Eleven-amino acid peptides that mimic the erythropoietin α-helix B increases cell survival in endotheliocyte culture

El péptido de once-aminoácidos que mimetiza la α-hélice B de la eritropoyetina aumentan la supervivencia celular en el cultivo de endoteliocitos

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Abstract

Aim. To study the cytoprotective activity of peptides that mimic the spatial structure of the erythropoietin α-helix B *in vitro*.

Material and methods. The experiment was performed on the primary cell culture of human endotheliocytes (HUVEC). Hydrogen peroxide (H_2O_2) was used to mimic oxidative stress. Cells were scattered in 96-well gelatin-coated plates with a density of 5 thousand cells per well. After 24 hours of incubation, the studied peptides were introduced - the basic peptide - Helix B surface peptide HBSP (QEQLERALNSS) and its derivatives EP-11-1 (UEHLERALNSS), EP-11-2 (UEQLERALNCS), EP-11-3 (UEQLERALNTS) in 3 concentrations - 5 μg/ml, 30 μg/ml and 50 μg/ml. Cell viability was measured using the MTT test.

Results. Initial screening of the cytoprotective activity of innovative peptides that mimic the erythropoietin α-helix B *in vitro* was performed. Our results demonstrate that the original HBSP peptide and its derivatives EP-11-1, EP-11-2, EP-11-3 have a pronounced ($p<0.05$, compared with the control) cytoprotective effect. Derivatives EP-11-1, EP-11-2 does not differ from the base peptide HBSP in terms of the level of detected activity. When comparing the density of the formazan solution and the percentage of surviving endotheliocytes between the series of experiments with the base peptide HBSP and its derivatives, EP-11-3 showed significant superior cytoprotective effects when compared with HBSP ($p<0.05$), all over the entire range studied concentrations.

Conclusion. Derivatives of the original peptide with laboratory code EP-11-1, EP-11-2 do not differ in their cytoprotective action from the base peptide HBSP. While the derivatives with laboratory code EP-11-3 presented a higher and significant cytoprotective activity when compared to the base peptide HBSP.

Keywords: erythropoietin, α-helix B, cytoprotection, endotheliocytes, cell culture, HUVEC, MTT-test.

Resumen

Objetivo. Estudiar la actividad citoprotectora de péptidos que imitan la estructura espacial de la eritropoyetina **α-**hélice B *in vitro*.

Materiales y métodos: el experimento se realizó en cultivo celular primario de endoteliocitos humanos (HUVEC). El peróxido de hidrógeno (H_2O_2) se utilizó para inducir el estrés oxidativo. Las células se dispersaron en placas recubiertas de gelatina de 96 pocillos con una densidad de 5 mil células por pocillo. Después de 24 horas de incubación, se introdujeron los péptidos estudiados, el péptido básico, el péptido de superficie Helix B HBSP (QEQLERALNSS) y sus derivados EP-11-1 (UEHLERALNSS), EP-11-2 (UEQLERALNCS), EP-11-3 (UEQLERALNTS) en 3 concentraciones: 5 μg/ml, 30 μg/ml y 50 μg/ml. La viabilidad celular se midió utilizando la prueba MTT.

Resultados. Se realizó el cribado inicial de la actividad citoprotectora de péptidos innovadores que imitan la α-hélice B de la eritropoyetina *in vitro*. Nuestros resultados demostraron que el péptido HBSP original y sus derivados EP-11-1, EP-11-2, EP-11-3 tienen un efecto citoprotector pronunciado ($p<0,05$ en comparación con el control). Los derivados EP-11-1, EP-11-2 no difieren del péptido base HBSP en términos del nivel de actividad detectada. Al comparar la densidad de la solución de formazán y el porcentaje de endoteliocitos supervivientes entre la serie de experimentos con el péptido base HBSP y su derivado con el código de laboratorio EP-11-3, se encontró que EP-11-3 presenta un efecto significativo superior en comparación con HBSP ($p<0,05$), en todo el rango de concentraciones estudiadas.

Conclusión. Los derivados del péptido original con el código de laboratorio EP-11-1, EP-11-2 no difieren en la acción citoprotectora del péptido base HBSP. Mientras que, el derivado con el código de laboratorio EP-11-3 tiene una actividad citoprotectora significativamente superior que el péptido base HBSP.

Palabras clave: eritropoyetina, α-hélice B, citoprotección, endoteliocitos, cultivo celular, HUVEC, prueba MTT.

Introduction

It is now known that vascular endothelium has a wide range of functions, and maintenance of endothelial homeostasis is a multidimensional active process, disruption of which has a potentially deleterious consequence if not reversed[1]. Endothelial cells injury leads to the development of endothelial apoptosis and dysfunction, the consequences of which can be local and general. Currently, the role of vascular endothelium in the pathogenesis of acute and chronic inflammatory processes is attracting increasing attention, especially in relation to cardiovascular diseases[2].

Endothelial cell dysfunction (ECD) manifested in lesion-prone areas of the arterial vasculature, results in the earliest detectable changes in the life history of an atherosclerotic lesion — the focal permeation, trapping, and physicochemical modification of circulating lipoprotein particles in the subendothelial space. Changes in the spectrum of molecules secreted and expressed by endothelium and smooth muscle cells such as chemokines, IL-1, monocyte chemoattractant protein-1, granulocyte-monocyte stimulating factor, and IL-8, disruption of endothelial barrier function, and ultimately leads to infiltration of the vascular wall by atheromatous masses, and the formation of atherosclerotic plaques[3]. In turn, chronic and acute overproduction of reactive oxygen species (ROS) develops into cardiovascular diseases (CVD) and, in particular, atherosclerosis[4]. The emerging pathogenetic cascade becomes a relevant target for the pharmacological effects of drugs.

In the search for compounds with endothelio-protective activity allows, substances with a pronounced cytoprotective effect play an important role in reducing oxidative stress manifestations, acute and chronic inflammation leading to atherogenesis.

One of these compounds with pronounced universal cytoprotective activity is erythropoietin (EPO). Our previous studies have shown that recombinant erythropoietin and **c**arbamylated EPO (CEPO) have tissue-protective, endothelial, and cardioprotective effects in various experimental models[5,6,7,8]. It has been reported that EPO can bind the tissue-protective receptor (TPR, namely EPOR/CD131 heterodimer) and plays an important role in tissue protection and immune regulation. It was suggested that the helix B of EPO, which is exposed to aqueous medium away from the binding sites of EPO and $(EPOR)_2$, is critical for the recognition of TPR, and it was confirmed that the helix B peptide had similar tissue-protective effects for EPO and CEPO. Based on these observations, an eleven-amino acid linear peptide, QEQLERALNSS, mimicking the three-dimensional structure of the external aqueous face of the helix B peptide was developed and named helix B surface peptide (HBSP)[9].

In the present study, we assessed the cytoprotective effect on the HUVEC cell culture of the following derivatives of the 11-membered peptide HBSP (QEQLERALNSS): EP-11-1 (UEHLERALNSS), EP-11-2 (UEQLERALNCS), EP-11-3 (UEQLERALNTS), peptides that mimic the spatial structure of the erythropoietin helix B *in vitro*. Amino acid sequences were obtained by homologous analysis to determine groups of related peptides of the parent compound using the BLAST program.

Material and Methods

The experimental study was conducted at the Research Institute of Pharmacology of Living Systems of Belgorod State University. The study was performed in compliance with the requirements of General Requirements for the Competence of Testing and Calibration Laboratories 17025-2009, GOST R ISO 5725-2002 and the Rules of Laboratory Practice, approved by Order of the Ministry of Healthcare and Social Development of the Russian Federation dated August 23rd, 2010 № 708n.

The study was performed using the primary culture of human endotheliocyte cells HUVEC (Sigma-Aldrich, Merck). Endothelial cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 20 mM HEPES buffer, 5 U/ml heparin, 200 µg/ml E CG F (Sigma-Aldrich), 10% fetal calf serum (FBS) at 37°C in a humid atmosphere containing 5% CO_2 [10,17].

Cells were scattered in 96-well gelatin-coated plates with a density of 5 thousand cells per well. After 24 h of incubation, the studied peptides (HBSP, EP-11-01, EP-11-02, EP-11-03) were added in 3 concentrations - 5 µg/ml, 30 µg/ml and 50 µg/ml. Thus, 3 experimental plates were formed, one for each studied peptide concentration.

Table 1. Protocol for the plate wells in the study of the cytoprotective activity of innovative peptides (concentration 5 µg/ml).

	1	2	3	4	5	6	7
A	Blank	HUVEC	HUVEC + H2O2	HBSP + HUVEC + H2O2	EP-11-01+ HUVEC + H2O2	EP-11-02+ HUVEC + H2O2	EP-11-03+ HUVEC + H2O2
B	Blank	HUVEC	HUVEC + H2O2	HBSP+ HUVEC + H2O2	EP-11-01+ HUVEC + H2O2	EP-11-02+ HUVEC + H2O2	EP-11-03+ HUVEC + H2O2
C	Blank	HUVEC	HUVEC + H2O2	HBSP+ HUVEC + H2O2	EP-11-01+ HUVEC + H2O2	EP-11-02+ HUVEC + H2O2	EP-11-03+ HUVEC + H2O2
D	Blank	HUVEC	HUVEC + H2O2	HBSP+ HUVEC + H2O2	EP-11-01+ HUVEC + H2O2	EP-11-02+ HUVEC + H2O2	EP-11-03+ HUVEC + H2O2
E	Blank	HUVEC	HUVEC + H2O2	HBSP+ HUVEC + H2O2	EP-11-01+ HUVEC + H2O2	EP-11-02+ HUVEC + H2O2	EP-11-03+ HUVEC + H2O2
F	Blank	HUVEC	HUVEC + H2O2	HBSP+ HUVEC + H2O2	EP-11-01+ HUVEC + H2O2	EP-11-02+ HUVEC + H2O2	EP-11-03+ HUVEC + H2O2
G	Blank	HUVEC	HUVEC + H2O2	HBSP+ HUVEC + H2O2	EP-11-01+ HUVEC + H2O2	EP-11-02+ HUVEC + H2O2	EP-11-03+ HUVEC + H2O2
h	Blank	HUVEC	HUVEC + H2O2	HBSP+ HUVEC + H2O2	EP-11-01+ HUVEC + H2O2	EP-11-02+ HUVEC + H2O2	EP-11-03+ HUVEC + H2O2

Three hours after the addition of the peptides, hydrogen peroxide (H_2O_2) was added to the plate wells at a final concentration of 200 µM. After 24 hours, the culture medium containing H_2O_2 was replaced with normal.

Cell viability was measured using the MTT test[19] developed by Mosman (1983), which consists of the formation of formazan purple from a tetrazolium salt, MTT (3- (4,5-dimethyl thiazol-2-yl bromide) -2,5-diphenyl tetrazolium), where this salt is reduced by the action of the mitochondrial enzyme succinate dehydrogenase. Thus, the level of mitochondrial respiration of the cell was assessed by the rate of accumulation of formazan crystals in the cytoplasm, which is an indicator of its viability. The amount of formazan formed in the cell monolayer is proportionally related to the number of living cells in the test sample.

At the end of the experiment, the medium was selected, a solution of 3- (4,5-dimethylthiazol-2-yl) -2,5-diphenyl-tetrazolium bromide (MTT, Sigma) in PBS (Sigma) containing 0.9 mM $CaCl_2$ was added to the wells of the plate and 0.5 mM $MgCl_2$, based on 10 mg MTT per well. After a 4-hour incubation at 37°C in a humid atmosphere containing 5% CO2, the plate was unscrewed at 600 g on a R4810 centrifuge (Eppendorf, Germany). The supernatant was carefully aspirated.

DMSO, 250 µl/well was used to dissolve the formed crystals of formazan. After the complete dissolution of the crystals, 150 µl, without affecting the cell layer on gelatin, was transferred to a new plate. The optical density of a solution of formazan in DMSO was measured on a Multiscan EX spectrophotometer at a wavelength of 570 nm. Assessment of the results of the MTT test was carried out by comparing the optical density in the experimental and control wells. The number of viable cells was calculated by the formula N = AU experimental / AU control * 100% Statistical data processing was performed using the Kruskal-Wallis test followed by Dunn test (ANOVA). Data are presented as mean ± s.d. Data were considered significant at p≤ 0.05.

Results and Discussion

To model oxidative stress, using hydrogen peroxide (H_2O_2) (200 µM), stimulates programmed cell death — apoptosis, which is accompanied by several biochemical and morphological changes in the cell: chromatin condensation, DNA fragmentation, increased membrane permeability and cell compression[11,18].

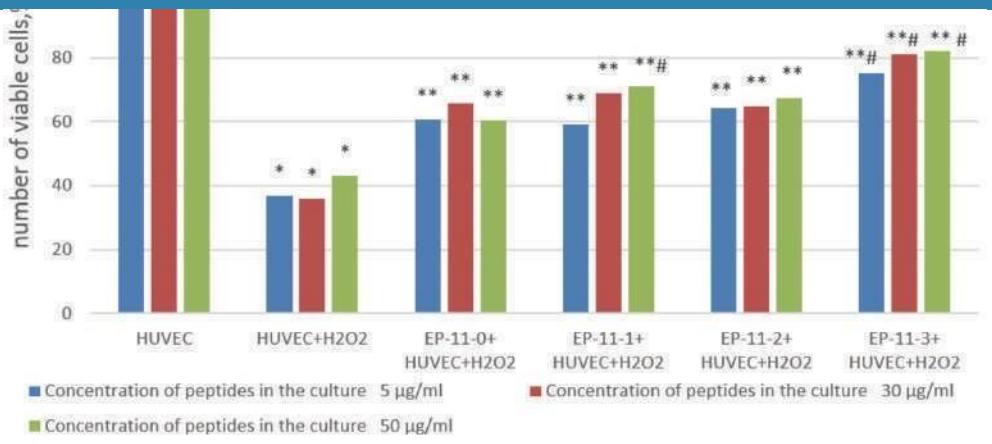
The average optical density in the experimental groups is shown in Table 1.

Our results show that oxidative stress induced by H_2O_2 produces a significant decrease in the optical density of the medium and the percentage of viable cells of the HUVEC (Table 1, figure 1).

Analysis of Table 1 in which are shown the medium optical density values, with a HUVEC cell culture, against the background of oxidative stress and in the presence of the different peptides at three concentrations, show that the studied compounds have a statistically significantly cytoprotective effect, increasing mitochondrial activity and cell survival in cell culture. Compound EP-11-1A showed a dose-dependent increase in the level of mitochondrial activity of cells in the culture. The increase in the level of cytoprotective activity with the innovative peptides EP-11-1 and EP-11-2 were similar to the percentage of viable cells in the HUVEC culture with the addition of hydrogen peroxide, while EP-11-1 at a concentration of 50 µg/ml was statistically significantly higher ($p<0.05$) than in the experimental series with the base peptide HBSP (Table 1, figure 1).

Evaluation of the results obtained in the analysis of the MTT test in a series of experiments with the EP-11-3 peptide, comparing the density of the formazan solution and the percentage of surviving endotheliocytes between the series of experiments with the HBSP base peptide and its derivative with laboratory code EP-11-3 allows us to conclude that the compound has the highest cytoprotective activity. Despite the

Figure 1. The number of viable cells in the experimental groups with the addition of innovative peptides at concentrations of 5, 30, and 50 µg/ml. *p <0.05 when compared with HUVEC; **p<0.05 when compared with HUVEC + H_2O_2; #<0.05 when compared with HBSP + HUVEC + H_2O_2.



absence of a pronounced dose-dependent effect, a statistically significant ($p<0.05$) increase in cell survival was found over the entire range of studied EP-11-3 concentrations (5, 30, and 50 μg/ml) (Table 1, figure 1).

As part of the possible approaches to the pharmacological treatment of endothelium-associated pathology, we previously studied the endothelio-protective activity of several compounds, drugs, and their combinations on various experimental models of endothelial dysfunction[12,13,14].

In effect, Michael Brines et al. (2004) demonstrated that the non-hematopoietic effects of erythropoietin are mediated through the heterodimeric EPOR/CD131 complex. The finding that the erythropoietic and tissue-protective properties of EPO are mediated through two different receptor systems has led to the creation of prerequisites for a fundamentally new direction in the search for innovative molecules with cytoprotective activity. In 2008, the same authors demonstrated the cytoprotective activity of an 11-amino acid peptide based on the α-helix B of erythropoietin that mimics the spatial part of a molecule that interacts with a heterodimeric EPOR/CD131 receptor but does not interact with a homodimeric EPOR/EPOR receptor. This peptide HBSP demonstrated the ability to significantly improve the morphofunctional state of tissues in diabetic retinal edema, renal ischemia-reperfusion, and significantly improve cognitive functions in the galantamine-induced amnesia model, in the absence of any effect on erythropoiesis[9].

Our previous results on the study of the endothelioprotective activity of the HBSP base peptide with the amino acid sequence QEQLERALNSS, using the L-NAME treatment to induceendothelial dysfunction, we demonstrated that HBSP has a pronounced endothelial-protective and potentially atheroprotective effect due to its ability to prevent the death of endothelial cells, as well as to reduce remodeling and pro-inflammatory activation of the vascular wall. Nevertheless, the prothrombotic activity of the peptide limits its use as a preventing and treating agent for atherosclerosis-associated diseases[15]. Thus it is advisable to search for peptides related to the original HBSP which that does not have prothrombotic activity, or that have anticoagulant properties. The search for such compounds can be solved in several ways, including adding amino acid motifs with anticoagulant properties to the amino acid sequence, or by searching for groups of related peptides of the parent compound using the BLAST program. The peptides analyzed in this study were obtained using the second approach - screening using the BLAST program[16].

At the first stage of the study of the pharmacological activity of the obtained derivatives. They were used to study of the cytoprotectionactivity of HBSP derivatives under laboratory codes EP-11-1 (UEHLERALNSS), EP-11-2 (UEQLERALNCS), EP-11-3 (UEQLERALNTS). Assessment of cell survival in a culture of human endotheliocytes under conditions of oxidative stress in the presence of the studied peptides showed that the original HBSP peptide and its derivatives EP-11-1, EP-11-2, EP-11-3 have a pronounced ($p<0.05$ in compared with control) cytoprotective action. Derivatives EP-11-1, EP-11-2 do not differ from the base peptide HBSP in terms of the level of detected activity. When comparing the density of the formazan solution and the percentage of surviving endotheliocytes between the series of experiments with the base peptide HBSP and its derivative with the laboratory code EP-11-3, a statistically significant ($p <0.05$ compared with HBSP) superiority of EP-11-3 was found over the entire range studied concentrations. Thus, we confirmed that our proposed compounds that mimic the erythropoietin B α-helix have pronounced cytoportective activity, and further studies are required to answer the questions of using these compounds as peptides with endothelioprotective and antiplatelet activity.

Conclusion

11-amino acids derivatives of EPO with laboratory codes EP-11-1, EP-11-2 do not differ in the cytoprotective action capacity when compared with the base peptide HBSP. The derivative with laboratory code EP-11-3 presents a higher significantly cytoprotective activity compared to the base peptide HBSP.

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