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Molecular mechanisms of LDL-cholesterol induced endothelial dysfunction

Molekulární mechanismy LDL-cholesterol indukované endoteliální dysfunkce

Bachelor's thesis

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Poděkování:

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Prohlášení:

Prohlašuji, že jsem závěrečnou práci zpracovala samostatně a že jsem uvedla všechny použité informační zdroje a literaturu. Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

V Praze, 6.5.2021 Tereza Rojková

Abstract:

Hypercholesterolemia, defined as elevated LDL-cholesterol levels in the blood, develops either as a result of genetic predispositions, unhealthy lifestyle, underlying diseases, or as a result of a combination of these factors. LDL-cholesterol is sometimes called "bad" cholesterol because its excess negatively affects the vessel`s innermost layer - endothelium, Endothelium is unique for its vasodilatory, vasoconstrictive, anti-inflammatory, and anti-coagulant function but also for its ability to control vascular permeability. In the case of hypercholesterolemia, cholesterol gradually accumulates in the subendothelial space and reduces by several mechanisms levels of the main modulatory molecule of the endothelium - nitric oxide. In endothelial dysfunction, oxidative stress increases, LDL-cholesterol is oxidized, endothelial cells are activated and produce proinflammatory cytokines and adhesive molecules. Endothelial dysfunction is considered the first stage of atherosclerosis, as monocytes enter the site of inflammation and differentiate into macrophages, which subsequently turn into foam cells by endocytosis of oxidized LDL-cholesterol. In this way, atherosclerotic plaques are formed, which not only narrow the blood vessels, but plaques can erupt, causing subsequent thrombosis, which can result in another very serious cardiovascular disease and even death. This thesis summarizes the main mechanisms by which LDL-cholesterol modulates endothelial dysfunction and subsequent formation of atherosclerotic plaques.

Keywords: endothelial dysfunction, nitric oxide, atherosclerosis, endothelial cell, cardiovascular disease, cholesterol, hypercholesterolemia, endothelium

Abstrakt:

Hypercholesterolémie neboli zvýšená hladina LDL-cholesterolu vzniká ať už jako důsledek genetických predispozic, nevhodného životního stylu, přidružených chorob či kombinací těchto faktorů. LDL-cholesterolu se někdy přezdívá „zlý“ cholesterol, neboť jeho nadbytek negativně ovlivňuje funkčnost výstelky cév-endotelu, který se vyznačuje svými různorodými účinky, zejména vazodilatační, vazokonstrikční, protizánětlivou a protisrážlivou funkcí, ale také kontrolou propustnosti cév pro látky potřebné tělu a pro buňky imunitního systému. V případě hypercholesterolémie cholesterol postupně kumuluje v subendotheliálním prostoru a vícero mechanismy snižuje hladinu hlavní modulační molekuly endotelu – oxidu dusnatého. Při endotelové dysfunkci vzrůstá oxidační stres, LDL-cholesterol je oxidován a endotelové buňky se aktivují a produkují prozánětlivé cytokiny a adhezivní molekuly. Endotelová dysfunkce je považována za první stupeň aterosklerózy, jelikož se monocyty dostávají do místa zánětu a diferencují v makrofágy, které se následně mění v pěnové buňky endocytózou oxidovaného LDL-cholesterolu. Tímto způsobem jsou vytvářeny aterosklerotické pláty, které nejen cévy zužují, ale také hrozí jejich erupce s následnou trombózou, která může vyústit v další velice závažné kardiovaskulární choroby až smrt. Tato práce shrnuje mechanizmy, jakými LDL-cholesterol moduluje dysfunkci endotelu a následně vznik aterosklerotických plátů.

Klíčová slova: endotelová dysfunkce, oxid dusnatý, ateroskleróza, endotelová buňka, kardiovaskulární onemocnění, hypercholesterolémie, endotel

TABLE OF CONTENTS

TABLE OF FIGURES

INTRODUCTION

According to World Health Organization, cardiovascular diseases are the number one cause of death at a global level, prevailing in the middle to high-income countries (World Health Organization, 2020). Even Czech Health Statistics from 2018 states that cardiovascular diseases were the most common cause of hospitalization, and in that year, the biggest part (34.8 %) of total distributed drugs were the ones to treat cardiovascular diseases (Ústav zdravotnických informací a statistiky ČR, 2019).

Endothelium, a monolayer of endothelial cells that lines vessels and the heart, plays a major role in maintaining normal cardiovascular functions. The endothelium produces various molecules with vasodilation, vasoconstriction, anti-inflammatory, and anti-thrombotic functions, and nitric oxide (NO) was found to be a pivotal molecule in maintaining vascular homeostasis by controlling these processes. Endothelial dysfunction, characterized by reduced NO bioavailability, is recognized as a key early step in the development of atherosclerosis and is an important prognostic marker for cardiovascular disorders. Risk factors including genetic predispositions and lifestyle choices like smoking, high-fat diet, or physical inactivity can cause impairment of the function of the endothelium (Mudau *et al.*, 2012).

Molecular mechanisms involved in the regulation of endothelium cell function are complex and are beyond the scope of this thesis to review each topic. Instead, a detailed view of the molecular basis behind endothelial dysfunction induced by hypercholesterolemia is presented. The main goal of this work is to describe endothelium, its physiological and pathophysiological roles emphasizing the principal events associated with oxidative stress and vascular inflammation resulting from chronic elevation of plasma LDL-cholesterol (LDL-C).

1 ENDOTHELIUM AND ITS PHYSIOLOGICAL FUNCTIONS

1.1 ENDOTHELIAL STRUCTURE

Endothelium, composed of approximately 1-6x10^{13} cells and extending over an area of 3000 - 6000 m^2 in the human body, is a monolayer of cells that are lining the lumen of blood and lymphatic vessels and the heart. The monolayer of cells is attached to the basement membrane by its basolateral surface. The basement membrane composes mainly of proteoglycans, type IV collagen, and laminin (Kramer *et al.*, 1985; Krüger-Genge *et al.*, 2019).

The individual endothelial cells (ECs) are of an oval shape with an approximate length of 30-50 µm, a width of 10-30 µm, and a thickness of 0.1-30 µm. The apical side of the cells is in direct contact with the blood. The endothelium is found in arteries, veins, and capillaries. While the thin capillary wall is composed only of endothelial cells and surrounding pericytes, in larger vessels, the endothelium is part of the much thicker vascular wall. In the latter, ECs represent the innermost layer called tunica intima, which is surrounded by tunica media (middle layer mainly composed of vascular smooth muscle cells) and tunica externa or so-called tunica adventitia (outer layer consisting mainly of collagen). Even though the thickness of tunica media differs between the blood vessel types like arteries and veins, the endothelium's thickness is similar all around the body (Krüger-Genge *et al.*, 2019).

1.2 ENDOTHELIAL FUNCTION

1.2.1 FROM INTACT BARRIER TO VARIOUS FUNCTIONS

In 1847, for the first time, a barrier between a blood flow and adjacent tissue was described by Schwann (Schwann *et al.*, 1847). For many years, it was thought that this layer is nothing more than just an intact barrier. However, the first successful isolation of ECs was done in 1973 by Eric A. Jaff, and that gave rise to research and discoveries about diverse endothelial physiological functions (Jaffe *et al.*, 1973). A groundbreaking discovery was that the ECs produced an endothelium-derived relaxing factor that caused vasodilatation (Furchgott and Zawadzki, 1980). After seven years, this molecule was identified as nitric oxide (NO) (Ignarro *et al.*, 1987). For these discoveries, Robert Furchgott, Ferid Murad and Louis Ignarro were awarded a Nobel prize in 1998. The fact that endothelium-derived relaxing factor stands for NO was confirmed in a follow-up work by Palmer, Ashton and Moncada in 1987 (Palmer *et al.*, 1988).

The endothelium is essential for maintaining vessel homeostasis. It works as an important endocrine organ that responds to chemical and physical impulses and controls blood flow via regulating vascular tone (vasodilation and vasoconstriction) together with coagulation. Regulation of permeability, inflammation, thrombosis, and fibrinolysis are other pivotal roles of the endothelium. The endothelium also plays a role in angiogenesis – the blood vessel formation process (Krüger-Genge *et al.*, 2019).

1.2.2 PERMEABILITY

The endothelium of blood and lymphatic vessels defines the barrier between the blood and lymph fluids, respectively, to the free diffusion of fluids and of small or large molecules. It determines the bidirectional transport of vital substances (i.e., nutrients, electrolytes, oxygen, and hormones) to supply body tissues and to discard metabolic waste products (i.e., urea, carbon dioxide). The transport involves paracellular (via the inter-endothelial connections) and transcellular (via the EC) pathways. Apart from the mentioned substances moved in and out of cells, plasma proteins like albumin, fibrinogen, insulin, and low-density lipoproteins are transported via caveolae (Vogel *et al.*, 2001; Wang *et al.*, 2006).

The luminal side of endothelium is protected by glycocalyx, of which function is to stabilize vascular endothelium, and it acts as a mechanotransducer of shear stress (Van Den Berg *et al.*, 2009). The glycocalyx is a thin layer of macromolecules, first visualized in 1966 by electron microscopy (Luft, 1966). Its major components are sialic acid and glycosaminoglycans linked to the subclass of membrane-bound glycoproteins – proteoglycans (Buonassisi, 1973; Pries *et al.*, 2000). The most abundant proteoglycans are syndecan and glypican. Glycosaminoglycans include heparan sulfate and chondroitin sulfate that are covalently bound to the proteoglycans. Meanwhile, another glycosaminoglycan, hyaluronic acid, is bound to its receptor CD44 (Aruffo *et al.*, 1990). The organization of heparan sulfate and hyaluronic acid gives glycocalyx the ability to work as a molecular sieve (Fan *et al.*, 2019).

The paracellular route, through the intercellular junctions, including tight and adherens junctions, enables passage to polar solutes with less than 3 nm of molecular radii like urea or ions (Mehta and Malik, 2006). Depending on the demand of organs for vascular permeability, the concentration of the various cell-cell junctions varies. Tight junctions are common in large arteries facing strong blood flow. They are also crucial in the blood-brain barrier, where the permeability is limited (Hori *et al.*, 2004).

Transcellular pathway or so-called transcytosis is a movement of large molecules or substances by way of vesicles that includes endocytosis – movement into the cell and exocytosis – out of the cell. The bigger molecules, such as albumin, LDL, or ox-LDL, travel via these pathways (Ghitescu *et al.*, 1986; Kima *et al.*, 1994; Mehta and Malik, 2006; Vasile *et al.*, 1983). The transcellular pathway includes both pinocytosis and receptor-mediated transcytosis. Receptor-mediated transcytosis is arranged mainly by cytoplasmic membrane invaginations called caveolaes (Yamada, 1955). The main component of caveolaes in ECs is a membrane protein caveolin-1 (Rothberg *et al.*, 1992).

1.2.3 LEUKOCYTE TRANSENDOTHELIAL MIGRATION

Under a variety of physiological conditions, interactions between leukocytes and endothelium occur. That accounts for the migration of leukocytes to lymphoid organs and in contrast to the migration of leukocytes to any other tissues, it does not cause inflammation. The leukocyte migration through endothelium is a carefully controlled

process consisting of a few steps in which different adhesive molecules have to be expressed by ECs and leukocytes. Firstly, the leukocytes are captured, and then they roll on the endothelium. For that, ECs express especially E-selectin, originally named endothelial-leukocyte adhesion molecule-1 (ELAM-1) (Bevilacqua *et al.*, 1987; Phillips *et al.*, 1990). E-selectin is a receptor containing a lectin domain that binds carbohydrates (Bevilacqua *et al.*, 1989). For strong adhesion that follows leukocyte's rolling, the ECs need to express intercellular adhesion molecules 1 and 2 (ICAM-1 and ICAM-2) and vascular cell adhesion molecule 1 (VCAM-1). It was found that ICAM-2 is expressed independently on the cytokine levels (Nortamo *et al.*, 1991). On the other hand, the expression of ICAM-1 and VCAM-1 is dependent on cytokine stimulation. ICAMs and VCAMs are counter receptors of integrins presented on the plasma membrane of leukocytes (Springer, 1990). Lastly, leukocytes travel through the endothelial cell-cell junctions. This process is called diapedesis and is controlled mainly by platelet endothelial cell adhesion molecule-1 and junctional adhesion molecules, accumulated at the intercellular junctions and components of tight junctions, respectively (Muller *et al.*, 1993; Ostermann *et al.*, 2002).

1.2.4 VASODILATATION

As mentioned before, NO, a small colorless gas, was identified as a molecule that causes vasodilatation (Furchgott and Zawadzki, 1980). NO activates guanylyl cyclase, leading to cGMP formation, resulting in vascular smooth muscle cells relaxation. NO in ECs is synthesized from L-arginine by a Ca^{2+}/calmodulin-dependent membrane-bound enzyme called endothelial nitric oxide synthase (eNOS) located in caveolaes (Palmer *et al.*, 1988; Pollock *et al.*, 1991; Radomski *et al.*, 1990; Shaul *et al.*, 1996). Activation of eNOS is caused by changes in intracellular calcium concentration, which mainly occur as a response to shear stress but is also mediated via receptors for acetylcholine, bradykinin, angiotensin II and insulin (Pueyo *et al.*, 1998; Ziegler *et al.*, 1998). The NO synthesis pathway is shown in Figure 1.

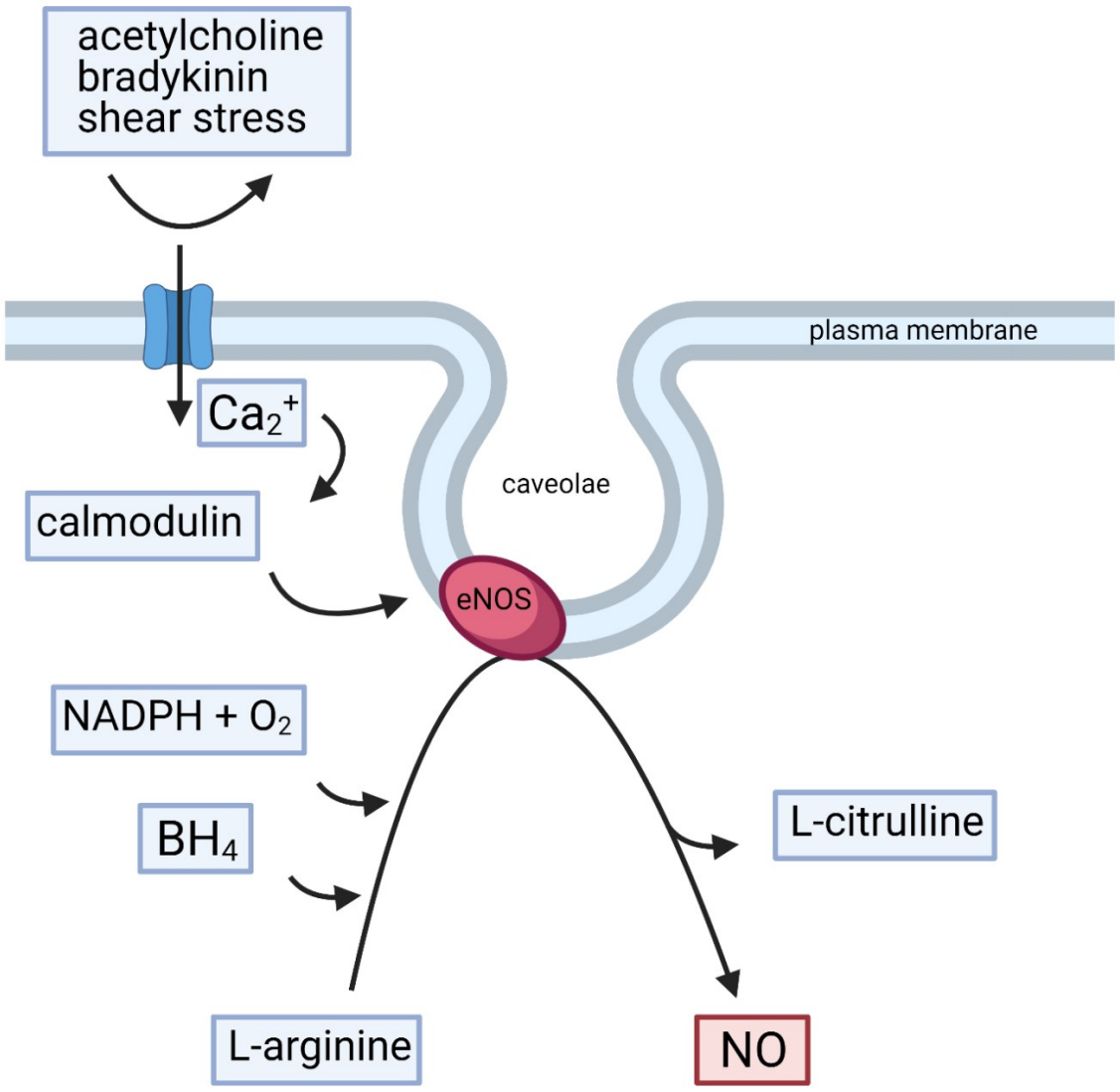

Figure 1: NO synthesis pathway

Acetylcholine, bradykinin, or shear stress were found to cause intercellular Ca^{2+} influx. Inside the cell, Ca^{2+} binds to calmodulin. This complex further activates eNOS. Activated eNOS produces NO and a side product L-citrulline from L-arginine. Important cofactors in this synthesis are BH_4 and NADPH; the presence of oxygen is necessary. Created with BioRender.com.

Vasodilatation is also mediated by prostacyclin (PGI2), a member of the eicosanoid family, synthesized during arachidonic acid metabolism. Its receptor is a G-protein-coupled receptor whose activation leads to the production of cAMP, a protein kinase A activator that causes vascular smooth muscle relaxation. PGI2 shows similar anti-thrombotic and anti-inflammatory functions as NO (Dusting *et al.*, 1977; Moncada *et al.*, 1977).

1.2.5 VASOCONSTRICTION

Vasoconstriction is mediated via peptide endothelin-1 (ET1) that was discovered in 1988 (Yanagisawa *et al.*, 1988). The presence of hypoxia, ischemia, shear stress or chemical stimuli like thrombin, angiotensin, insulin, LDL-C, or transforming growth factor β (TGF-β) activates the production of ET1. On the other hand, NO and PGI2 are inhibitors of ET1's function (Levin, 1996).

Angiotensin II is another peptide that causes vasoconstriction. It is converted from its precursor angiotensin I located in circulated blood by angiotensin-converting enzyme (ACE), an enzyme bound to the membrane of ECs (Cockcroft *et al.*, 1993).

1.2.6 ANTI-INFLAMMATORY AND ANTI-THROMBOTIC FUNCTION

Even though NO is known mainly for its function in the regulation of vascular tone, it plays a role in other critical processes that control vessel homeostasis. NO regulates platelet activity by inhibiting its aggregation and adhesion to endothelium (Radomski *et al.*, 1987). It was discovered that platelets also contain eNOS; therefore, platelets are NO producers (Cozzi *et al.*, 2015). In non-pathological states, ECs produce NO that establishes and maintains endothelium's anti-inflammatory function by stabilizing I-kappa B (IκB), an inhibitory factor of nuclear factor-kappa B (NF-κB) (Peng *et al.*, 1995). NF-κB, a proinflammatory transcription factor, mediates the synthesis of various inflammatory cytokines, chemokines, and adhesion molecules. Therefore, when NO is present, the synthesis of ICAM-1, VCAM-1, E-selectin, and cytokines like IL-6 and IL-8 is downregulated (De Caterina *et al.*, 1995). Inhibition of monocyte adhesion to endothelium and inhibition of vascular smooth muscle cell proliferation is another fundamental function of NO in the endothelium (Bath *et al.*, 1991; Garg and Hassid, 1989).

The glycocalyx is also involved in anti-inflammatory and anti-thrombotic processes, being a buffer that protects ECs from excessive bounding of leukocytes, platelets, or tumor cells and blocking the passage of plasma proteins to the ECs (Fan *et al.*, 2019).

2 ENDOTHELIAL DYSFUNCTION

2.1 CAUSES AND MECHANISMS OF ENDOTHELIAL DYSFUNCTION

Endothelial dysfunction can be described as changes in endothelial physiology that disrupt vessel homeostasis and normal stimuli responses. These changes are shifting the endothelium to reduced vasodilatation, vascular proinflammatory, and pro-thrombotic processes.

In a healthy state, vasodilators like acetylcholine, bradykinin, or shear stress producing NO predominate, whereas in endothelial dysfunction, reduced NO and an excessive release of vasoconstrictors result in an increased vascular tone. For the first time, impaired responses to the dilatating mediator acetylcholine were observed in rats with hypertension (Lockette *et al.*, 1986; Winquist *et al.*, 1984). The same effect was also observed during hypercholesterolemia and atherosclerosis (Ludmer *et al.*, 1986).

One of the many reasons for NO unavailability is the downregulation of eNOS synthesis after exposure to tumor necrosis factor α (TNF-α), hypoxia, or high concentration of oxidized low-density lipoprotein (ox-LDL) (Liao *et al.*, 1995; McQuillan *et al.*, 1994; Yan *et al.*, 2008). Another reason for NO unavailability lies either in inhibition of eNOS, for example, by caveolin-1 or by the altered function of eNOS (Ju *et al.*, 1997).

In normal conditions, eNOS is a dimeric enzyme, but in some conditions, for example, after exposure to peroxynitrite or in the presence of lower levels of tetrahydrobiopterin (BH_4), the dimer separates itself into monomers (Landmesser *et al.*, 2003; Zou *et al.*, 2002). This process is referred to as uncoupling of eNOS and represents the altered function of eNOS. Uncoupled eNOS produces reactive oxygen species (ROSs) instead of NO in a process so-called "oxidative stress" (Landmesser *et al.*, 2003).

ROS represent a group of free radicals that contain oxygen and are highly reactive with other molecules. A very common ROS is superoxide radical ($O_2^{\cdot -}$), which is a substrate for hydrogen peroxide production, another ROS. The levels of ROS in a body are balanced by antioxidants, reducing enzymes, and other molecules. The ROS are essential signaling molecules, but they can have a damaging effect when their amount exceeds (Bayir, 2005). Two different enzymatic sources of ROS are considered to be important - uncoupled

eNOSes and NAD(P)H oxidases. Overexpression of these oxidases leads to increased levels of ROSs (Rueckschloss *et al.*, 2001).

Activity and the effect of ROS play a role in impaired vasodilatation. There are several ways how ROSs disturb normal endothelial function. The first one is inhibition of NO by its reaction with ROS while forming peroxynitrite that contributes to more normal function disturbance like inhibiting soluble guanylate cyclase, a molecule essential for the NO signaling pathway, and oxidizing BH_4, whose importance was mentioned before (Gryglewski *et al.*, 1986; Weber *et al.*, 2001).

In addition, under oxidative stress, the oxidation of LDL occurs, which has a consequent negative impact on the ECs. This matter will be discussed in detail in the third chapter.

Elevated angiotensin II levels are another leading contributor to NO unavailability in endothelial dysfunction, with angiotensin II being a factor increasing arginase activity. Arginase is an enzyme that breaks down arginine, a substrate for NO synthesis (Berkowitz *et al.*, 2003).

Endothelial activation is part of endothelial dysfunction. Activation of endothelium is characterized by endothelial expression of ICAM-1, VCAM-1, and E-selectin, which promotes leukocyte adhesion and local inflammation. Endothelial activation is caused by activated NF-κB mediated by decreased bioavailability of NO and by proinflammatory cytokines, mainly by TNF-α and Interleukin-1 (IL-1) (Bevilacqua *et al.*, 1985; De Caterina *et al.*, 1995; Khan *et al.*, 1996).

2.2 RISK FACTORS OF ENDOTHELIAL DYSFUNCTION

Endothelial dysfunction has been associated with various risk factors, some of which are preventable by a healthy lifestyle, and some are independent of lifestyle choices; all are summarized in Figure 2.

The most preventable risk factor for developing endothelial dysfunction is smoking. It was confirmed that cigarette smoke elevates serum levels of cholesterol, triglycerides, very-low-density lipoprotein cholesterol (VLDL-C), and also LDL-C (Craig *et al.*, 1989). Next to that, cigarette smoke substances increase oxidative stress (Garbin *et al.*, 2009).

A sedentary lifestyle is another contributor to the development of endothelial dysfunction, and physical activity was proved to be protecting against developing endothelial dysfunction by many mechanisms, including upregulation of NO production by increased shear stress (Suvorava *et al.*, 2004; Wang *et al.*, 1993).

Hyperlipidemia and a high fat/cholesterol diet, as well as obesity and metabolic diseases like insulin resistance and diabetes, were all identified as initiators of endothelial impairment (Steinberg *et al.*, 1996; Vogel *et al.*, 1997). Aging and hypertension are also considered risk factors (Higashi *et al.*, 2012).

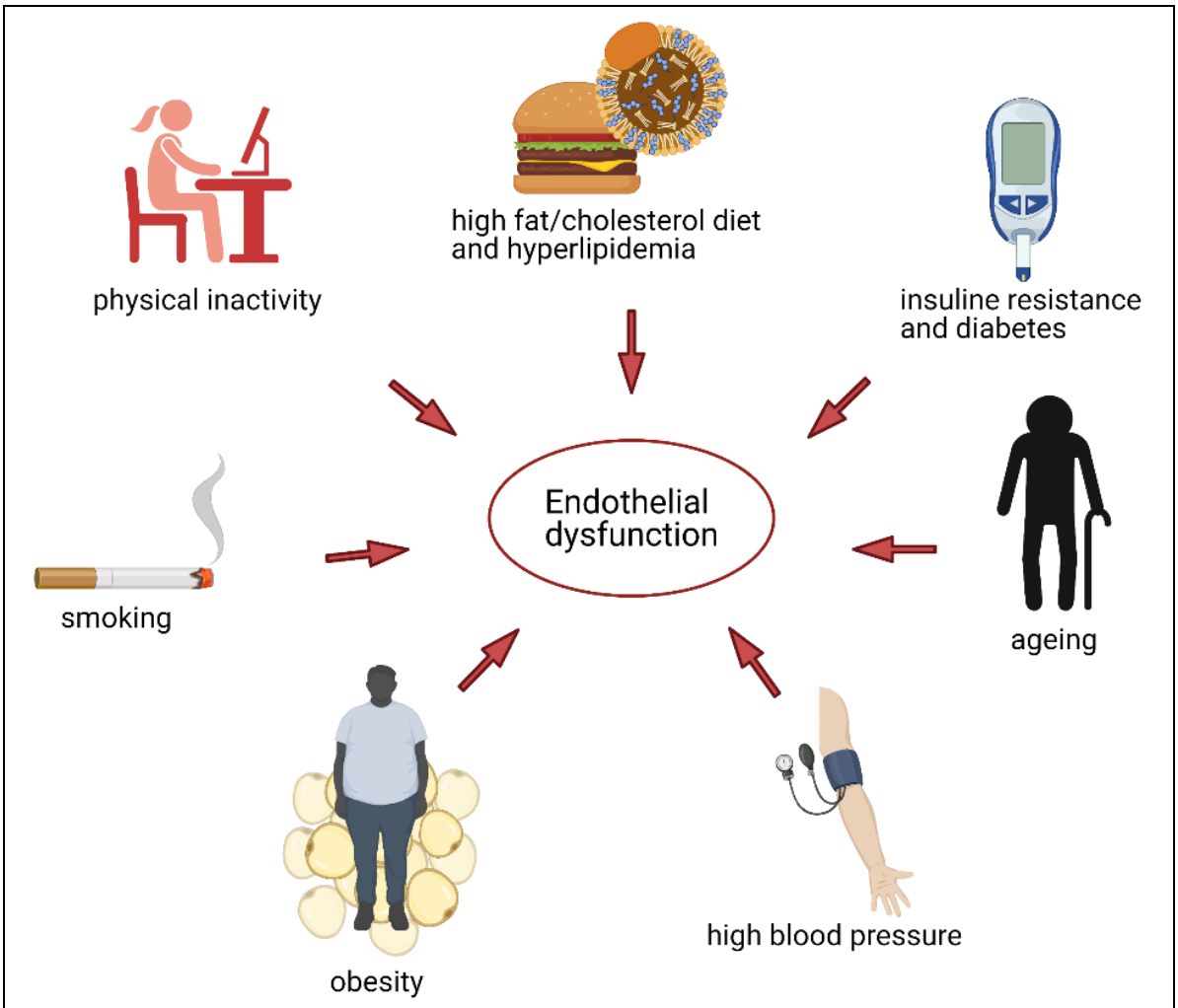

Figure 2: Risk factors of endothelial dysfunction

Physical inactivity, high fat/cholesterol diet, hyperlipidemia, insulin resistance, diabetes, ageing, high blood pressure, obesity, and smoking are all considered endothelial dysfunction risk factors. Created with BioRender.com.

3 LDL CHOLESTEROL AND ITS ROLE IN ENDOTHELIAL DYSFUNCTION

3.1 LDL-CHOLESTEROL

LDL-C is a micellar lipoprotein particle with only one apolipoprotein B-100 (Apo-B) molecule, consisting of 4536 amino acids. Apo-B100 is interlacing the outer monolayer composed of approximately 700 molecules of phospholipids, mainly phosphatidylcholine, sphingomyelin, and lysophosphatidylcholine, and about 600 molecules of cholesterol. The core of LDL-C is formed by triglycerides and an especially high number of cholesteryl esters. The average size of an LDL-cholesterol particle is about 22 nm (Esterbauer *et al.*, 1992). The LDL-C particle is shown in Figure 3.

LDL-C is found in blood serum as it is formed from VLDL when triglycerides are removed by an enzymatic reaction. Its function is to transfer lipids across the body to the place of need. On the one hand, cholesterol is essential for maintaining homeostasis in the body as it is a component of cell membranes, a precursor of bile acids, steroid hormones, and vitamin D, and it also plays an important role in the neural pathway. However, on the other hand, higher serum levels of cholesterol are causing vessel impairment.

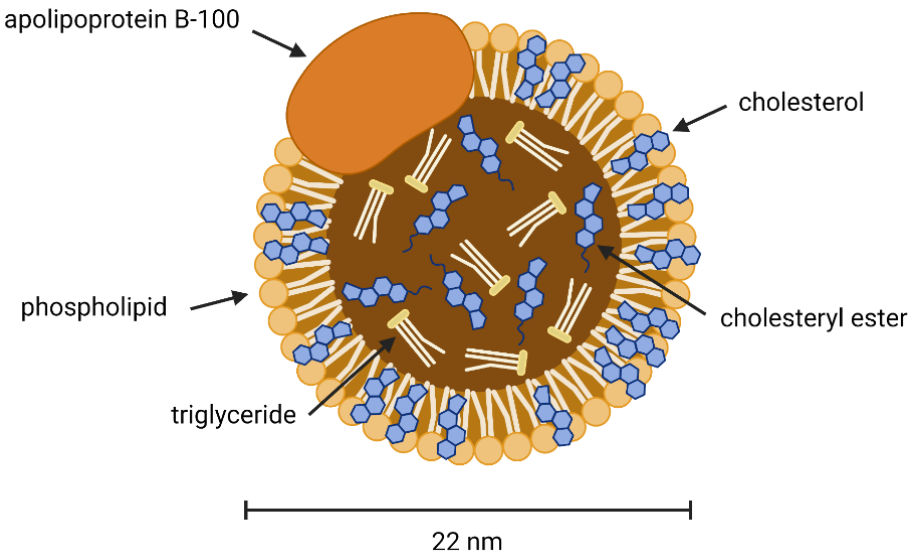

Figure 3: LDL-cholesterol

LDL-cholesterol particle with an average size of 22 nm consists of a single apolipoprotein B-100. Phospholipids and cholesterol molecules are coating triglycerides and cholesteryl esters located inside the particle. Created with BioRender.com.

3.2 HYPERCHOLESTEROLEMIA

Hypercholesterolemia, a form of dyslipidemia, refers to elevated levels of LDL-C in blood serum. According to the Czech Society for Atherosclerosis and their statement of the Committee from the year 2019, LDL-C levels over 4.9 mmol/l are considered to be a high-risk factor for developing cardiovascular diseases, especially atherosclerosis (Vrablík,*et al.*, 2019). Hypercholesterolemia typically develops due to a combination of environmental factors (obesity, dietary cholesterol intake, and stress) and genetic predispositions. Primary hypercholesterolemia is usually due to genetic mutations and is inherited, while secondary hypercholesterolemia is an acquired condition, and it develops from causes such as obesity or diabetes.

Familial hypercholesterolemia is an autosomal dominant genetic disorder caused by pathogenic gene variants in LDL-C receptor (*LDLR*), Apo-B (*APOB*), and proprotein convertase subtilisin/kexin type 9 (*PCSK9*) genes. Most cases are caused by variants in the gene for LDLR or in the gene for its ligand Apo-B. As a result of these variants, the uptake of LDL-C from the blood into hepatocytes where it is normally degraded is significantly slowed down (Ejarque *et al.*, 2008; Vrablík, *et al.*, 2020). Although less frequently, familial hypercholesterolemia may also be caused by gain-of-function types of a variant in the *PCSK9* gene as a result of diminished levels of LDLRs (Abifadel *et al.*, 2003).

However, the most common cause of hypercholesterolemia is polygenic, i.e., it results from an interaction of unidentified genetic factors in combination with increased intake of saturated fatty acids and a sedentary lifestyle. Another secondary causes identified as underlying causes of hypercholesterolemia include diseases like hypothyroidism, chronic renal diseases, nephrotic syndrome, diabetes, or anorexia nervosa (Ohwada *et al.*, 2006; Vodnala *et al.*, 2012).

3.3 HYPERCHOLESTEROLEMIA AND ENDOTHELIAL DYSFUNCTION

3.3.1 OXIDATIVE STRESS

The primary effect of hypercholesterolemia is increased oxidative stress, which is characterized by an imbalance between ROS and antioxidants where ROS prevail (Ohara *et al.*, 1993). A significantly reduced activity of superoxide dismutase (SOD; the major antioxidant enzyme reversing O_2^- to oxygen and hydrogen peroxide) was detected in patients with coronary artery disease. On the other hand, in young patients with familial hypercholesterolemia, the high ROS production was accompanied by increased activity of SOD, suggesting that the enzyme activity may partially counteract excess radical formation (Landmesser *et al.*, 2000).

There have been identified various sources of ROS in ECs, especially of the O_2^-. The main site of ROS generation are mitochondria. ROS are produced in a small amount as a byproduct during oxidative phosphorylation. Their production increases in hypercholesterolemia, which results from defective function of LDLRs and consequent accumulation of LDL-C to outside the cell. In response, cells supplement cholesterol by intracellular de-novo cholesterol synthesis that uses a significant amount of nicotinamide adenine dinucleotide phosphate (NADPH). NADPH is a reducing equivalent necessary in ROS-removal mechanisms, so when NADPH levels in cytoplasm and mitochondria are restricted due to lipogenesis, ROSs cannot be adequately removed. (Oliveira *et al.*, 2005)

A significant source of ROSs in ECs is considered a membrane-bound enzyme NADPH oxidase (NOX). In 2012, Keizer introduced a "mevalonate hypothesis," which can explain NOX activation in hypercholesterolemia. The mevalonate hypothesis is based on the fact that reduced LDL-C uptake in hepatocytes triggers the mevalonate pathway producing cholesterol and free radicals by NOX, which transforms LDL into ox-LDL. Intermediates of this pathway mediate activation of NOX by the small GTPase Rac1 (Keizer, 2012).

NOX activity in ECs is also stimulated by Angiotensin II, whose production is increased in hypercholesterolemia, by serine phosphorylation of NOX subunit $p47^{phox}$.

Phosphorylation of $p47^{phox}$ subsequently increases its binding to another subunit $p22^{phox}$, which promotes ROS production (Daugherty *et al.*, 2004; Li and Shah, 2003).

Generation of ROS in ECs is also associated with xanthine oxidase that is converted from xanthine dehydrogenase (White *et al.*, 1996). This conversion is mainly driven by previously induced oxidative stress by NOX and partially by hyperlipidemia-induced hypoxia (Landmesser *et al.*, 2007; Talbott and Frayser, 1963).

3.3.2 LDL-C OXIDATION

At present, there is a consensus that oxidation of LDL-C in ECs is an early event in the development of atherosclerosis, a disease demonstrated by elevated accumulation of LDL in the arterial wall. In order for LDL-C to become oxidized, it needs to be in contact with a vascular wall. LDL-C is transferred into the extracellular matrix of subendothelial space, where it is intercepted by proteoglycans. The major part of LDL-C is transported to sub-endothelial space in a receptor-independent transcytosis, a process allowing LDL-C to accumulate in tunica intima (Wiklund *et al.*, 1985). In recent years, a receptor activin-like kinase 1 (ALK1) was found to be responsible for the uptake of LDL-C into ECs, which occurs only at the hypercholesterolemic stage (Kraehling *et al.*, 2016). Oxidation occurs mainly in a vascular wall rather than in plasma, which is usually abundant in antioxidants.

With the higher levels of LDL-C in hypercholesterolemia, the chances of its oxidation increase. In the early stages of oxidation, LDL-C is minimally oxidized and can still bind to LDLRs. Esterified polyunsaturated fatty acids like phosphatidylcholine and cholesterol esters, components of LDL-C particles, were described as the main target of oxidation in the early stages (Itabe *et al.*, 2003). However, oxidized phospholipid molecules cause oxidation of apolipoprotein B through chain reactions. This ongoing oxidation leads to the formation of highly immunogenic ox-LDL, which loses the ability to bind to LDLR (Itabe, 2009).

Oxidation of LDL-C is initiated by ROS like O_2^- (Steinbrecher, 1988). Oxidation can additionally occur in non-enzymatic and enzymatic ways. Non-enzymatic ways include copper and iron ions, and enzymatic ways include mainly lipoxygenases and myeloperoxidases (Parthasarathy *et al.*, 1989). The mechanisms of LDL-C oxidation are

extensive and are summarized in a review "Mechanism of LDL oxidation" from Yoshida and Kisugi (Yoshida and Kisugi, 2010).

3.3.3 EFFECTS OF ROS

ROS affect many cellular functions of the endothelium. Some of them, like direct effects of ROSs on NO bioavailability by oxidation of BH_4, conversion of NO into peroxynitrite, or oxidation of LDL-C, were mentioned before.

ROS also promote inflammation by oxidation of LDL-C but also by activating transcriptional factor NF-κB, a key regulator of the inflammatory process and adaptive immunity. Hydrogen peroxide generated during oxidative stress was found to activate NF-κB for the first time in 1991 (Schreck *et al.*, 1991). Hydrogen peroxide was described in macrophages as an initiator of PI3K/PTEN/Akt and NIK/IKK pathways that activate IκB kinases that further phosphorylate IκB, which results in its degradation. As mentioned before, degraded IκB allows NF-κB to translocate to the cell nucleus and initiate gene expression of inflammatory genes (Kim *et al.*, 2008).

3.3.4 EFFECTS OF NATIVE LDL AND MODIFIED LDL

Native, unmodified LDL-C not only ignites endothelial dysfunction by promoting oxidative stress, but its direct effect on NO bioavailability was also described. The elevated levels of LDL-C were found to upregulate caveolin-1 that further binds to eNOS and inhibits its activation (Feron *et al.*, 1999; Michel *et al.*, 1997).

Even more substantial inhibitory effect on eNOS was described in the case of ox-LDL, which causes displacement of eNOS from the plasma membrane caveolae by binding to the endothelial scavenger receptors CD36 causing depletion of caveolae cholesterol content. The reduction of cholesterol levels leads to eNOS disassociation from caveolae and its transfer to intracellular segments where eNOS activity is muted (Blair *et al.*, 1999; Uittenbogaard *et al.*, 2000).

Both native LDL-C and ox-LDL upregulate the expression of protein arginine N-methyltransferases that form asymmetric-dimethylarginine, an eNOS uncoupling mediator (Böger *et al.*, 2000; Tang *et al.*, 2000).

The difference between native LDL-C and ox-LDL lies in the fact that increasing levels of ox-LDL upregulate its receptor compared to native LDL-C that in its abundance downregulates its receptor. The main receptor of ox-LDL in endothelial cells is lectin-like oxidized low-density lipoprotein receptor 1 (LOX-1) (Sawamura *et al.*, 1997).

The signaling via LOX-1 activates GTPase protein RhoA, which further activates arginase II that not only hydrolyses L-arginine but also mediates eNOS uncoupling (Ryoo *et al.*, 2011; Shin *et al.*, 2012). All the mechanisms leading to eNOS uncoupling cause secondary ROS production and intensify vascular oxidative stress. LOX-1-induced oxidative stress mediates activation of NF-κB (Sawamura *et al.*, 2000).

The inflammation is further promoted via the expression of one of the key chemokines - chemokine monocyte chemoattractant protein-1 (MCP-1), whose expression depends on the reaction of ox-LDL with LOX-1 and further activation of mitogen-activated protein kinase (MAPK) (Li and Mehta, 2000). Activated NF-κB as a result of hypercholesterolemia induces inflammatory processes by promoting the expression of various cytokines and adhesion molecules like VCAM-1, ICAM-1, E-selectin, as well as P-selectin (Calara *et al.*, 1998; Gebuhrer *et al.*, 1995; Schmidt *et al.*, 1995).

4 ENDOTHELIAL DYSFUNCTION AS A PREDICTOR OF CARDIOVASCULAR DISEASES

Endothelial dysfunction is directly responsible for various cardiovascular diseases, such as atherosclerosis, arterial hypertension, ischemic heart disease, stroke, venous thrombosis, and others. Increasing evidence based on genetic studies and epidemiological observations unequivocally show that elevated plasma concentrations of LDL-C, often called "bad" cholesterol, play detrimental roles in atherosclerosis, which is a dominant cause of heart attacks, stroke, and peripheral vascular disease.

Atherosclerosis is a pathological condition in which the arteries lose their elasticity and become narrow as a result of atherosclerotic plaque formation in the tunica intima. The atherosclerotic plaque, also called atheroma, is made up mainly of accumulated foam cells developed from macrophages (Howell *et al.*, 2011; Little, 1990) Its formation can be divided into several steps, as shown in Figure 4.

The endothelial activation and chronic inflammation with typical chemokine and cytokine production recruit the monocytes into the site of endothelial dysfunction, especially MCP-1 is essential for this process as it binds to its receptor C-C chemokine receptor type 2 (CCR2) expressed by monocyte (Gerszten *et al.*, 1999; Maus *et al.*, 2002). Monocytes adhere to ECs via adhesive molecules and migrate into subendothelial space, where they differentiate into macrophages (Poon *et al.*, 1997; Swirski *et al.*, 2007).

Macrophages express scavenger receptors on their surface, mainly class A1 and class B1 scavenger receptors (SR-A1 and SR-B2/CD36) and LOX1, which allow them to bind and intake cholesterol, but only in the form of ox-LDL. Ox-LDL upregulates scavenger receptors and simultaneously mute mechanisms that ensure cholesterol efflux leading to intracellular cholesterol accumulation and foam cell formation (Yan and Hansson, 2007). Next to scavenger receptors, toll-like receptors (TLR), particularly TLR 4, were described as an essential part of foam cell formation because ox-LDL by binding to TLRs causes receptor upregulation. Recognition of ox-LDL by TLRs also initiates macrophage activation and differentiation into type 1 phenotype that was found to be proinflammatory and

accelerating plaque formation as they produce inflammatory cytokines and ROS (Lee *et al.*, 2018).

Atherosclerotic plaque formation is a complex process including both innate and adaptive immunity with various interactions, which can eventually lead to vascular smooth muscle cell proliferation, apoptosis, or necrosis, creating a necrotic core and a fibrous cap (Herrero-Fernandez *et al.*, 2019).

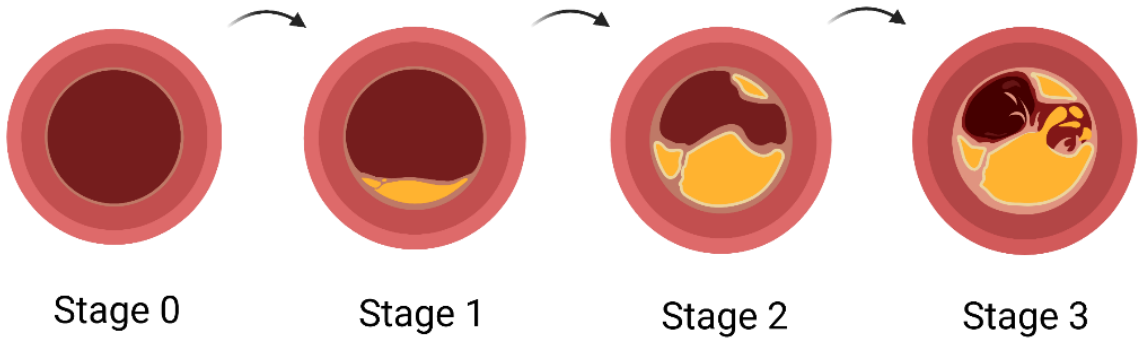

Figure 4: Stages of atherosclerosis impairment

Stage 0: Healthy endothelium with no cholesterol accumulation in a subendothelial space. Stage 1: Cholesterol begins to accumulate in the subendothelial space, oxidative stress is increased and inflammatory processes are activated. Stage 2: Atherosclerotic plaque is advanced and the fibrotic cap is covering the core. Stage 3: Atherosclerotic plaque ruptures releasing its content into blood circulation and causing thrombosis. Created with BioRender.com.

CONCLUSION

Hypercholesterolemia as a consequence of genetic predisposition, high-cholesterol diet, or underlying diseases, was found to be the main contributor to endothelial dysfunction. Increased levels of LDL-C promote ROS production and oxidative stress development. ROS decrease NO bioavailability, activate the NF-κB pathway and promote oxidation of LDL-C accumulated in the subendothelial space. Reduced availability of NO leads to increased production of vasoconstricting factors, resulting in impaired endothelium-dependent vasodilatation. Furthermore, activation of the NF-κB pathway and production of ox-LDL promote the expression of proinflammatory cytokines and adhesion molecules, creating local inflammation.

A deep understanding of the mechanisms implicated in endothelial dysfunction is essential for targeting treatment as it can develop into atherosclerosis, which can lead to ischemic heart disease and death. Even though the main pathways and mechanisms were uncovered, there are still interactions that remain unknown and need further investigation (Wang *et al.*, 2019).

For nearly a century, a high level of LDL-C has been considered the causative factor of atherosclerosis and cardiovascular disease. However, only in the last few decades, it was discovered that the events could be reciprocal, with endothelial dysfunction following inflammation. Patients with chronic inflammatory diseases such as rheumatoid arthritis, systemic lupus erythematosus, the seronegative spondyloarthropathies, psoriasis, and inflammatory bowel diseases, are predisposed to the development of endothelial dysfunction and atherosclerosis. However, the exact mechanisms underlying the high inflammatory burden in atherosclerosis are not clear. Therefore, a search for direct evidence of systemic inflammation on vascular inflammation is an important topic for future research, which might bring potential atheroprotective mechanisms that may be applicable to the general population (Steyers and Miller, 2014).

ABBREVIATIONS

ALK-1: activin-like kinase 1

APOB: gene for apolipoprotein B100

ApoB: apolipoprotein B100

CCR2: C-C chemokine receptor type 2

EC: endothelial cell

ELAM-1: endothelial-leukocyte adhesion molecule-1

eNOS: endothelial nitric oxide synthase

ET1: endothelin 1

ICAM: intercellular adhesion molecule

IL-1: interleukin-1

Iκ-B: I kappa-B

LDL-C: low density lipoprotein-cholesterol

LDLR: gene for low density lipoprotein receptor

LOX-1: lectin-like oxidized low-density lipoprotein receptor 1

MAPK: mitogen-activated protein kinase

MCP-1: monocyte chemoattractant protein-1

NF-κB: nuclear factor-kappa B

NO: nitric oxide

NOX: NADPH oxidase

PCSK9: gene for proprotein convertase subtilisin/kexin type 9

PGI2: prostacyclin

ROS: reactive oxygen species

SOD: superoxide dismutase

TGF-β: transforming growth factor β

TLR: toll-like receptor

TNF-α: tumor necrosis factor α

VCAM: vascular cell adhesion molecule

VLDL-C: very low density lipoprotein-cholesterol

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