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Artículos

The anti-aggregation activity of new 11-amino acid of erythropoietin derivate containing tripeptide motifs

Actividad antiagregante de nuevos derivados de 11 aminoácidos de la eritropoyetina, que contienen motivos de tripéptidos

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Abstract:

Objective. To study the platelet antiaggregant activity of new 11-amino acid derivatives of erythropoietin, containing tripeptide motifs.
Materials and methods. The platelet aggregation degree was determined using the platelet-rich plasma of male Wistar rats. Formation of platelet aggregates results in an increase in light transmission through the sample; the kinetics of the responses and maximal aggregation provide a quantitative assessment of platelet aggregation The degree of platelet aggregation is estimated by the magnitude of the amplitude of aggregatograms, and the time of onset of light transmission level as well the time of onset of 85% of light transmission.
Results. When blood was incubated with the studied peptides P-αB1, P-αB3, and P-αB4, a pronounced antiplatelet effect was observed. This is evidenced by a decrease in the maximum light transmittance of the plasma to 28.4±1.00%, 29.7±1.13% and 30.1±0.97%, respectively, and a delay in its onset to 134.4±2,90, 135.8±3.72 and 132.0 ± 3.59 seconds, respectively. In this case, a shift in the plasma light transmission curve to the right was observed which characterizes the platelet aggregation process.
Conclusion. The addition of P-αB1, P-αB3, and P-αB4 produces antiplatelet activity, which is reflected by the shifting to the right of the platelet aggregation curve. The data obtained indicate a prolongation of platelet aggregation time and a decrease in its degree.
Keywords: erythropoietin, α-helix B, thrombocytes, aggregation, 11-amino acid peptide.

Resumen:

Objetivo. Estudiar la actividad antiagregante plaquetario de los nuevos derivados de 11 aminoácidos de la eritropoyetina, que contienen motivos de tripéptidos.
Materiales y métodos. El grado de agregación plaquetaria se determinó usando el plasma rico en plaquetas de ratas Wistar macho. La formación de agregados de plaquetas resulta en un aumento en la transmisión de luz a través de la muestra. La cinética de las respuestas y la agregación máxima proporcionan una evaluación cuantitativa de la agregación plaquetaria. El grado de agregación plaquetaria se estima por la magnitud de la amplitud de los agregados y el tiempo de inicio del nivel de transmisión de luz, así como el tiempo de inicio del 85% de la luz. Transmisión.
Resultados. Cuando se incubó sangre con los péptidos estudiados P-αB1, P-αB3 y P-αB4, se observó un efecto antiplaquetario pronunciado. Esto se evidencia por una disminución en la transmitancia máxima de luz del plasma a 28,4±1,00%, 29,7±1,13% y 30,1±0,97%, respectivamente, y un retraso en su inicio a 134,4±2, 90 s, 135,8±3,72 s y 132,0±3,59 segundos, respectivamente. En este caso, se observo que la curva de transmisión de luz plasmática se desplazo a la derecha, lo que caracteriza el proceso de agregación plaquetaria.
Conclusión. La adición de P-αB1, P-αB3 y P-αB4 produce actividad antiplaquetaria, que se refleja al desplazarse a la derecha de la curva de agregación plaquetaria. Los datos obtenidos indican una prolongación del tiempo de agregación plaquetaria y una disminución en su grado.
Palabras clave: eritropoyetina, α-hélice B, trombocitos, agregación, péptido de 11 aminoácidos.

Introduction

The high contribution of cardiovascular pathology to the general structure of the causes of mortality and disability in the population of developed countries necessitates their in-depth study and improvement of correction methods. Moreover, as the main task of research in this area, one can designate a precise search for ways to prevent and treat the atherosclerotic process, as the main cause of cardiovascular mortality.

Atherosclerosis is a chronic arterial disease and a major cause of vascular death. Fatty streaks in arterial walls gradually develop into atheroma and characteristic plaques. The acute rupture of these atheromatous plaques causes local thrombosis, leading to partial or total occlusion of the affected artery. The clinical consequences of these plaques depend on their site and the degree and speed of vessel occlusion. The most significant from

the epidemiological point of view is the defeat of the coronary and cerebral vessels, leading to such nosologies as coronary heart disease (CHD) and ischemic stroke [1].

Activation of pro-inflammatory cascades in macrophages and endothelium is an important link in atherogenesis. Accumulation of excess lipids within the artery due to the presence of increased circulating LDL promotes endothelial dysfunction and activation, which results in increased production of pro-inflammatory cytokines and reactive oxygen species, overexpression of adhesion molecules, chemokines, CRP, and decreased NO bioavailability. These processes contribute to the recruitment and infiltration of monocytes, which differentiate into macrophages and following the uptake of modified-LDL via scavenger receptors, become foam cells, which are essential steps in atherogenesis [2-7]. Oxidative stress, modified lipoproteins, and other factors (bioactive lipids, molecular patterns associated with damage, cytokines) stimulate inflammation through their receptors [2,3,8].

Based on the available information on the pathogenesis of atherosclerosis, one of the approaches for influencing atherogenesis is the use of drugs with cytoprotective and mitochondrial-oriented activity. Recent preclinical *in vitro* and *in vivo* studies demonstrated that an endogenous erythropoiesis stimulator with a mass of 34 kDa erythropoietin (EPO) has a high cytoprotective activity, which is mainly associated with effects on the mitochondrial unit and has been confirmed in experimental models of ischemic and traumatic lesions, including endothelium, myocardium, and brain [9-11,18]. Our previous studies have shown that recombinant erythropoietin and carbamylated darbepoetin have tissue-protective, endothelial, and cardioprotective effects in various experimental models [12-14].

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 α-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 α-helix B peptide had similar tissue-protective effects for EPO and CEPO. Based on these observations, an eleven-amino acid linear peptide mimicking the three-dimensional structure of the external aqueous face of the α-helix B peptide was developed and named helix B surface peptide. In the present study, we assessed in vitro the antiaggregant activity of new 11-amino acid derivatives of erythropoietin, P-αB1, P-αB3, and P-αB4, containing tripeptide motifs.

Material and Methods

The experimental study was conducted at the Research Institute of Pharmacology of Living Systems of Belgorod State National Research 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 platelet aggregation degree was determined using the platelet-rich plasma of male Wistar rats. Blood was taken from the abdominal aorta into a test tube with a 3.8% sodium citrate solution in a 9:1 ratio, followed by the addition of the test polypeptides at a final concentration of 30 μg/ml.

The amino acid sequences of the studied peptides are presented in Table 1.

TABLE 1
Amino acid sequences and laboratory ciphers of innovative peptides studied in this study.

Lab Code	Amino Acid Sequence
P-αB	QEQLERALNSS
P-αB1	RGDQEQLERALNSS
P-αB2	UEQLERALNSSRGD
P-αB3	KGDQEQLERALNSS
P-αB4	UEQLERALNSSKGD
P-αB5	PGPQEQLERALNSS
P-αB6	UEQLERALNSSPGP

The compounds were incubated for 30 minutes. Then it was centrifuged at 1000 rpm for 10 minutes. After that, 270 μl of platelet-rich plasma was added to a 0.3 ml aggregometer cuvette. Then, 30 μl of adenosine-5-diphosphoric acid disodium salt (ADP) was added at a final concentration of 5 μM. The antiplatelet activity was determined by the G. Born method on a two-channel laser platelet aggregation analyzer ALAT-2 Biola. Reagents manufactured by RENAM. The analysis was carried out no later than 2 hours after the extraction of the blood. Graphically registering platelet aggregation for 5 minutes obtained curves reflecting a decrease in the optical density of the plasma. The degree of platelet aggregation was evaluated by the magnitude of the maximum amplitude of the aggregatogram and the time of onset of maximum light transmission and the time of onset of the level of 85% of the maximum light transmission.

Statistical processing was performed using the software environment of calculations R. The nature of the distribution of characters in the statistical sample was determined using the Shapiro-Wilk test and the Spiegelhalter criterion, the equality of variances was assessed using the Levene criterion. Depending on the type of distribution of attributes and the equality of variances, the significance of the results was evaluated using parametric (ANOVA) or non-parametric (Kruskal-Wallis test) one-way analysis of variance and unpaired Student t-test was used as a post-hoc analysis to identify differences in intergroup comparisons or the Mann-Whitney test, respectively, adjusted by Benjamini-Hochberg for multiple hypothesis testing. The results were considered significant for $p \leq 0.05$.

Results and Discussion

When using ADP as an inducer, light transmission in the group of intact animals occurs at 120.1±4.03 s and is 37.2±1.55%. P-αB induced statistically significant increase in platelet aggregation, since after adding ADP the maximum level of light transmission increased to 46.4±2.40%, and on average it reached 104.3±4.46 s. Therefore, the basic peptide with the protein sequence QEQLERALNSS has pronounced pro-coagulant properties (Fig. 1, Table 2).

When incubating blood with the studied derivatives having tripeptide motifs under the codes P-αB1, P-αB3, and P-αB4, a pronounced antiplatelet effect was observed. This indicates a decrease in plasma light transmittance to 28.4±1.00%, 29.7±1.13%, and 30.1±0.97%, respectively, and an increase in the onset time leads to platelet aggregation to 134.4±2, 90 s, 135.8±3.72 s and 132.0±3.59 s, respectively (Fig. 1). At the same time, a natural shift in the light transmission curve of the plasma is observed, which indicates a platelet aggregation process that is eligible for a group of intact animals.

P-αB2 did not affect the aggregation ability of platelets in the conducted experiment (Table 2 and Figure 1).

Incubation of blood with P-αB5 and P-αB6 led to an increase in the aggregation ability of platelets to the level of comparative analysis. At the same time, a natural shift to the left in the light transmission curve of the plasma is observed, which indicates a platelet aggregation process, depending on the group of intact animals.

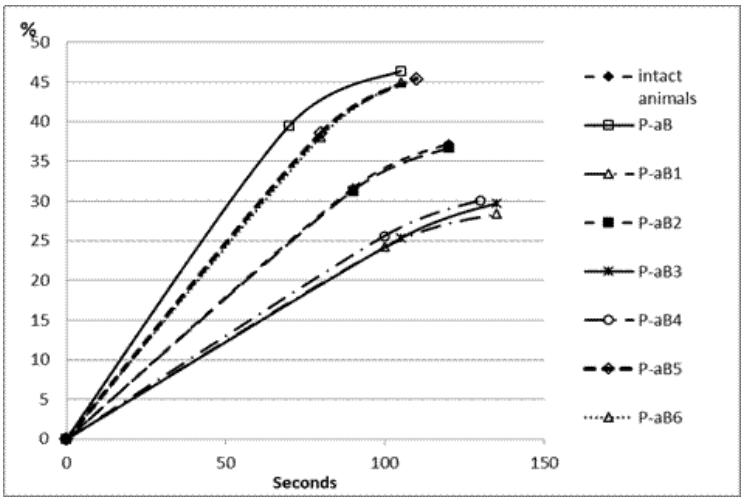

FIGURE 1
The effect of innovative peptides that mimic the spatial structure of the erythropoietin α-helix B on platelet aggregation ability. Not flooded marker at p<0.05 compared with intact animals.

A logical trend is observed when analyzing the onset time of 85% of the maximum level of light transmission. In the samples: P-αB1, P-αB3, and P-αB4, the time of its onset are longer with respect to the group of intact animals, and this circumstance also contributes to a shift of the platelet aggregation ability curve to the right (Table 2).

Table 2. The effect of innovative peptides that mimic the a-helix of erythropoietin B on the aggregation ability of platelets.

TABLE 2
The effect of innovative peptides that mimic the a-helix of erythropoietin B on the aggregation ability of platelets.

Group	Onset time		Maximum transmittance, %
	85 % of the maximum, seconds	Maximum aggregation, seconds	
Intact	90.1±3.01	120.1±4.03	37.2±1.55
P- αB	74.0±3.83*	104.3±4.36*	46.4±2.40*
P- αB1	101.4±1.85*	134.4±2.90*	28.4±1.00*
P- αB2	90.8±2.56	119.8±119.8	36.7±1.32
P- αB3	104.1±2.88*	135.8±3.72*	29.7±1.13*
P- αB4	99.5±2.84*	132.0±3.59*	30.1±0.97*
P- αB5	80.8±2.94*	107.8±3.87*	45.4±2.04*
P- αB6	79.0±3.34*	105.6±4.17*	45.0±1.64*

* p 005 compared with intact animals

In our previous study we found that the basic 11-amino acid peptide P-αB (QEQLERALNSS), which mimics the structure of erythropoietin α-helix B has a pronounced endothelial-protective and potentially atheroprotective effect due to its ability to prevent the death of endothelial cells, however, it shows pro-thrombotic activity in rats [15], suggesting the requirement for necessitates further modifications of this molecule.

Is proposed a modification of P-αB by attaching peptide motifs with antiplatelet activity. Our work showed that modifications of P-αB by attachment of peptide motifs with antiplatelet activity leads to the appearance of antigaggregant activity in the resulting peptides that mimic the spatial structure of erythropoietin α-helix B. As the 3 peptide motifs used in this study, the possibilities of including RGD, KGD,

and PGP motifs in the amino acid sequence were studied. It is known that these motifs have pronounced antiplatelet properties [16,17,19].

In our study, we incubated blood samples with the studied pharmacological and received a pronounced effect to different degrees. It is noteworthy that the modification of the initial peptide by attaching tripeptide motifs to its n-terminal and c-terminal led to different changes in the antiplatelet properties of the obtained compounds. The results of our study indicate that the RGD motif attached to the n-terminal terminal leads to the appearance of pronounced antiplatelet properties in the obtained P-αB1 peptide, which resulted in a statistically significant increase in platelet aggregation time by 11% ($p<0.05$) and a decrease in its degree by 8.8% ($p<0.05$). At the same time, the P-αB2 compound containing the RGD motif at the c-terminal portion of the peptide does not have antiplatelet properties. The KGD motif determined the statistically significant antiplatelet properties of the obtained peptides P-αB3 and P-αB4 in both the n-terminal and c-terminal regions of the peptide. But a more pronounced activity was still found in the P-αB3 compound containing the KGD motif at the n-terminal. The PGP motif included in the peptides at the n-terminal (P-αB5) and c-terminal (P-αB6) did not change the antiplatelet properties of the base peptide, and the compounds P-αB5 and P-αB6 showed procoagulant activity.

Thus, we confirmed that our proposed compounds P-αB1, P-αB3 and P-αB4 that mimic the erythropoietin α-helix B have pronounced antiagregant activity, and further studies of the obtained molecules will answer the questions of using these compounds as peptides with endothelioprotective and cytoprotective activity.

Conclusion

Based on the study, antiplatelet activity was detected in the samples: P-αB1, P-αB3, and P-αB4, which is expressed in the shift of the platelet aggregation curve to the right. The data obtained indicate an increase in platelet aggregation time and a decrease in its degree.

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