward: GATGACTGAGTACCTGAACCG, Reverse: CAGAGACAGCCAGGAGAAATC) and β -actin (Forward: CACTTTCTACAATGAGCTGCG, Reverse: CTGGATGGCTACGTACATGG). The comparative threshold cycles values were normalized for β -actin reference gene and the results were expressed as CT relative quantification by $2^{-\Delta\Delta CT}$ method [43].

2.11. Preparation of rat hippocampal slices

Adult male Sprague-Dawley rats (275–325 g) were used to prepare hippocampal slices. Rats were decapitated under sodium pentobarbital anesthesia (60 mg/kg, i.p.); brains were removed from the skull and placed into ice-cold Krebs bicarbonate dissection buffer (pH 7.4, containing (in mM): NaCl 120, KCl, CaCl₂ 0.5, NaHCO₃ 26, MgSO₄ 10, KH₂PO₄ 1.18, glucose 11 and sucrose 200). The chamber solutions were pre-bubbled with either 95% O₂/5% CO₂ gas mixture, for 45 min before slice immersion to ensure O₂ saturation. The hippocampi were dissected and slices (300 μ m) using a McIlwain Tissue Chopper (Campden Instruments, UK) and transferred to vials of sucrose-free dissection buffer, bubbled with 95% O₂/5% CO₂ in a water bath for 45 min at 34 °C to allow tissue recovery from slicing trauma before starting the experiments (equilibration period). Slices pertaining to basal group were incubated throughout the experiment with Krebs solution (pH 7.4, containing (in mM): NaCl 120, KCl 2, CaCl₂ 2, NaHCO₃ 26, MgSO₄ 1.19, KH₂PO₄ 1.18 and glucose 11; and equilibrated with 95% O₂/5% CO₂). Slices belonging to the control OGD group were exposed for 15 min to oxygen and glucose deprivation (OGD) solution, which was previously, equilibrated with a gas mixture containing 95% N₂/5% CO₂ and 2-deoxyglucose instead of glucose. After this OGD period, slices were returned back to an oxygenated normal Krebs solution containing glucose for 2 h (re-oxygenation period). Slice groups treated with different concentrations of Epo, EpoL or EpoL+Ab (1:200) were exposed for 15 min to OGD and thereafter; the compounds were added to the slices only during the 2 h re-oxygenation period

2.12. Determination of cell survival by MTT assay on slices

Hippocampal slices were collected immediately after the re-oxygenation period and were incubated with MTT (0.5 mg/mL) in Krebs bicarbonate solution at 37 °C for 30 mins. The formazan products generated in the hippocampal slices were solubilized adding 200 μ L dimethyl sulfoxide (DMSO), and were thereafter absorbance was measured in a microplate reader at 540/670 nm (Thermo iEMS reader MF, USA). Absorbance values obtained in basal slices were considered as 100% of viability.

2.13. Measurement of cell death by Propidium Iodide/Hoechst double staining

At the end of the experiment, the hippocampal slices were loaded with 1 μ g/mL Propidium Iodide (PI) and Hoechst during the last 5 min of incubation. Mean PI/Hoechst fluorescence in CA1 region in

each slice, after treatment, was analyzed in a fluorescence inverted NIKON eclipse T2000-U microscope (USA). Wavelengths of excitation and emission for PI/Hoechst were 530/350 or 580/460 nm, respectively. Images were taken at CA1 region using magnifications of 100 \times , and the fluorescence analysis was performed using ImageJ software. The cell death was calculated as mean PI/Hoechst fluorescence. Data were normalized with respect to basal values, which were considered as 100%.

2.14. Statistical analysis

Data in graphics is expressed as mean \pm SEM. Statistical analysis of the results was performed using one-way ANOVA with Tukey test or Kruskal-Wallis and Dunns test, as appropriate. It was considered statistically significant results: *p < 0,05, **p < 0,01, ***p < 0001, ****p < 0,0001 versus stressed cells (FCCP or H₂O₂) or OGD slices; +p < 0,05, ++p < 0,01 and +++p < 0001, ++++p < 0,0001 versus control group; #p < 0,05, ##p < 0001, ###p < 0001, ####p < 0,0001 versus treated cells (+Epo, +EpoL or +Inhibitor).

3. Results

3.1. The EpoL variant did not show in vivo hematopoietic activity

The in vivo hematopoietic activity of EpoL was compared with that of recombinant human Epo in normocytic C57BL/6 mice at different times for 3 weeks, at days 0, 5, 10, 15 and 20, as scheme shown in Fig. 1. Significant differences were observed in the time-course of hematocrit percentage from animals treated with both Epo variant (Fig. 1). This result confirms that EpoL variant does not show hematopoietic activity, respect to control rates (6,4% \pm 0,82; n = 10); according to previous results [35].

3.2. Neuroprotective effect of EpoL on PC12 cells treated with FCCP depends on the activation of its receptor

PC12 cells were used as neuronal model [24,44]. PC12 cells exposed to the mitochondrial un-coupler FCCP (60 μ M) for 3 h reduced their viability to 57,4% (\pm 4,5), and we used this in vitro model as slight oxidative stress assay. Under these experimental conditions, cells were

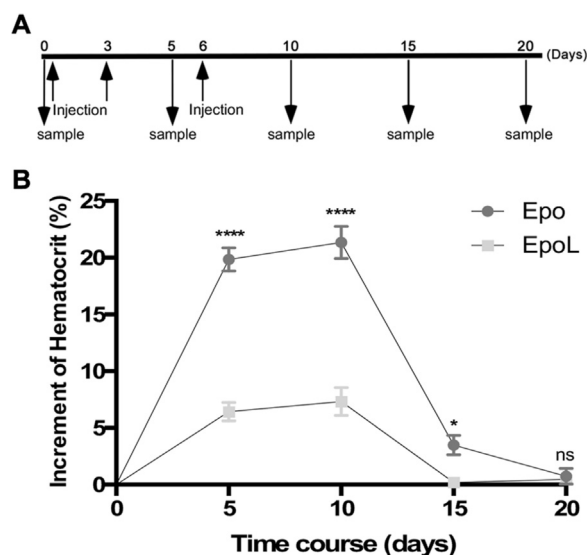


Fig. 1. *In vivo* Hematopoietic activity assay. Epo and EpoL were administered in C57BL/6 normocytic mice (88 μ g/kg). (A) Experimental design timeline. (B) Time course of hematocrit percent for each treatment respect to t = 0. Values are mean \pm SEM of increment of hematocrit. n = 10, two-way ANOVA and Sidak test, *: p < 0,05; ***: p < 0001; *****: p < 0,0001 versus EpoL treatment.

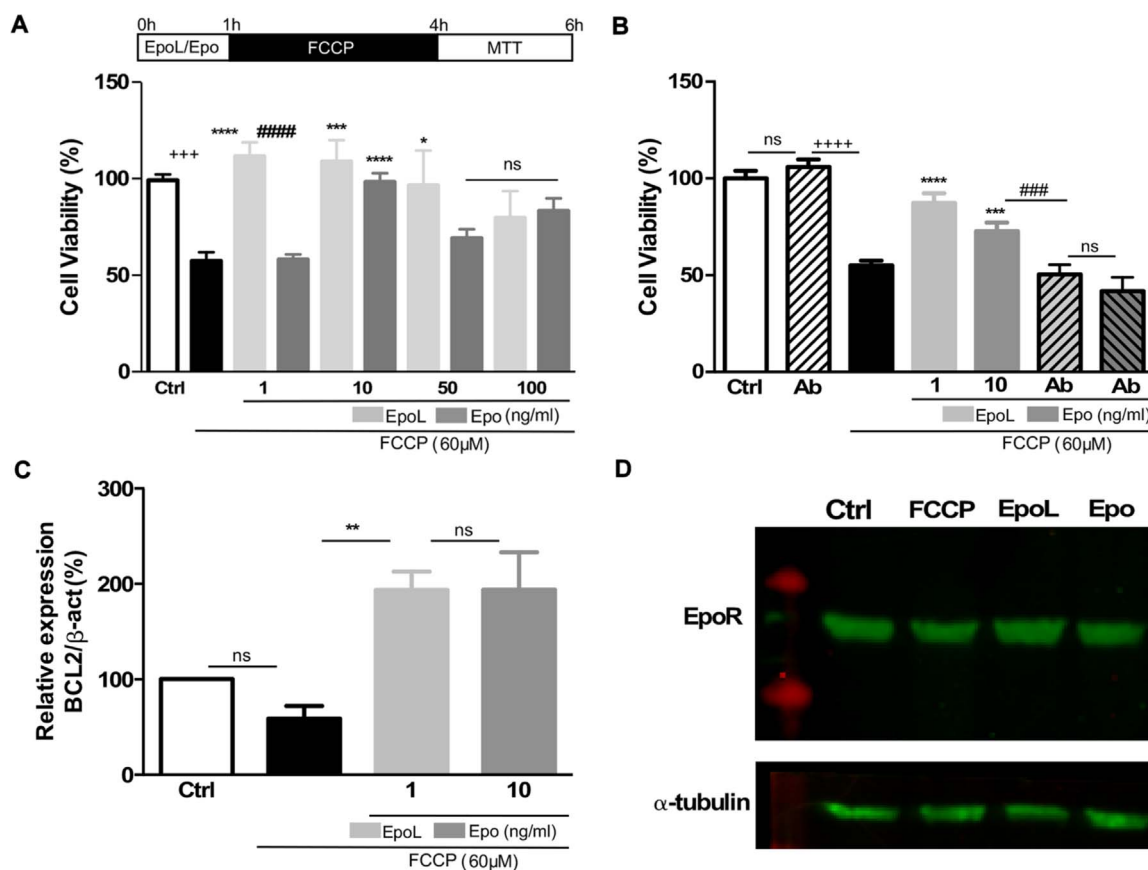


Fig. 2. Neuroprotective effect of EpoL on PC12 cells treated with FCCP depends on the activation of its receptor. (A) Viability assay in PC12 cells incubated with Epo or EpoL at different concentrations for 1 h, and treated with FCCP for 3 h. The percentage of viability response was evaluated by MTT. (B) The same experiment, which included the co-incubation with a specific antibody against Epo-R (1:200). (C) Evaluation of the relative expression of BCL2 gene using β-actin as a housekeeping gene after 3 h of treatment with FCCP and co-treatment with Epo or EpoL, at the most efficient concentration observed to each one (D). Evaluation of EpoR expression by western blot at the same conditions of C. Values are mean ± SEM. n = 5, using one-way ANOVA and Dunns test. *: p < 0,05; **: p < 0,01; ***: p < 0001;****: p < 0,0001 versus PC12 cells treated with FCCP; + + + +: p < 0,0001; + + +: p < 0001 versus control cells, #: p < 0,05; ##: p < 0,01; ###: p < 0001; ####: p < 0,0001 versus Epo or EpoL treatment.

pre-treated with EpoL or Epo at increasing concentrations (1, 10, 50, 100 ng/mL) for 1 h prior to the cytotoxic stimuli to determine the minimal effective concentration of both variants. EpoL variant showed a significant neuroprotective effect of 54% (± 2,8) at a minimal concentration of 1 ng/mL, while Epo generated a similar effect (40,91% ± 0,0) with a concentration 10 times greater (10 ng/mL). Also, at concentrations of 50 ng/mL of EpoL, maximal protective effects

were observed (Fig. 2A). To analyze the relation between neuroprotection and EpoR activation, PC12 cells were incubated with a specific-EpoR antibody (1: 200 dilution) under the same assay conditions, using the minimum effective concentration of EpoL (1 ng/mL) and Epo (10 ng/mL) in pre-treatment. The protective effect mediated by Epo variants was blocked in the presence of a specific antibody anti-EpoR (Fig. 2B). These results suggest that the protective effects exerted by the

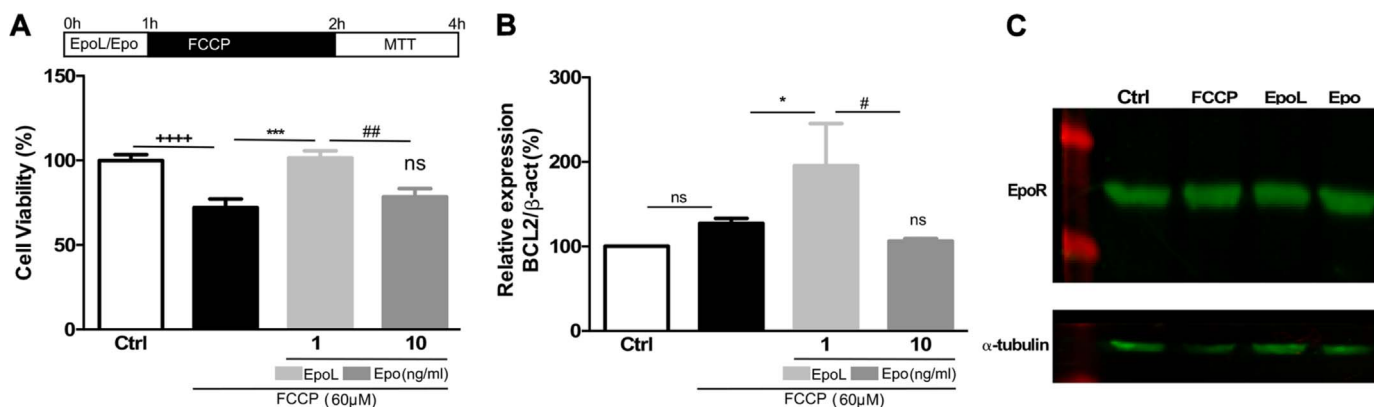


Fig. 3. Neuroprotective effect of EpoL on PC12 cells treated for 1 h with FCCP: (A) Cell viability assay in PC12 cells incubated with Epo or EpoL at the neuroprotective concentration previously established and PC12 were treated with FCCP for 1 h. (B) Evaluation of relative expression of BCL2 gene observed at the same treatments as in A (C). Evaluation of protein expression for EpoR at the same conditions used as in A. Values are mean ± SEM. n = 5, using one-way ANOVA and Dunns test *: p < 0,05; **: p < 0,01; ***: p < 0001;****: p < 0,0001 versus PC12 cells treated with FCCP; + + + +: p < 0,0001; + + +: p < 0001 versus control cells, #: p < 0,05; ##: p < 0,01; ###: p < 0001; ####: p < 0,0001 versus Epo or EpoL treatment.

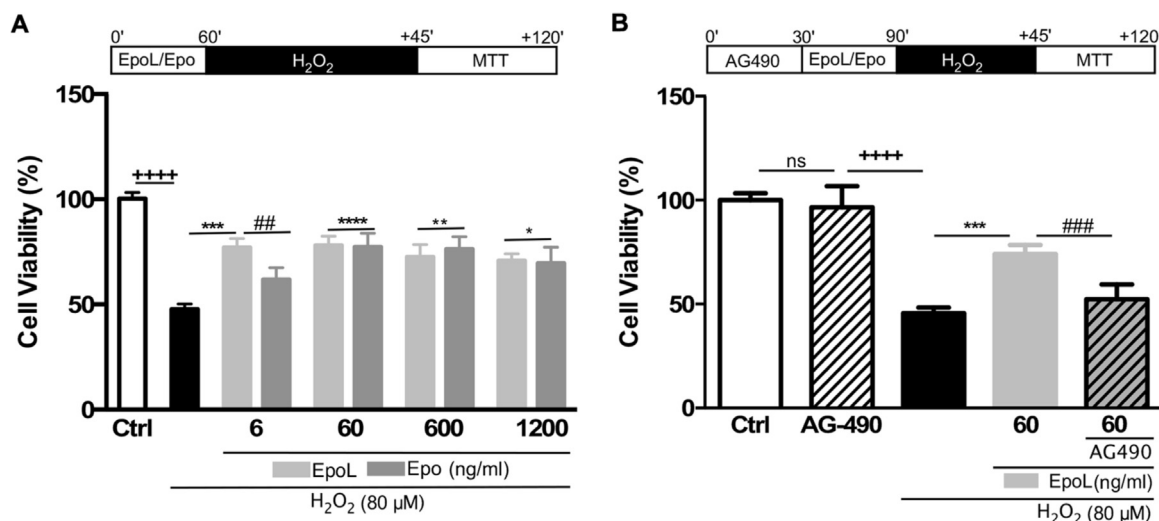


Fig. 4. Neuroprotective effect of EpoL on PC12 cells treated with H₂O₂ depends on the activation of its receptor: (A) PC12 cells were incubated with Epo or EpoL at different concentrations for 1 h and then were treated with H₂O₂ for 45 mins. (B) Cells treated using the most effective concentration of EpoL and the inhibitor to JAK/STAT pathway. Values are mean ± SEM. n = 3, using one-way ANOVA and Dunns test *: p < 0,05 versus PC12 cells treated with H₂O₂; + + + +: p < 0,0001; + + +: p < 0001 versus control cells, #: p < 0,05; ##: p < 0,01; ###: p < 0001; ####: p < 0,0001 versus Epo, EpoL or AG490 treatment.

Epo variants could be mediated by activation of the same receptor.

Additionally, we assessed the activation of antiapoptotic signaling pathways associated with EpoR under the same treatment conditions described above. The neuroprotective effect against FCCP as oxidative stress model, was corroborated using the lowest effective concentrations previously obtained for each variant of erythropoietin. Both glyco hormones induced a significant increase in BCL2 gene transcript expression, a well-known component of anti-apoptotic signaling pathways. Furthermore, BCL2 expression levels correlated with the protective effect observed under the same treatment conditions (Fig. 2C). However, no changes in EpoR expression were observed in the same treatment conditions (Fig. 2D). Also, under pre-treatment conditions and acute stress with FCCP for 1 h, statistically significant differences between the neuroprotective effects of both variants were observed. Specifically, EpoL, and not Epo, induced a protective effect of 29,47% (± 1) at 10 times lower concentration on PC12 cells, against the oxidative agent (Fig. 3A). This result was correlated with the overexpression of BCL2 transcript observed only in treatments with EpoL (Fig. 3B). However, no differences in EpoR expression were observed using the same treatment conditions (Fig. 3C).

3.3. Neuroprotective effect of EpoL on PC12 cells treated with H₂O₂ depends on the activation of EpoR

When hydrogen peroxide treatment for 45 min (80 μM) in PC12 cells was used, a reduction of viability on 52,71% (± 0,07) was observed. Using these experimental conditions, the minimum concentration of Epo and EpoL required to achieve neuroprotection against a strong oxidative stress condition was determined by 1 h pre-treatment of increasing concentrations (6, 60, 600, 1200 ng/mL) with both Epo variants. The low glycosylation variant showed a significant neuroprotective effect of 24,5% (± 2,3) at a concentration of 6 ng/mL. This value was 10 times lower than that required by Epo (60 ng/mL) to obtain the same neuroprotective effect (Fig. 4A). To assess whether this effect was related to the activation of signaling pathways downstream of the hormone receptor activation, we replicated the same experiment with the lowest effective concentration observed with EpoL in the presence of an inhibitor of JAK/STAT pathway (AG490; 0,5 μM) [24,45]. The results showed that the protective effect induced by EpoL was abolished in the presence of the JAK/STAT inhibitor (Fig. 4B).

3.4. Neuroprotective effect of post-treatments using EpoL on PC12 cells pretreated with H₂O₂

To determinate the protective effect on PC12 cells after an event of oxidative stress, we treated the cells with H₂O₂ (80 μM) for 45 min, and thereafter we incubated EpoL (60 ng/mL) at different time points (0, 15, 30 or 60 min) (Fig. 5A). When comparing the percentage of living cells after oxidative stress versus cells with post-treatment, we observed that cells treated with EpoL for 15, 30 or 60 mins after the oxidative stress, maintained a constant percentage of live cells respect to the dead cells (Fig. 5B). This percentage was calculated as the difference of treated cells, and we called it Ct = % of living cells (untreated/ treated with EpoL) for each point of treatment. All Ct observed had a similar value, with an average of 1.27 (SD ± 0,2), suggesting that although EpoL is applied 15 or 60 mins after the oxidative stress event, the percentage of protected cells remained constant (Fig. 5C).

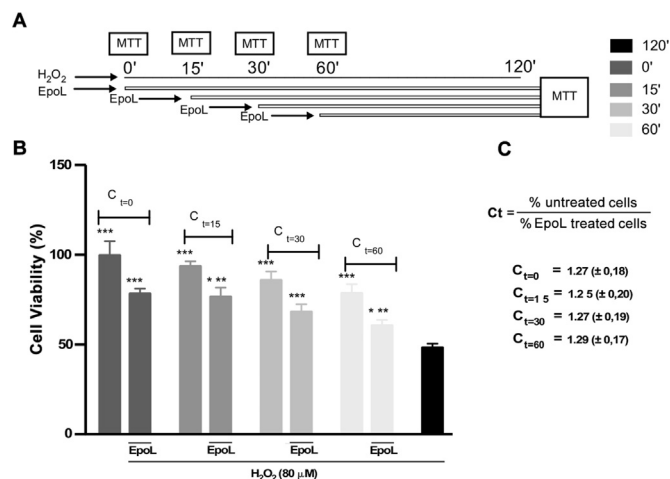


Fig. 5. Neuroprotective effect of post-treatments using EpoL on PC12 cells against H₂O₂. (A) PC12 cells were treated with H₂O₂ (80 μM), and then treated with EpoL (0,06 μg/mL) at different times, as the scheme shown. (B) Then we evaluate the neuroprotective effect of post-treatment with EpoL and we compare with H₂O₂ treated cells. (C) We observed a constant difference between EpoL treated cells and H₂O₂ treated group, independent of post-incubation time. Values are mean ± SEM. n = 3, using one-way ANOVA and Dunns test ***: p < 0001 versus cells treated with H₂O₂.

3.5. *In silico* interaction of human Epo glyco-variants with Epo-receptors

To explore the relation between the neuroprotective function of Epo variants and EpoR activation, were generated two N-glycosylated Epo models that contain: a tetra (Epo)- and bi-antennary (EpoL) glycosylation profiles. The presence of the oligosaccharides did not compromise the

binding region for the EpoR predicted by docking, which is conserved with the Epo-EpoR structure (Fig. 6A, left) [46]. Meanwhile, docking predictions using a model of the nonHem-EpoR, that contains the β common receptor subunit [15,17] and glycosylated Epo variants showed a binding site allowing the generation of a stable complex with both Epo variants (Fig. 6A, right).

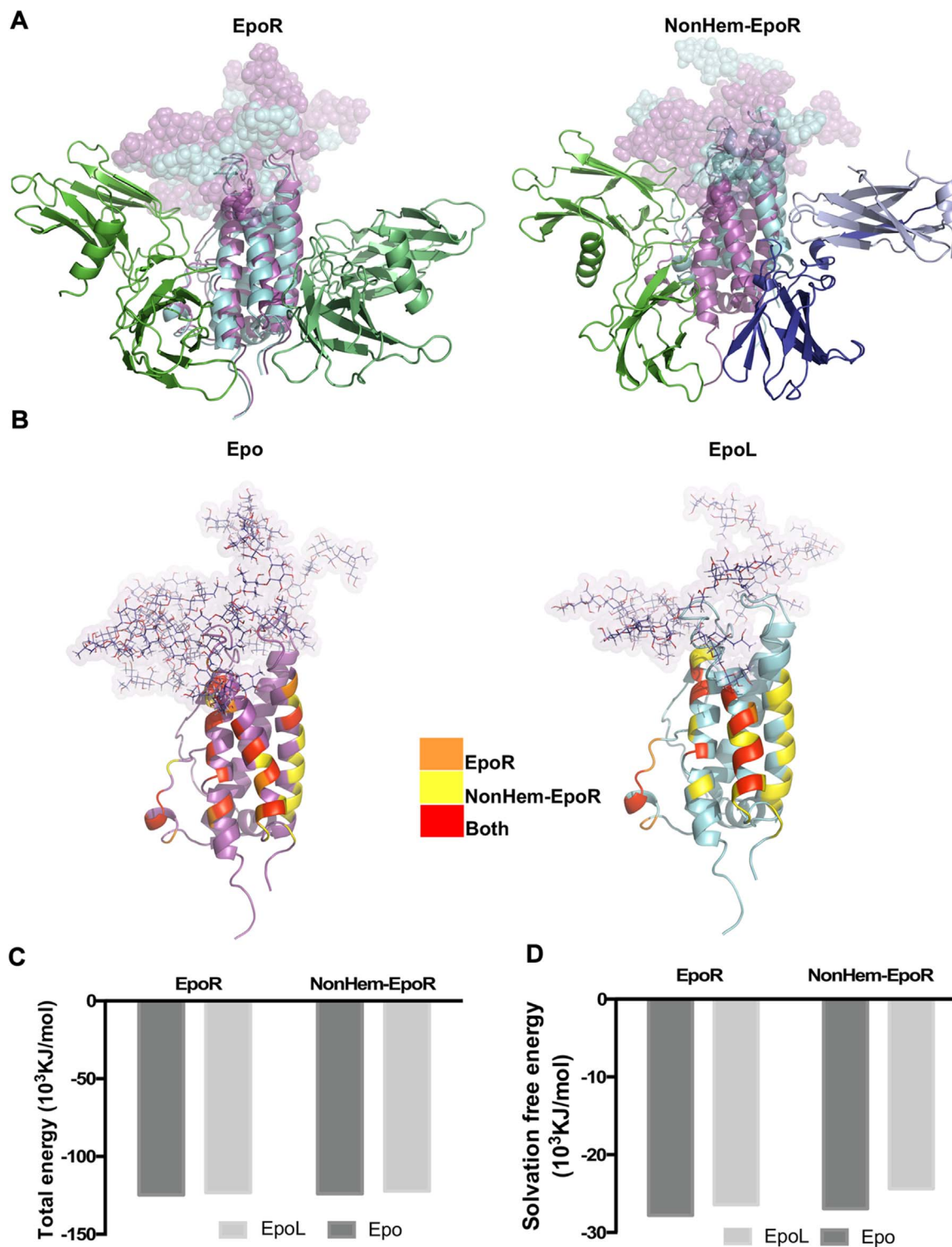


Fig. 6. *In silico* interaction between erythropoietin and its receptor in different states of glycosylation: (A) Models of Epo (magenta) and EpoL (cyan) variants generated using the structure of the Epo (PDB: 1EER) forming a complex with EpoR (right panel, both subunits are colored green) and NonHem-EpoR (left panel, chain A and B of β common receptor are colored in blue tones), respectively. (B) Amino acids important on Epo variant, to form the complex with both conformations of the receptors. For each Epo variant the amino acids detected in the interface with EpoR or NonHem-EpoR are highlighted in orange or yellow, while the repeated residues between the two receptors are colored in red. (C, D) The graphs summarize the total energy and Solvation free energy calculated from complexes of erythropoietin variants and EpoR or NonHem-EpoR.

Table 1

Energetic characterization of glycosylated erythropoietin and the complexes formed by the interactions with their respective receptors (EpoR and NonHem-EpoR) obtained using Prime.

	Total energy (kJ/mol)	Stretch (kJ/mol)	Bend (kJ/mol)	Torsion (kJ/mol)	Improper torsión (kJ/mol)	VdW (kJ/mol)	Electrostatic (kJ/mol)	Solvation (kJ/mol)
Epo	- 37,674.4	703.2	2629.9	8062.8	54.1	- 1637.3	- 36,047.7	- 11,439.4
Epo L	- 36,402.5	514.4	1824.3	4805.5	52.9	- 1857.3	- 31,488.1	- 10,254.3
Epo -EpoR	- 124,672.5	1554.5	6125.1	13,055.1	171.3	- 7585.4	- 110,209.7	- 27,783.4
Epo L -EpoR	- 123,130.2	1381.0	5250.5	9952.3	177.5	- 7556.0	- 105,918.0	- 26,417.5
Epo - NonHem-EpoR	- 123,991.2	1553.2	6078.2	12,594.6	177.2	- 7521.1	- 109,934.7	- 26,938.5
Epo L - NonHem-EpoR	- 122,187.7	1379.6	5248.7	9392.5	175.4	- 7668.4	- 106,345.6	- 24,369.9

A more detailed analysis shows that the amino acids involved in the complexes formed between two glycosylated variants of the hormone, and both conformations of the receptor, define a conserved region of interaction independent of glycosylations or receptor type (Fig. 6B). The complex Epo-NonHem-EpoR presents similar total energy compared to that containing the EpoR ($- 122.2 \times 10^3$ kJ/mol EpoL-NonHem-EpoR, $- 124.0 \times 10^3$ kJ/mol Epo-NonHem-EpoR versus $- 123.1 \times 10^3$ kJ/mol EpoL-EpoR, $- 124.7 \times 10^3$ kJ/mol Epo-EpoR), confirming that the structure of the NonHem-EpoR is capable of stably interacting with Epo and EpoL (Fig. 6C). Also, both receptors have similar solvation energy values ($- 24.4 \times 10^3$ kJ/mol EpoL-NoHem-EpoR, $- 26.9 \times 10^3$ kJ/mol Epo-NonHem-EpoR versus $- 26.4 \times 10^3$ kJ/mol EpoL-EpoR, $- 27.8 \times 10^3$ kJ/mol Epo-EpoR) (Fig. 6D), related with favorable interaction between ligand-receptor. In addition to the results described above, these findings indicate that the non-hematopoietic receptor would present an activation mechanism similar to that found for the classic hematopoietic receptor of Epo. The details of energies calculated are shown in Table 1.

3.6. Neuroprotective effect of EpoL on hippocampal slices depends on the activation of its receptor

According with the *in silico* analysis, where Epo and EpoL can form a stable complex with NonHemEpoR and EpoR, the neuroprotective effect of EpoL was evaluated using an *ex vivo* model of CNS, i.e., rat hippocampal slices. Slices were stressed with oxygen and glucose deprivation (OGD) for 15 min with a subsequent re-oxygenation for 2 h. To determine the minimum effective neuroprotective concentration of

EpoL or Epo, these drugs were incubated during the re-oxygenation period. The EpoL variant had a significant neuroprotective effect at a concentration of 50 ng/mL, whereas Epo did not show the same effect at this concentration. Moreover, maximum protection for EpoL was achieved at 100 ng/mL, a concentration 10 times lower than that required with the Epo variant (1 μ g/mL) to achieve maximum protection (Fig. 7A). In addition, this effect was directly related to the interaction of EpoL and EpoR, because inhibiting receptor activation with an EpoR-specific binding antibody or using an inhibitor of the JAK/STAT signaling pathway, the neuroprotective effect observed is lost for both isoform (Fig. 7B).

An increase in oxidative stress has been reported in this model of hippocampal slices exposed to OGD [47,48]. Therefore, we evaluated oxidative stress variations and cell death in the hippocampal CA1 region by the fluorescent DCFDA probe or propidium iodide, respectively (Fig. 8A and C). The results obtained showed that, unlike Epo, EpoL treatment during re-oxygenation caused a significantly decreased in ROS production represented as a reduction of DCFDA fluorescence (Fig. 8B). Also, the percentage of dead cells observed by PI signal (Fig. 8D) with EpoL treatment diminished; these results correlated with the increase in viability observed with the MTT measurements. Moreover, the fluorescence intensity (both DCFDA and PI) increased significantly when hippocampal slices were incubated with an EpoR specific antibody, which indicates that the neuroprotective effect of EpoL in hippocampal slices is lost when EpoR activation is blocked. EpoL presents structural characteristics similar to, human Epo present in the central nervous system (neuro-Epo), as we expected.

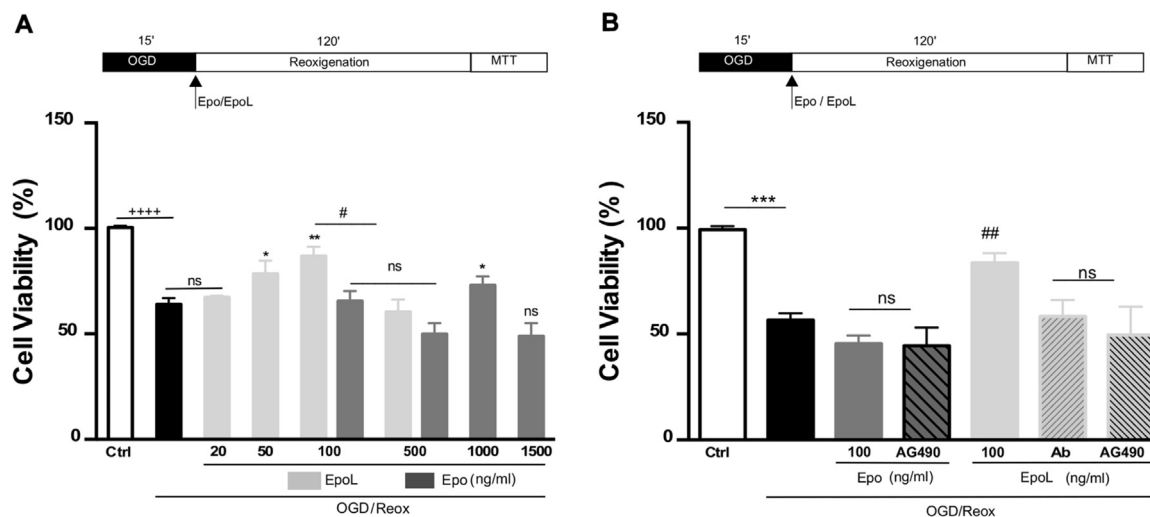


Fig. 7. Neuroprotective effect of EpoL on hippocampal slices depends on the activation of its receptor: (A) Hippocampal slices of rat were incubated with Epo or EpoL at different concentrations during the reoxygenation time to determinate the most efficient concentration of EpoL or Epo, using MTT assay. (B) Evaluation of Epo/EpoR interaction and the neuroprotector effect using a specific antibody against EpoR (Ab), or JAK/STAT inhibitor (AG490). Values are mean \pm SEM. n = 5, using one-way ANOVA and Dunns test *: p < 0,05 versus hippocampal slices with OGD. n = 5, + + + + : p < ,0001; + + + : p < 0001 versus control slices, #: p < 0,05; ##: p < 0,01; ###: p < 0001; ####: p < 0,0001 versus Epo, EpoL, Ab or AG490 treatment.

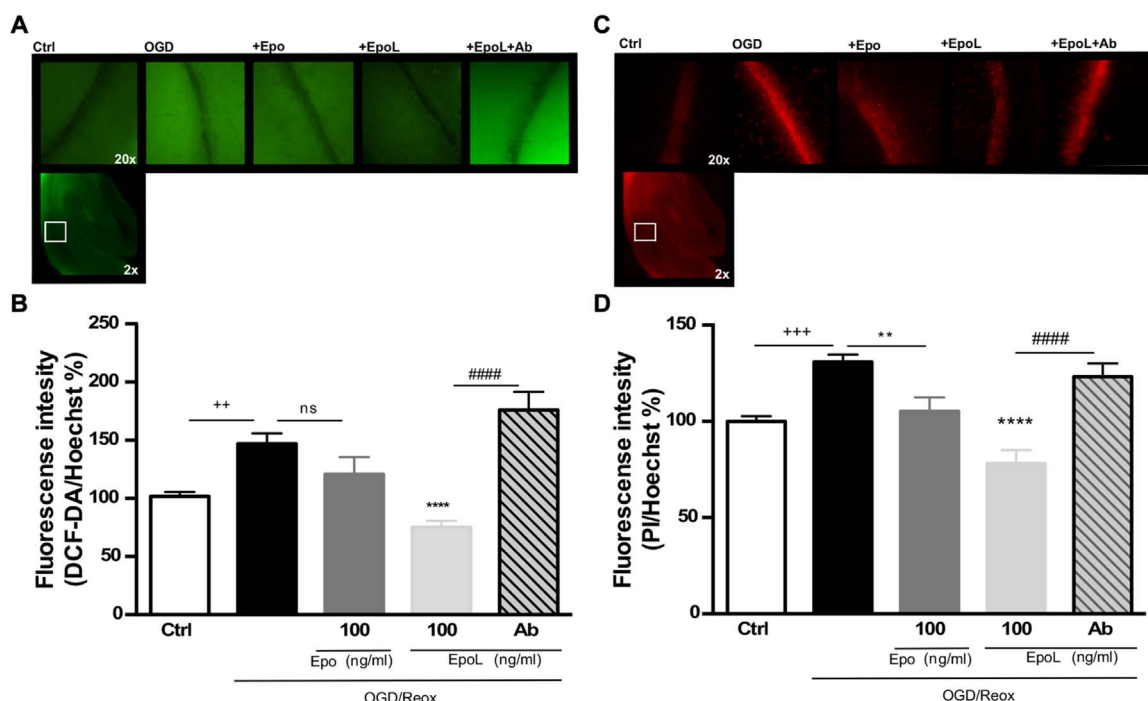


Fig. 8. Neuroprotective effect of EpoL on hippocampal slices is related to decrease ROS: (A and C) Fluorescence images of rat hippocampal slices incubated with Epo or EpoL post OGD treatment and incubated with DCFDA dye (green) and PI stain (red). (B) Evaluation of oxidative stress in the CA1 area using (DCFDA/Hoechst) signal, analyzed by Image J software. (D) Quantification of [dead cells/total cells] using (PI/Hoescht) staining, and analyzed by Image J software. Values are mean \pm SEM. $n = 7$, using one-way ANOVA and Dunns test *: $p < 0,05$ versus hippocampal slices treated with OGD. +: $p < 0,05$ versus EpoL treatment.

4. Discussion

The production method for erythropoietin used in this work gives rise to a new variant of Epo with a complex glycosylation pattern, which is characterized mostly by neutral biantennary structures (EpoL). This characteristic provides a new Epo with decreased *in vivo* hematopoietic activity; this loss of activity was previously described [35,36,42] and, confirmed in this study. The aforementioned characteristics have a direct relation with glycoprotein half-life. Human urinary Epo has a treta-antennary, fucosylated and treta-sililated glycosilation pattern, which increases the molecular size, lowering its glomerular filtration and prevents its recognition by the liver pentose receptors [49,50]. Therefore, in the EpoL molecule, with low terminal sialic acid, we must expect a shorter half-life, which would explain why it lacks hematopoietic activity [20,50,51]. In spite of this, both molecules, EpoL as well as Epo, interact with EpoR and activate the JAK/STAT signaling pathway, inducing an up-regulation of the antiapoptotic gene BCL2, a gene that is associated to EpoR activation (Fig. 2). Thus, EpoL demonstrates a potential as a neuroprotective agent as previously reported for recombinant human Epo produced by CHO cells and hematopoietically active [24,52,53].

Our results showed that EpoL presents higher neuroprotective effect against oxidative stress induced by FCCP than Epo, when equal concentration was used for both proteins (10 ng/mL). Moreover, this effect of EpoL was observed earlier than Epo, in the same conditions, with over-expression of antiapoptotic genes as BCL2 (Fig. 3). The activation of this antiapoptotic pathway has been reported for Epo [54,55], but not the early effect that we observed in EpoL treatment. Nevertheless, we have not detected a mitogenic effect, which suggests that EpoL did not activate signaling pathways that lead to a proliferative response.

Additionally, hydrogen peroxide treatment has been established as an *in vitro* model of neuronal diseases with high oxidative stress [56]. Using this model, we demonstrate that EpoL induces a neuroprotective effect 10 times more potent than Epo in PC12 cells (Fig. 4A). Also, this effect was abolished using an inhibitor of EpoR activation, which indicates that this new variant also exerts its effect through receptor

binding, as well as the Epo effect previously reported [54]. Moreover, treatments with EpoL after H_2O_2 -oxidative stress showed very similar percentage of protection, independently of the previous time of stress for the culture, maintaining an average Ct = 1.27.

In order to evaluate the interaction probabilities of EpoL variant to EpoR and to the non-hematopoietic receptor expressed in CNS [55] we performed *in silico* modeling and evaluated interaction regions between both variants and both receptors. Interaction residues reported for Epo-EpoR are conserved for EpoL-EpoR models, and corroborates our *in vitro* results, related to the abolishing of neuroprotective effects by blocking receptor/ligand interaction. Besides, EpoL residues that interact with non-EpoR are sufficient and similar to those observed for EpoR (Fig. 6B). A more detailed energetic analysis of complexes revealed that both receptors (nonHem-EpoR or Hem-EpoR) stably interact with Epo or EpoL generating complexes with similar total energy and solvation energy values (Fig. 6C and D). Taken together, these results confirm that the non-hematopoietic receptor is also capable of interacting with glycosylated Epo and would present an activation mechanism similar to that found for the classic hematopoietic receptor of Epo [54,55].

Considering *in silico* evidence of EpoL interaction to non-hematopoietic brain receptor and *in vitro* results of neuroprotection, we decided to use hippocampal slices as a *ex vivo* model of CNS to assay the neuroprotective actions of Epo and EpoL in an ischemic model induced by oxygen glucose deprivation [57]. Under experimental brain ischemic conditions, EpoL given as post-OGD treatment provided a higher neuroprotective action compared to Epo. Moreover, when the hippocampal slices were treated with the anti-EpoR monoclonal antibody, the protective effect afforded by EpoL significantly decreased.

The neuroprotective effect of both Epo and EpoL against oxidative stress is directly related to EpoR activation, through JAK/STAT phosphorylation, up-regulation of anti-apoptotic genes such as BCL2, and the decrease of ROS induced by OGD. Other authors described similar effects in anti-apoptotic signal and diminished oxidative stress, induced by different neuroprotective compounds like Curcumine or Melatonine [58,59] hat can activate different genes related to survival, such as the

nuclear factor (erythroid-derived 2)-like 2 (Nrf2). This is a member of the transcription factors group that binds to the Antioxidant Response Element (ARE) to regulate the oxidative stress response, inducing the activation of phase II genes as Hemeoxygenase-1, which is related to maintain the redox homeostasis and cell survival [58–61]. Our results showed an increase in BCL2 gene expression, and a decrease of ROS induced by EpoL against oxidative stress, suggesting that this Epo variant could activate the same pathway through Nrf2 and a group of vital genes to preserve cell survival, prevent apoptotic signals and promote the inflammation decrease [62,63].

These results strongly suggest that the different affinity between Epo and NonHem-EpoR previously described and supports the idea of neuroprotective actions afforded by EpoL are directly related to the nonHem-EpoR binding and activation of survival signaling pathways in neuronal tissues [12,50].

5. Conclusions

The new variant EpoL is devoid of hematopoietic activity, but it binds to the NonHem-EpoR present in neuronal tissues, causing activation of antiapoptotic pathways to provide neuroprotection under brain ischemia/oxidative stress related conditions. Compared to classical Epo, EpoL was more potent and faster in exerting its neuroprotective action. Taken together, our results indicate a potential use of EpoL as a biopharmaceutical to treat CNS diseases endowed with an oxidative stress component in its pathogenesis.

Acknowledgments

Grant Innova-Corfo “13IDL2-18688”; Conicyt Grant “Beca Doctorado Nacional no. 21130386”; Fondecyt Grant “1161078”.

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