EPOR/CD131-MEDIATED ATTENUATION OF ROTENONE-INDUCED RETINAL DEGENERATION IS ASSOCIATED WITH UPREGULATION OF AUTOPHAGY GENES

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Mitochondrial dysfunction is a key driver of neurodegeneration. This study aimed to evaluate the protective potential of EPOR/CD131 (heterodimeric erythropoietin receptor) stimulation in the neurodegeneration caused by rotenone-induced mitochondrial dysfunction. The effects of erythropoietin (EPO) and an EPO mimetic peptide pHBSP were assessed using *in vivo* and *in vitro* models. Single injections of 10 μg/kg EPO or 5 μg/kg pHBSP significantly alleviated the degeneration of ganglion cells of the retina in a rotenone-induced retinopathy in rats ($p < 0.05$). Consistently, in vitro exposure of rotenone-treated murine primary neuroglial cultures to 500 nM EPO or pHBSP significantly rescued the survival of the cells ($p < 0.005$). The observed enhancement of LC3A, ATG7, Beclin-1, Parkin and BNIP3 mRNA expression by EPOR/CD131 agonists implicates the autophagy and mitophagy activation as a plausible mitoprotective mechanism.

Keywords: mitochondrial dysfunction, rotenone-induced retinopathy, EPOR/CD131 receptors, erythropoietin, pHBSP

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EPOR/CD131-ОПОСРЕДОВАННАЯ РЕТИНОПРОТЕКЦИЯ ПРИ РОТЕНОН-ИНДУЦИРОВАННОЙ НЕЙРОТОКСИЧНОСТИ СВЯЗАНА С УВЕЛИЧЕНИЕМ ЭКСПРЕССИИ ГЕНОВ АУТОФАГИИ

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Митохондриальная дисфункция является ключевым драйвером развития нейродегенерации. Целью исследования было оценить протективный потенциал стимуляции EPOR/CD131, гетеродимерного рецептора эритропоэтина (EPO), при нейродегенерации, вызванной нарушением функции митохондрий. В качестве агонистов EPOR/CD131 были использованы EPO или pHBSP, эффективность которых оценивали в условиях *in vivo* и *in vitro*. В модели ротенон-индуцированной ретинопатии однократная инъекция 10 мкг/кг EPO или 5 мкг/кг pHBSP привела к значительному снижению дегенерации ганглионарных клеток сетчатки ($p < 0,05$). Кроме того, инкубация в 500 нМ растворах EPO и pHBSP резко увеличила выживаемость первичной мышиной нейроглиальной культуры, обработанной ротеноном ($p < 0,005$). Примечательно, что применение агонистов EPOR/CD131 привело к увеличению экспрессии мРНК LC3A, ATG7, Beclin-1, Паркина и BNIP3, что свидетельствует об активации аутофагии и митофагии как потенциальном механизме митопротективного действия.

Ключевые слова: митохондриальная дисфункция, ротенон-индуцированная ретинопатия, рецептор EPOR/CD131, эритропоэтин, pHBSP

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Despite the remarkable advances in our understanding of brain pathophysiology, the progress in handling neurodegenerative disorders remains modest. With the continued increase in human lifespan, the social and medical burden of neurodegenerative disorders is growing ever so fast [1], while etiotropic approaches in their management are missing and the available pathogenetic and symptomatic options are of limited efficacy with regard to outcomes. Obviously, this situation dictates the urgent need for new neuroprotection strategies. In ophthalmology, neurodegeneration is of special relevance, as many retinal abnormalities have strong neurodegenerative component [2].

Mitochondrial dysfunctions are often considered as one of the key pathophysiological links in the context of neuronal degeneration [3]. Rather than being a passive indicator, mitochondrial defects have been shown to drive neurodegeneration [4]. Considering the vital importance of the oxidative energy metabolism in nervous tissue, the maintenance of healthy mitochondrial pools is essential for its functioning. Mitophagy is the key cellular mechanism ensuring the timely elimination of defective mitochondria [5]. The term 'mitophagy', introduced by Lemasters [6], stands for selective degradation of unfit mitochondria by macroautophagy [7] involving two major pathways known as PINK1/Parkin-mediated and receptor-mediated autophagy [8].

The timely elimination of defective mitochondria alleviates the oxidative stress and boosts the energy metabolism efficiency [9]. Accordingly, mitophagy stimulation can be considered an efficient neuroprotective strategy. Moreover, the overall fitness of autophagy mechanisms prevents the overloading of neurons with misfolded proteins and counteracts the accumulation of dysfunctional bulk protein aggregates — the common pathomorphological substratum in neurodegeneration [10].

Pharmacological activation of EPOR/CD131, the heterodimeric erythropoietin (EPO) receptor, is a promising neuroprotection strategy apparently related to the enhancement of mitochondrial function and autophagy [11]. The neuroprotective capacity of EPOR/CD131 agonists [12–14] and their stimulating effect on autophagy [15] have been demonstrated previously. This study aimed to evaluate the neuroprotective capacity of EPO and its peptide analog pHBSP in experimental neuronal damage.

METHODS

Rotenone toxicity was chosen as a model background for the assessment of mitotropic effects *in vivo* and *in vitro*. Pesticide rotenone interrupts the mitochondrial respiratory chain by blocking the electron transfer complex I. The action of rotenone can induce cellular changes characteristic of mitochondrial dysfunctions and neurodegeneration [16].

Animals

The experiments involved 24 male rats purchased from 'Stolbovaya' breeding facilities (Moscow region, Russia) and 3 female CD-1 mice (purchased from Pushchino facilities; Moscow region, Russia) with 24 newborn pups. The animals were housed in rooms with artificial lighting (12/12 h mode) at 21–23 °C, 38–50% humidity and *ad libitum* access to food and water.

The rotenone-induced retinal degeneration model

To induce retinal degeneration 18 male rats (age 20 weeks, body weight 250–275 g) were intravitreally injected by 5 μL of 0.4 mM rotenone in 5% DMSO in Dulbecco's phosphate-buffered saline (D-PBS) (2 nmol/eye) under local novocainamide anesthesia [17].

Table. Primers used in qPCR target gene expression assay

Gene (encoded protein)	Primer sequence	Product length (b.p.)
Retinal degeneration marker		
Nefl (NEFL)	F: 5'-GGAGTACCAGGACCTCCTCA-3'	102
	R: 5'-CTGGTGAAACTGAGCCTGGT-3'	
Autophagy/mitophagy regulating genes		
Becn1 (Beclin 1)	F: 5'-CAGCTGGACACTCAGCTCAA-3'	99
	R: 5'-CTGTTCACTGTCGCCCTCAT-3'	
Map1lc3a (LC3A)	F: 5'-TTGGTCAAGATCATCCGGCG-3'	104
	R: 5'-TCAGCGATGGGTGTGGATAC-3'	
Atg7 (ATG7)	F: 5'-TCCTGGCCAAGGTGTTTAACT	104
	R: 5'-ACTCATGTCCCAGATCTCAGC-3'	
Prkn (Паркин)	F: 5'-TGCCCATTGAAAAGAATGGAGG-3'	95
	R: 5'-GTTCCACTCACAGCCACAGT-3'	
Bnip3 (BNIP3)	F: 5'-AACAGCACTCTGTCTGAGGA-3'	100
	R: 5'-GCCGACTTGACCAATCCCA-3'	
Inflammatory response genes		
Il1b (IL1b)	F: 5'-GGCTGACAGACCCCAAAAGA-3'	101
	R: 5'-TGTCGAGATGCTGCTGTGAG-3'	
Il6 (IL6)	F: 5'-CTCATTCTGTCTCGAGCCCAC-3'	105
	R: 5'-AGAAGGCAACTGGCTGGAAG-3'	
Housekeeping gene		
Actb (B-actin)	F: 5'-CCACCCGCGAGTACAACC-3'	95
	R: 5'-GACGACGAGCGCAGCGATA-3'	

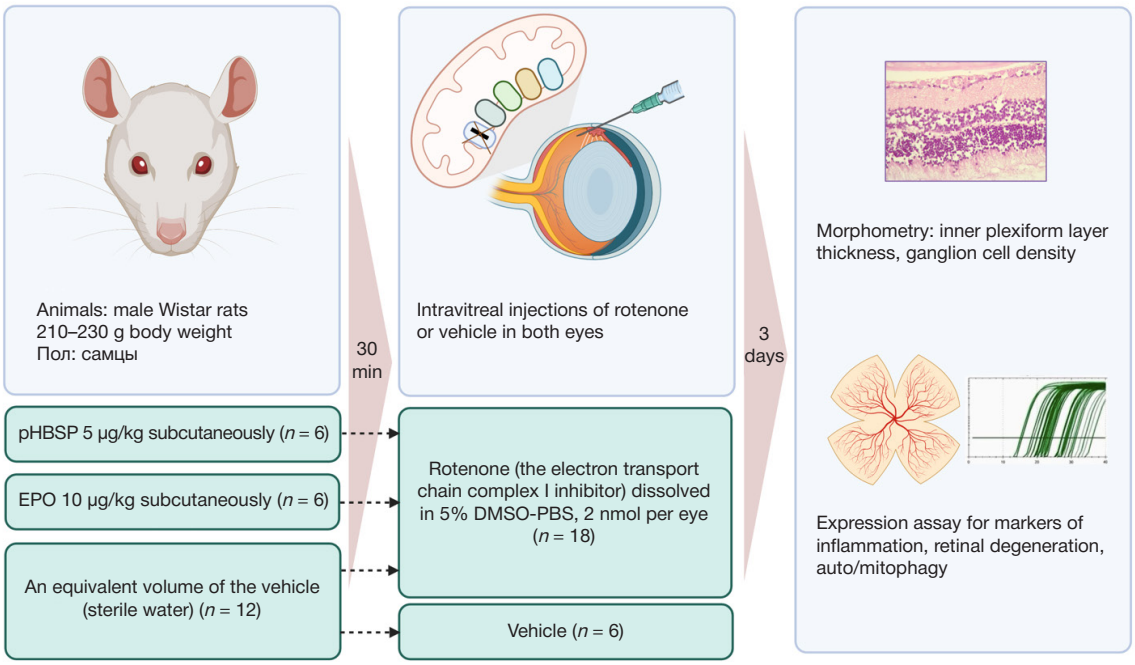

Fig. 1. A scheme of *in vivo* experiments to assess retinoprotective effects of EPOR/CD131 agonists in rotenone-induced retinopathy

The animals were distributed into three equal groups:

1) 'control' (sterile water subcutaneously + rotenone intraorbitally);

2) 'pHBSP' (pHBSP 5 μg/kg subcutaneously + rotenone intraorbitally);

3) 'EPO' (EPO 10 μg/kg subcutaneously + rotenone intraorbitally).

Additionally, the group of 'intact' animals (n = 6; matching by age and weight) received identical injections of the vehicle instead of rotenone.

The studied agents (EPO and pHBSP) were injected subcutaneously 30 min before the rotenone administration; the 'control' and 'intact' animals received similar injections of sterile water instead of the agents. On day 3, the eyes were enucleated for histomorphological examination and gene expression analysis (Fig. 1).

Morphological study

The eyeballs were embedded in paraffin after fixation in buffered formalin (pH about 7.0) with 0.002% picric acid followed by gentle aspiration of the vitreous humor and its replacement with molten wax as described previously [18]. All sections were standardized for the area (1 mm above the blind spot) and thickness (7 μm) to enable a proper comparison. The slides were stained by standard protocols [19] for morphometric examination including the inner plexiform layer (IPL) thickness measurements and the nuclei counts per 100 μm of ganglion cell layer.

Quantitative polymerase chain reaction (qPCR) assay

Extraction of the total RNA and reverse transcription were carried out as previously described [20]. The expression of target genes was analyzed by qPCR in a CFX96 real-time PCR thermal cycler (BioRad; USA) using the commercial SYBR® Green Master Mix (Bio-Rad Laboratories, Inc.; USA) and oligonucleotide primers (Evrogen; Russia).

The primers were designed using Primer-BLAST tool (NCBI) with the following stipulations: 1) melting temperature 59–61 °C; 2) each primer must span an exon-exon junction or the two primers must fall into different exons; 3) forward and reverse primers in a pair must not form auto- and cross-dimers; 4) the size of PCR product must be 95–107 b.p. (Table).

Processing of the raw qPCR data was carried out using the delta-delta Ct method. Following the amplification, ΔCt value (the difference in threshold cycles between the reference housekeeping gene and threshold cycle for the gene of interest) was calculated for each sample in the Bio-Rad CFX Manager software (Bio-Rad; USA).

The data were transformed by formula (1) [21]:

$$2E\ \Delta\Delta Ct = \Delta Ct - \Delta Ct_{cont} \quad (1)$$

where ΔCt is the cycle at which the logarithmic curve of the SYBR Green fluorescence intensity reaches the threshold level (threshold cycle) when running the reaction with housekeeping gene as a reference target;

ΔCt_{contr} is the difference in threshold cycles between the gene of interest and the reference gene.

Cytoprotective effects of pHBSP against rotenone toxicity *in vitro*

The cultures were obtained from brain tissues of CD1 mice collected on postnatal day 1. The animal was decapitated and the head was transferred to a Petri dish placed on ice. The dissection of the skull from skin and fascia was carried out under ice cooling. The brain was extracted and placed in a Petri dish with chilled PBS. The hippocampus, cortex and midbrain portions were dissected under a Leica binocular (magnification ×10) on a cooled glass slide placed in a Petri dish.

The tissues were collected in a tube, washed with D-PBS, trypsinized, and seeded at 20,000 cells in poly-D-lysine-treated plates with Neurobasal medium (PanEco; Russia). After 16–18 h, half of the medium was replaced with Neurobasal™ Plus (Thermo Fisher; USA). The cultures were examined microscopically every 2 days and half of the medium was replaced with a fresh portion; the cultures were maintained like this for 10 days. Rotenone to a final concentration of 2.5 μM

was added to the wells 24 h prior to cell viability assay. After 20 h exposure to rotenone, a putative cytoprotectant (pHBSP or EPO) was added to the suspension in a final concentration of 50 nM or 500 nM. The viability was accessed after staining with [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT) in an automated Cell Counter with cell viability analyzer function (Corning; USA) using CytoSmart software (Axion; the Netherlands).

Statistics

The normality of distributions was challenged by Shapiro–Wilk test. Normally distributed data are presented as M ± SD; for distributions other than normal the data are presented as Me [Q1; Q3]. Statistical processing and visualization of the data were carried out in GraphPad Prism 9.2.0 (Graphpad Software Inc; USA). Significance of the differences was accessed by one-way ANOVA with Kruskal–Wallis test and post-hoc Dunn's test with Benjamini-Hochberg procedure. The heat maps involved statistically unprocessed data.

RESULTS

Cytoprotective effects *in vivo*

The intravitreal injections of rotenone led to specific morphological changes in the retina: its overall thinning, a decrease in IPL thickness and a decrease in ganglion cell numbers. The IPL thickness decreased from 41.92 μm [38.74; 43.70] in the vehicle injection group to 23.59 μm [21.37; 25.62] in the rotenone injection group, whereas the nuclei counts per 100 μm of the ganglion cell layer (GCL) decreased from 8.22 [7.75; 8.83] to 4.78 [4.42; 4.67]. These changes are indicative of ganglion cell degeneration revealing a rotenone-induced neurodegenerative process. The gene expression assay revealed elevated mRNA levels for *Il6* and *Il1b* and reduced expression of the retinal ganglion cell marker *Nefl* additionally confirming the retinal damage.

Both pHBSP and EPO significantly alleviated the rotenone-induced degenerative changes as assessed histologically (Figs. 2A–C) or by gene expression measurements (Fig. 2D). Of note, the effects of pHBSP in reducing retinal degeneration were stronger compared with EPO. The IPL thickness and the nuclei counts per 100 μm of GCL in rats receiving pHBSP constituted 34.34 [31.52; 36.02] and 6.89 [7.00; 7.33], respectively.

Moreover, rats receiving pHBSP or EPO on the background of rotenone had elevated expression levels of the mitophagy/autophagy markers *Map1lc3a*, *Atg7*, *Becn1*, *Prkn* and *Bnip3* in the retina compared with rotenone only (Fig. 2D). Both agents demonstrated similar cytoprotective activity as assessed through the elevated expression of *Nefl* and autophagy protein-encoding genes, and their suppressive effects on the pro-inflammatory cytokine expression were comparable as well.

Thus, single subcutaneous systemic administration of 5 μg/kg pHBSP or 10 μg/kg EPO promoted a reduction in the degree of retinal degeneration induced by intravitreal injections of rotenone. The EPOR/CD131 stimulation-mediated retinoprotective effects are likely to involve a decrease in pro-inflammatory component and an increase in mitophagy, as indicated by a decrease in the expression of inflammation markers and an increase in the expression of auto/mitophagy markers.

Cytoprotective effects *in vitro*

The 24 h incubation of the primary neuroglial cultures in 2.5 μM rotenone killed over 50% of the cells, as reflected by a

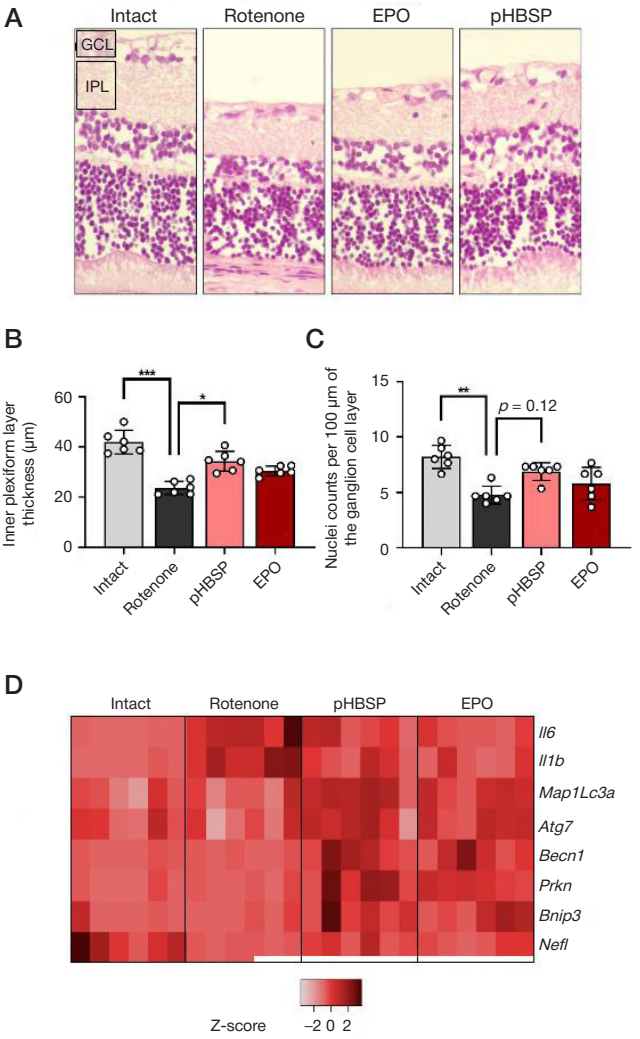

Fig. 2. The therapeutic effect of pHBSP and EPO in the rotenone-induced retinopathy in rats. **A**. Representative histological images of the retina. **B**. The inner plexiform layer thickness measurements. **C**. Nuclei counts in the ganglion cell layer. **D**. Normalized heat map of gene expression levels for inflammation (*Il6*, *Il1b*), autophagy (*Map1Lc3a*, *Atg7*), mitophagy (*Becn1*, *Prkn*, *Bnip3*) and retinal degeneration (*Nefl*) markers. GCL — ganglion cell layer; IPL — inner plexiform layer; * — $p < 0.05$; ** — $p < 0.005$; *** — $p < 0.0005$ (Kruskal–Wallis test, post-hoc Dunn's test).

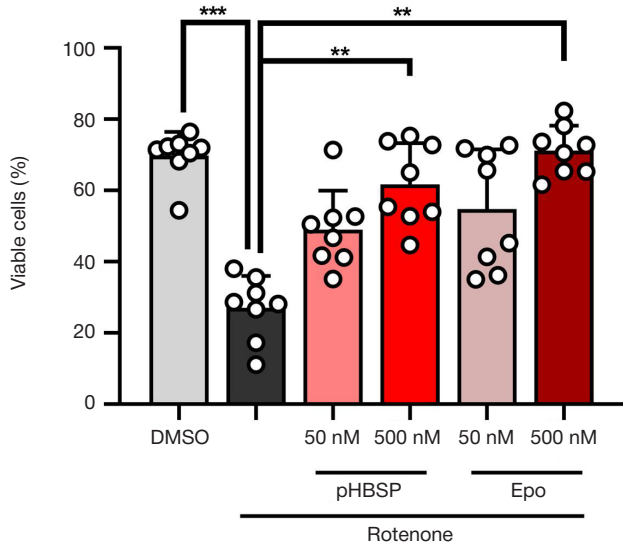

Fig. 3. Cytoprotective effects of pHBSP and EPO on the primary neuroglial cultures treated with 2.5 μM rotenone. ** — $p < 0.005$; *** — $p < 0.0005$ (Dunn's test)

69.80 ± 6.62% to 27.06 ± 8.98% decrease in viability observed in the 'rotenone-only' cultures (Fig. 3). The use of EPOR/CD131-binding agents at 500 nM concentrations significantly rescued the viability of rotenone-treated cultures to 61.73 ± 11.56% ($p < 0.005$) and 71.20 ± 6.97% ($p < 0.005$) for pHBSP and EPO, respectively. At concentrations reduced to 50 nM, both agents showed a similar rescuing tendency, albeit the effects lacked statistical significance.

DISCUSSION

The functional state of mitochondria is considered one of the main factors defining cellular homeostasis. Tissues with the top energy demands, such as the brain and the retina, show the highest sensitivity to mitochondrial abnormalities [2].

In this study, we demonstrate that EPOR/CD131 stimulation can improve the functional state of neuronal cells of the retina in vivo and the survival of primary neuroglial cultures *in vitro* under conditions of chemically induced mitochondrial dysfunction. As the neurotoxic effect of rotenone is associated with the respiratory chain disruption in mitochondria, the observed beneficial effects of EPOR/CD131 agonists are most likely due to their mitoprotective action. This assumption is consistent with the previously reported beneficial influence of EPO on mitochondrial function [22]. The results of gene expression analysis support the view of auto- and mitophagy stimulation as a potential mechanism of mitoprotective effects exerted by EPOR/CD131 agonists, indicated by increased mRNA expression levels for LC3A, ATG7, Beclin-1, Prkn and BNIP3. These results also agree with our previous research on the effects of pHBSP on the autophagy gene expression under conditions of ethanol-induced neurodegeneration in rats [23]. Although we provided no quantitative or semi-quantitative assessment of the autophagy protein phosphorylation levels, some of the identified factors had been already recognized as neuroprotective mediators in mitochondrial dysfunctions [16, 24, 25]. In addition, there is a line of evidence showing a connection of the positive effects of pHBSP with autophagy stimulation. For example, the hepatoprotective activity of pHBSP is accompanied by elevated expression of LC3II, LC3I, and Beclin 1, while being sensitive to autophagy inhibition [15]. Consistently, autophagy has been identified as one of major links mediating the neuroprotective effects of EPO [26].

CONCLUSIONS

Thus, both erythropoietin and pHBSP, a selective peptide agonist of EPOR/CD131, reveal pronounced neuroprotective activity during the rotenone-induced damage. The data implicate the autophagy/mitophagy stimulation as a likely mechanism of the observed pharmacological effects. The use of EPOR/CD131 agonists is a promising direction for the treatment of neurodegenerative processes in the central nervous system and retina. Further studies using specific models of particular neurodegenerative disorders, such as Parkinson's and Alzheimer's diseases or amyotrophic lateral sclerosis, may help to determine the clinical prospects of pHBSP administration.

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