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Research Article

# Non-hematopoietic erythropoietin-derived peptides for atheroprotection and treatment of cardiovascular diseases

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## Abstract

**Relevance:** Cardiovascular diseases continue to be the leading cause of premature adult death.

**Lipid profile and atherogenesis:** Dislipidaemia leads to subsequent lipid accumulation and migration of immunocompetent cells into the vessel intima. Macrophages accumulate cholesterol forming foam cells – the morphological substrate of atherosclerosis in its initial stage.

**Inflammation and atherogenesis:** Pro-inflammatory factors provoke oxidative stress, vascular wall damage and foam cells formation.

**Endothelial and mitochondrial dysfunction in the development of atherosclerosis:** Endothelial mitochondria are some of the organelles most sensitive to oxidative stress. Damaged mitochondria produce excess superoxide and $H_2O_2$, which are the main factors of intracellular damage, further increasing endothelial dysfunction.

**Short non-hematopoietic erythropoietin-based peptides as innovative atheroprotectors:** Research in recent decades has shown that erythropoietin has a high cytoprotective activity, which is mainly associated with exposure to the mitochondrial link and has been confirmed in various experimental models. There is also a short-chain derivative, the 11-amino acid pyroglutamate helix B surface peptide (PHBSP), which selectively binds to the erythropoietin heterodymic receptor and reproduces its cytoprotective properties. This indicates the promising use of short-chain derivatives of erythropoietin for the treatment and prevention of atherosclerotic vascular injury. In the future, it is planned to study the PHBSP derivatives, the modification of which consists in adding RGD and PGP tripeptides with antiaggregant properties to the original 11-member peptide.

## Keywords

Atherosclerosis, cytoprotection, epor/cd131, erythropoietin.

## Relevance

The high contribution of cardiovascular pathology to the overall structure of the causes of death and disablement in developed countries calls for in-depth study and improvement of correction methods. At the same time, the main task of research in this area is to find ways to prevent and treat atherosclerotic processes as the main cause of cardiovascular mortality.

Atherosclerotic vascular occlusion, as well as thrombosis and embolism following plaque rupture, lead to partial or complete ischemisation of the affected basin, the clinical consequences of which depend on the localisation and calibre of the vessel. The most significant from an epidemiological point of view is coronary and cerebral vascular lesions, leading to such nosologies as coronary heart disease (CHD) and ischemic stroke (Herrington et al. 2016).

Since the middle of the 20$^{th}$ century, most high-income countries have experienced a sharp decline in mortality due to CHD and brain stroke. For example, in the United Kingdom, cardiovascular mortality rates for middle-aged men (35-69 years old) fell from around 700 per 100,000 per year in 1950 to <200 by 2010, and for middle-aged women from ≈450 in 1950 to <100 by 2010. Most low- and middle-income countries have also reported a decline in stroke-related deaths over the past few decades. However, trends in CHD mortality in these regions have been less consistent: some countries have reported a decline, while others have reported an increase (especially in some Eastern European and Asian countries) in CHD mortality (Bennett et al. 2014; Moran et al. 2014).

*The Global Burden of Disease 2010 Study* showed that overal, global age-standardized death rates for both CHD and stroke decreased between 1990 and 2010, with a much higher rate in developed countries than in developing countries (GBD 2013 Mortality and Causes of Death Collaborators 2015). Nevertheless, despite relatively reassuring trends, CHD and ischaemic stroke remain the leading causes of premature adult mortality worldwide, indicating the highest importance of research into the underlying atherosclerotic process.

## Lipid profile and atherogenesis

The introduction of effective methods for preventing and correcting the atherosclerotic process directly depends on a multi-level understanding of the pathophysiology of the vessel wall lesion. Molecular-biological, instrumental and other research methods provide consistent information on new aspects of the progression of atherosclerosis and its clinical complications. These advances have not only deepened the understanding of the details of atherogenesis, but have also led to the discovery of many unexpected facts that have called into question many previously dominant concepts, and allowed the development of new therapeutic approaches to combat cardiovascular disease (Libby et al. 2016).

It is now known that the initial event in the development of atherosclerosis is endothelial damage. Endothelial dysfunction (ED) causes infiltration and accumulation of low-density lipoprotein cholesterol (LDL) in the subendothelial space. In the pathological conditions, LDLs can interact with ROS to form oxidized LDL (oxLDLs) (Ketelhuth and Hansson 2011). Modified lipoprotein particles increase the expression of cell adhesion molecules (VCAM-1, P, and E-selectin) in endothelial cells, resulting in the recruitment of white blood cells (mainly monocytes and T-lymphocytes) into the subendothelial space. These inflammatory cells migrate into the intima during the interaction of protein-chemoattractants, such as of monocyte MCP-1, eotaxin, and interferon-γ. Monocytes are differentiated into macrophages, express scavewnger receptors CD36, SRA, and LOX-1 and internalize modified lipoproteins. Due to their appearance, macrophages loaded with lipids are called foam cells, and their presence in the arterial wall is a sign of an early atherosclerotic lesion. T-lymphocytes and mast cells that migrate to the intima, along with the foam cells, secrete a wide profile of cytokines, which contribute to inflammation and the formation of reactive oxygen species (ROSs). The growth factors released by these cells and ROSs stimulate the migration of smooth muscle cells and collagen deposition, which leads to the development of atheromatous plaque (Kattoor et al. 2017).

In 1912, at a meeting of the Society of Russian Physicians in St. Petersburg, a major Russian scientist, N.N. Anitschkow, whose name is now used for the prize for the most outstanding research in the field of atherosclerosis, together with S.S. Khalatov, presented the first results of his revolutionary research to identify the relationship between nutritional factors, blood cholesterol levels, and atherosclerosis.

The main environmental factors determining blood cholesterol concentration are the consumption of saturated and polyunsaturated fats as well as cholesterol in food (Keys et al. 1957; Hegsted et al. 1993; Clarke et al. 1997), although metabolic, genetic and other factors also influence cholesterol levels (Dattilo and Kris-Etherton 1992). The geographical prevalence of atherosclerosis-associated diseases demonstrates their high dependence on national diets around the world. Atheroprotective diets associated with a lower cardiovascular risk have been identified

separately (Mozaffarian et al. 2011). Blood cholesterol (in particular, low-density lipoprotein cholesterol) is a widely recognised risk factor for CHD, as confirmed by a large number of prospective observational studies from around the world (Lewington et al. 2007; Baigent et al. 2010).

During the initial phase of atherosclerosis, the process of fat infiltration of the vascular wall (fatty streak stage) is initiated. Macrophages absorb apoB-containing lipoproteins, which break down in lysosomes, whereas excess free cholesterol enters the endoplasmic reticulum and is esterified by a cholesterol enzyme – cholesterol acyltransferase. The resulting cholesterol ether is packaged in cytoplasmic lipid droplets, which are characteristic of foam cells (Brown et al. 1979; Brown and Goldstein 1983; Rogacev et al. 2012). The chemical modification of ApoB lipoproteins in the form of oxidation and glycation enhances their absorption through a number of receptors (CD36, acceptor receptor A and a family of lectin-like receptors) that are not affected by negative feedback from excess cholesterol (Moore and Freeman 2006; Younis et al. 2008; Polonikov et al. 2017). Enzyme-mediated ApoE lipoprotein aggregation increases lipid absorption through phagocytosis (Torzewski et al. 2004; Torzewski and Lackner 2006), while native lipoprotein residues can stimulate the formation of foam cells through the ApoE receptor family (LRP1 and VLDLR) (Fujioka et al. 1998; Schwartz and Reaven 2012). Native LDL uptake through liquid-phase pinocytosis may also stimulate the formation of foam cells (Anzinger et al. 2012; Kruth 2013).

In addition to atherogenic lipoproteins, there are also atheroprotective ones, which are responsible for transporting cholesterol away from the vascular wall, thus reducing its lipid infiltration. The greatest importance was demonstrated for the ApoA-I and HDL proteins that transport cholesterol to the liver. In addition to ApoA-I and HDLs, ApoE production by macrophages is crucial in preventing the formation of atherosclerotic lesions. Most ApoE in plasma is produced by the liver, but about 5-10% is synthesized by macrophages (Linton et al. 1998). ApoE serves as a ligand for hepatic capture and clearance of all the lipoproteins in the ApoB group, except LDLs. Knocking out the ApoE gene in mice leads to hypercholesterolemia and spontaneous development of an atherosclerotic lesion (Zhang et al. 1992; Plump et al. 1992). Therefore, ApoE-deficient mice are some of the most routine models for studying the mechanisms of development of atherosclerotic lesions. In studying the role of macrophage ApoE in lipoprotein metabolism, its crucial role in lipoprotein metabolism between the vascular wall and blood was demonstrated, making ApoE one of the most important targets for atherosclerotic treatment. Interestingly, the atheroprotective effect of ApoE is implemented through several mechanisms. First, the expression of ApoE by hematopoietic stem cells reduces the proliferation and infiltration of monocytes into the intima (Murphy et al. 2011). In addition, ApoE reduces lysosomal accumulation of cholesterol by increasing lipase expression (Wu et al. 2007). It is important that ApoE secretion by macrophages stimulates outflow in the absence and presence of exogenous acceptors, including HDLs and ApoA-I, which do not contain lipids (Mazzone and Reardon 1994; Langer et al. 2000; Huang and Mazzone 2002). Recent studies have shown that macrophatic ApoE facilitates the reverse transport of cholesterol in vivo (Zanotti et al. 2011). It also stimulates the outflow of phospholipids and cholesterol through ABCA1, ABCG1, and SR-BI (Chroni et al 2005; Yancey et al. 2007a; Kim et al. 2007; Lammers et al. 2009). In addition to cholesterol outflow, macrophage ApoE prevents inflammation (Yu et al. 2004; Ali et al. 2005; Jofre-Monseny et al. 2007; Li et al. 2015) and oxidative stress (Miyata and Smith 1996; Maor et al. 2000; Rosenblat et al. 2002; Rosenblat and Aviram 2002). Local ApoE production is likely to be a critical atheroprotective mechanism, given that the content of atherosclerotic plaques has limited access to ApoA-1 and HDLs dissolved in plasma (Fazio et al. 1997; Fazio et al. 2002). Three common polymorphisms of ApoE have been identified in the human population, which are of predictorial value in assessing the risk of CHD regardless of plasma cholesterol levels (Davignon et al. 1988). ApoE3 is the most common isoform and is functionally similar to mouse ApoE. In contrast to isoforms ApoE3 and ApoE2, ApoE4 isoform is incapable of either stimulating cholesterol outflow (Cullen et al. 1998; Gong et al. 2002; Okoro et al. 2012) or preventing inflammatory and oxidative activation (Rosenblat and Aviram 2002; Yu et al. 2004; Huebbe et al. 2010). The dysfunction of ApoE4 in the host leads to an increased risk of CHD compared to people expressing ApoE3 or ApoE2 (heterozygotes) (Utermann et al. 1984; Davignon et al. 1988; Song et al. 2004).

Thus, the experience gained from N.N. Anichkov's first work demonstrates that in the pathogenesis of atherosclerosis, correlated (environmental) factors must be considered inextricably linked with genetic predisposition (Zárate et al. 2016). This fact leads to a high proportion of research on cardiovascular diseases in the Genome-Wide Association Studies (GWAS) programme and the introduction of the found associations in the early genetic diagnosis panel. The emergence of GWAS and Mendelian randomization analysis opened a new era in the study of the pathobiology of atherosclerosis. Modern genetics has confirmed the causal role of LDLs in atherogenesis, but has also identified new targets that could revolutionize the treatment of the atherosclerotic process.

## Inflammation and atherogenesis

Activation of pro-inflammatory cascades in macrophages and endothelium is an essential link in atherogenesis. Activated macrophages are prone to an increased generation of reactive oxygen species, increased cholesterol capture and cytokine/chemokine secretion, resulting in greater LDL oxidation, endothelial cell activation, monocyte recruitment, and foam cell formation (Brand et al.

1993; Wang et al. 2002; Bolick et al. 2009; Adamson and Leitinger 2011; Colin et al. 2014; Peled and Fisher 2014). Oxidative stress, modified lipoproteins and other factors (bioactive lipids, molecular patterns associated with damage, cytokines) stimulate inflammation through their own receptors (Adamson and Leitinger 2011; Chinetti-Gbaguidi and Staels 2011; Colin et al. 2014). In addition, plasma membrane cholesterol in foam cells increases the transmission of signals through inflammatory receptors (Ye et al. 2011; Tall and Yvan-Charvet 2015). The atherogenic role of IL-1β- and IL-18-mediated inflammatory activation has recently been confirmed (Duewell et al. 2010; Lu and Kakkar 2014): the clinical study has shown that subjects receiving a monoclonal antibody to IL-1β, kanakinumab, had a significantly lower frequency of repeated cardiovascular events, which did not depend on cholesterol reduction (Ridker et al. 2018).

The formation of macrophage foam cells and transmission of cholesterol-dependent inflammatory signals can be reduced by means of removing cholesterol by atheroprotective HDLs and ApoA-Is using a number of mechanisms, including ABCA1-, ABCG1-, and SR-BI-mediated elimination and water diffusion (Ye et al. 2011; Yancey et al. 2007b; Kellner-Weibel and de la Llera-Moya 2011). In addition, HDLs and ApoA-Is protect against atherosclerosis by reducing inflammation using mechanisms independent of cholesterol outflow (Tang et al. 2009; Kratzer et al. 2014). In addition, small non-coding RNAs have been found to influence the development of atherosclerosis by regulating inflammation and/or homeostasis of cholesterol in different types of cells in lesion foci (Michell and Vickers 2016; Donaldson et al. 2018). MiR-33a and MiR-33b contribute to atherosclerosis by disrupting cholesterol outflow and promoting inflammatory macrophage conversion (Rayner et al. 2011; Horie et al. 2012; Nishino et al. 2018). Other microRNAs, including MiR-223 and MiR-93, have an atheroprotective effect by increasing cholesterol outflow and facilitating the conversion of macrophage phenotype into anti-inflammatory M2 (Donaldson et al. 2018; Zhuang et al. 2012; Vickers et al. 2014; Ganta et al. 2017).

## Endothelial and mitochondrial dysfunction in the development of atherosclerosis

Currently, studying the pathobiological processes occurring in endothelial cells in atherosclerosis has led to the understanding that one of the most significant factors in atherogenesis is mitochondrial dysfunction. Mitochondria are a cell metabolic centre that synthesises the cell's main energy currency, adenosine 5'-triphosphate (ATP), through an oxidative phosphorylation reaction. However, the role of mitochondria in eukaryotic cells goes beyond their ability to act as metabolic mediators. These cellular organelles regulate various cellular processes, including proliferation (Mitra et al. 2009), an immune response (Zhou et al. 2011a), apoptotic cell death (Kroemer et al. 2007), and also participate in the transmission of intracellular signals to the nucleus (Al-Mehdi et al. 2012). Cells in mitochondria vessels are involved in the regulation of a wide range of biological processes. Transgenic mice devoid of a number of mitochondrial proteins usually die during embryogenesis, which coincides with the development of the cardiovascular system, or are prone to the development of a cardiovascular pathology (Miller et al. 2010; Shenouda et al. 2011; Dong et al. 2013; Kröller-Schön et al. 2013).

Direct contact between endothelial cells and the bloodstream means that they are particularly vulnerable to damage from molecules circulating in the blood, on the one hand, and that they play an essential "protective" role, on the other hand. ED is associated with the development of almost all vascular diseases. Compared to cells of other types with higher energy needs, the content of mitochondria in endotheliocytes is relatively low. In rat endothelials, for example, mitochondria account for 2-6% of cell volume, whereas in hepatocytes and cardiomyocytes – 28% and 32%, respectively (Dromparis and Michelakis 2013; Kluge et al. 2013). The low content of mitochondria in endothelium indicates that mitochondrial-dependent oxidative phosphorylation does not contribute significantly to the energy supply of this tissue. In fact, endotheliocytes receive most of their energy from anaerobic glycolytic glucose metabolism. For example, in cultured endothelial cells, more than 75% of pig aorta ATPs is provided by glycolysis (Culic et al. 1997). Similar data indicate that mitochondria mainly serve as important signaling organelles in endothelial and vascular vessels (Quintero et al. 2006).

A large number of studies show a causal relationship between mitochondrial and endothelial dysfunctions. Phenotypic endotheliocyte dysfunction is highly correlated with mitochondrial biogenesis dysfunction, reduced mitochondrial mass and altered expression of electron transport chain (ETC) components (Ungvari et al. 2008; Dai et al. 2012). Such processes correspond to natural processes occurring in endotheliocytes during aging. Damaged mitochondria produce excess superoxide and $H_2O_2$, which are the main determinants of cellular aging (Passos et al. 2007).

Mitochondria are an important source of ROSs and at the same time have an antioxidant function. Although the majority of electrons passing through the redox gradient of the ETC eventually reach the V complex, 1-3% of electrons react prematurely with oxygen in complexes I and III to form superoxide and other types of ROSs known as mROSs (Quintero et al. 2006). Mitochondrial ROSs (mROSs) play a signal role in initiating cellular responses to stress. Changing transmembrane potential is an important factor that triggers excess mROS production under pathological conditions, including hypercholesterolemia, hyperglycemia, smoking, infections, and hypoxia. In ad-

dition to complexes I and III, other sources of mROSs have been identified in endotheliocytes. One of them is NADPH oxidase 4 (Nox4) (Lassègue et al. 2012).

Factors that modulate the production of endothelial mROSs have been proved to participate in atherosclerosis. One of such examples is a protein adapter, p66Shc. The fact that the expression of p66Shc may be relevant to the cardiovascular function is evident from the observation that mice knocked out by p66Shc are protected from ROS-dependent age-related ED (Francia et al. 2004), ED caused by hyperglycemia (Zhou et al. 2011b), and alimentary atherosclerosis (Napoli et al. 2003; Martin-Padura et al. 2008).

Thus, according to the modern understanding of the pathogenesis of cardiovascular diseases, mitochondrial dysfunction plays a significant role in the development of atherosclerosis and related complications (Marzetti et al. 2013; Chistiakov et al. 2018). Such changes in endotheliocytes and myocytes of the vessel wall lead to structural and functional disorders, manifested in the progression of atherosclerosis, hypertension and susceptibility to thrombosis (Peng et al. 2019). Dysfunction of mitochondria in other cells, including neurons and cardiomyocytes, leads to a decrease in their resistance to ischaemia, which is manifested in an increase in lethal outcomes against the background of brain stroke, coronary artery occlusion, infarction of kidneys and other organs. The emerging pathogenetic cascade is becoming a relevant target for a pharmacological intervention.

## Short erythropoietin-based non-hematopoietic peptides as innovative atheroprotectors

Based on available information on the pathogenesis of atherosclerosis, one approach to treating atherogenesis is to use drugs with cytoprotective and mitochondrial activities. The current arsenal of drugs with a mitochondrial-oriented mechanism of action includes metabolic enchancers (emoxypine and its derivatives, racetames), metabolic regulators (trimetazidine, mildronate), and antioxidants (Skulachev ions, SkQ). Preclinical studies in vitro and in vivo in recent decades have shown that a 34-kDa erythropoietin (EPO), an endogenous stimulant of erythrogenesis has a high cytoprotective activity, which is mostly associated with exposure to the mitochondrial link and has been confirmed in experimental models of ischemic and traumatic lesions of virtually all organs, including endothelium, myocardium, and the brain (Qin et al. 2014; Millet et al. 2016; Nekoui and Blaise 2017). These properties make it possible to consider EPO as an agent with a universal cytoprotective orientation. The remote (hematopoiesis) and paracrine action is associated with the ability of EPO to influence the cell cycle and to reduce the pro-apoptic orientation of cells, but the erythropoietic action is mediated by the homodymeric receptor complex of the erythropoietin receptor (EPOR/EPOR), while the cytoprotective, antioxidant and anti-inflammatory actions are mediated by the heterodymeric complex of EPOR/CD131 (Brines et al. 2004).

Despite the promising results of preclinical studies, information about the EPO antiapoptic activity received by hundreds of research teams is poorly translated into clinical reality. As a result, the search for molecules that selectively mimic the cytoprotective effects of EPO is one of the important tasks of modern pharmacology. To date, compounds with an amino acid sequence identical to that of EPO, but differing in carbohydrate fragment, have become very common. Such modifications improve the pharmacokinetics and reduce the ability to influence hemopoiesis, preserving the tissue-protective activity of the molecule. However, these drugs have not demonstrated high clinical efficacy as cytoprotectors either, possibly due to their low ability to penetrate histogematic barriers. A principally novel approach is the search for low-molecular peptide derivatives of EPO, which have an antiapoptic activity and freely penetrate through biological barriers. The first of such derivatives is an 11-amino acid peptide PHBSP, which is an amino acid chain "Pyr-Glu-Gln-Leu-Glu-Arg-Ala-Leu-Asn-Ser-Ser" and has a selective affinity for the heterodymeric complex EPOR/CD131 (Brines et al. 2004). In developing this peptide, the authors relied on the amino acid sequence of alpha-spiral B erythropoietin, since this is the only structural part of the molecule that does not participate in the spatial interaction with the homodymeric complex EPOR/EPOR. Several studies have shown that PHBSP has more pronounced antiapoptic properties than the basic molecule, exhibits an analgesic activity and does not have a hemopoietic effect intrinsic to EPO (Brines et al. 2004; Swartjes et al. 2014; Zhang et al. 2017; Yang et al. 2019). However, a significant disadvantage of PHBSP is its very short half-life, approaching several minutes. In addition, studies carried out in our laboratory in vivo and in vitro have revealed a pro-thrombotic activity in this peptide, which is to some extent characteristic of all erythropoietin molecules.

## Conclusion

In our view, a modification of PHBSP to improve the pharmacokinetic and pharmacodynamic parameters may be a further promising development of a short-chain-peptide-based CVD pharmacotherapy. To solve this problem, our team, together with the scientific team of A. Titze (University of Gothenburg), has worked on searching for the ways of modifying the base molecule. To eliminate the pro-thrombotic activity, it was decided to add the amino

acid sequence RGD (Arg-Gly-Asp) to PHBSP. It is known that this motif has pronounced antiaggregant properties (Pytela et al. 1986, Sheu et al. 1995, Hung et al. 2017). In parallel, the PGP motive (Pro-Gly-Pro) was added to a number of compounds to improve their pharmacokinetic characteristics. This amino acid sequence stabilizes the molecule in biological media by inhibiting the activity of proteolytic enzymes (Shevchenko et al. 2019). Moreover, PGP also has the ability to block the angiotensin-converting enzyme (Wang et al. 2011), one of the most important proatherogenic factors that catalyzes the reaction of angiotensin II formation and promotes vascular wall remodeling (Montezano et al. 2014). A number of Russian studies have also identified the antithrombotic and antiaggregant properties of PGP (Pastorova et al. 2001; Lyapina et al. 2007; Liapina et al. 2010), making its adherence to PHBSP even more promising. The key question remains the nature of embedding RGD and PGP motifs in the base molecule. A bioinformatic analysis has made it possible to determine the most optimal localizations for joining tripeptides, which do not affect the pharmacophores of interest, preserving both cytoprotective (Korokin et al. 2019; Korokin et al. 2020) and antiaggregant activities. Further in vitro and in vivo studies will open up the possibility of screening the most promising compounds.

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## Conflict of interest

The authors have no conflict of interest to declare.

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