Molecular mechanisms of endothelial dysfunction in diabetes mellitus type 2

Ružić, Karlo

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Karlo Ružić

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ABBREVIATIONS

ACE - angiotensin converting enzyme

AGEs - advanced glycation end products

AR - aldose reductase

BP - blood pressure

BH4 - tetrahydrobiopterin

cAMP - cyclic adenosine monophosphate

CRP - C-reactive protein

CVS - cardiovascular system

CVD - cardiovascular disease

DM - Diabetes mellitus

DMT1 - Diabetes mellitus type 1

DMT2 - Diabetes mellitus type 2

DPP 4 - dipeptidyl peptidase-4

DPH - dihydropyridines

EC - endothelial cell

ED - endothelial dysfunction

ET - endothelin

eNOS - endothelial nitric oxide synthase

GAPDH - glyceraldehyde-3-phosphate dehydrogenase

Glc - glucose

GLP-1 – glucagon-like peptide 1

HMG-CoA - hydroxy-3-methylglutaryl coenzyme A

ICAM - intercellular adhesion molecules

iNOS - inducible nitric oxide synthase, neuronal (nNOS).

NAD - Nicotinamide adenine dinucleotide

NADP - Nicotinamide adenine dinucleotide phosphate

nNOS - neuronal nitric oxide synthase

NF-κB - nuclear factor kappa-light-chain-enhancer of activated B cells

NO - nitric oxide

NOS - nitric oxide synthase

PGI2 - prostacyclin

PMNs - polymorphonuclear cells

PKC- protein kinase c

SMC- smooth muscle cell

RAGE - receptor for advanced glycation end products

ROS - reactive oxygen species

VEGF - vascular endothelial growth factor

VCAM 1 - vascular cell adhesion molecule-1

vWF - Willebrand factor

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SUMMARY

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Karlo Ružić

Endothelium is a thin layer of cells that coats the inner surface of the cardiovascular system and provides a link between the circulating blood and the vessel wall. Due to variety of functions and wide distribution in the body, endothelium can be thought of as an organ by itself. Endothelial dysfunction (ED) is a term which describes the state of damaged endothelium with related loss of function. ED consists of a wide range of pathophysiologic states, in span of localised intimal damage to global, persistent, inadequate endothelial activation important for development of numerous clinically different pathologic states. Major mechanisms of ED include: oxidative stress, endothelial NO synthase (eNOS) uncoupling, inflammatory pathways activation, differential expression of vascular endothelial growth factor, intracellular sorbitol accumulation. ED is a hallmark of impaired vascular integrity from which chronic complications of diabetes mellitus type 2 (DMT2) stem from. ED starts to appear in pre-diabetic state and worsens proportionally with duration of DMT2. Assessment of endothelial function in patients with risk of cardiovascular disease (CVD) development is of great importance because it enables us to detect vascular abnormalities and efficiency of therapeutic approach. Increasing quantities of therapeutic modalities have been shown to improve endothelial dysfunction, which has important implications for the treatment of patients at risk of developing chronic complications related to DMT2.

KEY WORDS - endothelium, endothelial dysfunction, diabetes mellitus

SAŽETAK

Molekularni mehanizmi endotelne disfunkcije u dijabetes mellitusa tipa 2

Karlo Ružić

Endotel je tanki sloj stanica koji oblaže unutarnje površine kardiovaskularnog sustava te služi kao poveznica između cirkulirajuće krvi i krvnih žila. S obzirom na raznolikost funkcija i široku rasprostranjenost u tijelu, na endotel se može gledati i kao na zaseban organ. Endotelna disfunkcija je pojam koji opisuje stanje oštećenog endotela te posljedično njegovu smanjenu funkciju. Sastoji se od niza patofizioloških stanja, počevši od lokalne ozljede intime do globalne, dugotrajne i neadekvatne endotelne aktivacije koja dovodi do brojnih patoloških stanja. Glavni mehanizmi nastanka endotelne disfunkcije uključuju povećan oksidativni stres, disfunkciju endotelne NO-sintaze (eNOS), upalna stanja, poremećeni izražaj vaskularnog endotelnog faktora rasta (VEGF) te unutarstanično nakupljanje sorbitola. ED je glavno obilježje poremećenog vaskularnog integriteta, iz kojeg se razvijaju kronične komplikacije diabetesa mellitusa tipa 2 (DMT2). Praćenje funkcionalnosti endotelnih stanica od velike je važnosti kod pacijenata s rizikom od razvitka kardiovaskularnih bolesti jer nam omogućuje praćenje stanja vaskularnog sustava te učinkovitosti terapije. Konstantan razvoj i povećanje broja terapijskih pristupa koji vode k poboljšanju ED, također su od velike važnosti u prevenciji, odgodi nastanka ili terapiji kroničnih komplikacija DMT2.

KLJUČNE RIJEČI- endotel, endotelna disfunkcija, diabetes mellitus

1. INTRODUCTION

Diabetes mellitus (DM) is one of the most widespread and morbid chronic diseases, affecting the health of millions of people in the world. The global prevalence of diabetes has risen in adults from 4.7% in 1980 to 8.5% in 2014. 90–95% of adults have DMT2 (1). According to International Diabetes Federation, in 2019 approximately 463 million adults were living with diabetes, of which 90% was DMT2. Long term effects of diabetes affect many organ systems which involve complex pathology, mainly on cellular and subcellular level. Changes in cerebrovascular system (CVS) are initiated by events that affect endothelium. Vascular endothelial cells line the entire circulatory system, from the heart to the smallest capillaries. These cells have very unique functions which include regulation of blood vessel tone, hemostasis, fluid filtration, such as in the glomeruli of the kidneys, neutrophil recruitment, and hormone trafficking (2). Endothelial dysfunction is one of the key factors that contribute to the development of diabetic complications, and tends to be main event in macrovascular complications such as coronary artery disease, peripheral arterial disease, stroke and microvascular complications such as neuropathy, retinopathy and nephropathy which impose a huge burden on management of this disease. The rate of cardiovascular disease (CVD) in adults with diabetes is 2-3 times greater than in adults without diabetes. CVD is additionally the leading cause of premature death in those with DMT2 (3)

2. ENDOTHELIAL CELLS AND VASCULAR FUNCTION

Although once considered a simple barrier between blood and vessel wall, today it is known that endothelium is a dynamic organ which coats the inner surface of the entire vascular system. By its structure endothelium it is simple, while by distribution it is ubiquitous. The single layer of endothelial cells (EC) that constitutes this barrier is in itself uniquely versatile, showing remarkable physiological and morphological heterogeneity across the vasculature

(4). It is a semipermeable monolayer of spindle-shaped endothelial cells that helps in constituting the innermost layer of blood vessels, known as the tunica intima, and helps in maintaining vascular health and metabolic homeostasis.

Total number of 1-6 x 10^{13} of endothelial cells, covers the inner walls of vascular system by area of 4000-7000m². Endothelium is a heterogenous epitelial structure of high biological dynamics which helps preserve metabolic homeostasis and vascular health (5). Cells of the endothelium have numerous functions of great importance which include recognition and adaptation to humoral, mechanical, and hemodynamic changes. Additionally the endotel is a highly active metabolic and endocrine organ producing a variety of different molecules, including vasoactive peptide hormones, growth factors, coagulation factors and adhesion molecules

2.1 Vascular tone

Blood vessel endothelium has a critical role in regulation of vascular tone, and due to that, changes of vascular flow are in complex interaction with endothelium. Ability to cause vasodilation in response to change in blood flow is one of the main functions of healthy endothelium. Upon change in shear stress or blood pressure, endothelial structures react in immediate manner, by performing changes in cellular membranes, reorganisation of internal structure and by programmed initiation of various biochemical processes. In this way EC shows the ability to change mechanical force into biological reactions which can cause long term variation in gene regulation with accompanying restructuring of vessel wall, as well as acute and fast reactions like change in vascular tone (6,7).

In homeostasis, the vascular tone is regulated by the balance of vasodilative and vasoconstrictive signals to normalize blood pressure and flow to current activity requirements. EC control vascular tone by sending paracrine signals to smooth muscle cells surrounding the

vessels, which can constrict vessels by contraction or dilate them by relaxation (8). The most potent vasoconstrictor is endothelin (ET), a 21 amino acid peptide existing in 3 isoforms mainly synthesized by EC (9). In normal, physiologic state, they are in balance with other mechanisms, but when overexpressed, they can significantly increase blood pressure. Endothelin functions through activation of two G protein- coupled receptors, endothelin$_A$ and endothelin$_B$ receptor. The two types of ET receptor are distributed across whole diversity of cells and organs but with different levels of expression and activity, indicating a multiple-organ ET system (10). The main counter-player against vasoconstriction is nitric oxide (NO), a transmitter, produced by NO synthases (NOS). It relaxes inner muscles of blood vessels, and in this way allows increased blood circulation. Nitric oxide production is essential for healthy organism as it allows flow of oxygen, blood and nutrients to all parts of the body. Insulin has a role in regulation in vascular tone, as it possesses the ability to induce release of both ET and NO (11).

2.2 Coagulation and fibrinolysis

In the vascular system of a healthy organism, EC secrete various agents and mediators which are important for the regulation of blood coagulation and platelet functions. In this way they are preventing the activation of thrombin and inhibiting platelet adhesion, thereby mediating anticoagulant activity. EC displays anticoagulant activity by increasing the number of receptors for protein C activation, by production of thrombin inhibitors and tissue factor. Prostacyclin (PGI2) and nitric oxide (NO) are the major antiplatelet agents which are constitutively secreted by EC (12). Both mediators synergistically increase the cyclic adenosine monophosphate (cAMP) content in platelets, thereby preventing their aggregation (13). Synthesis of NO and PGI2 is raised in response to agonists like thrombin or bradykinin or secreted by aggregating platelets and serves to limit the formation of thrombi.

3. ENDOTHELIAL DYSFUNCTION

Every cell in the organism of people with DM is exposed to abnormally high glucose concentration, but not all are affected. Some cells show an inversely proportional relation between Glc transport through the cell membrane and extracellular hyperglycemia. Cells of the vascular system are specifically acted upon by hyperglycemic damage, because they are unable to downregulate Glc transport under those circumstances, which results in intracellular hyperglycemia (14). Hyperglycemia enhances and prolongs inflammatory reaction by increasing the number of receptors on target cells and thus upregulating them. It also increases reactive oxygen species (ROS) production by various mechanisms some of which are PKC activation, eNOS dysfunction, NADPH oxidase activation (15).

Endothelial dysfunction describes the state of damaged endothelium, its interrupted functionality and is an early event in development of vascular impairment (16). This kind of disturbance comprises a variety of pathophysiological states, from localised injury of intima to widespread and persistent endothelial activation which is important for emergence of numerous clinically manifesting pathological states (17). The basis for its presence is inappropriate activation of endothelium – which is a term that comes from results of in vitro studies that came to conclusion of various harmful stimuli being the trigger for nonspecific expression of endothelial activatory antigens. Endothelial activation can be caused by genetic factors, but in the majority of cases it is the result of bad health habits like smoking, alcohol overconsumption, lack of physical activity, intense physical and psychological stress, bad nutrition. While phenotype of nonactivated, healthy EC is anticoagulant, vasodilatory and antiinflammatory, ED is change which is characterised by prothrombotic, vasoconstrictory and proinflammatory phenotype(18,19).

ED is present in DMT2, in both peripheral and coronary circulation (20). In patients with DMT2, circulating biomarkers which are indirect indices of activation and damage of EC,

with vascular inflammation are found to be increased. These include intercellular adhesion molecule (ICAM)-1, vascular cell adhesion molecule (VCAM)- and E selectin. Von Willebrand factor (vWF) level, that can be a measure of EC activation and damage (21–24). Presence of slightly increased levels of albumin in urine (ref. range 30-300mg/24h urine), referred to as microalbuminuria, can be used as an predictor of EC damage and global endothelial dysfunction. Even in earlier stages of DMT2, it can be pronounced and additionally serves as an indicator of diabetic nephropathy (21,25).

3.1 ROS production, oxidative stress

In physiological conditions, reactive oxidative species (ROS) act as important second messengers which regulate cell growth and differentiation. They may also act as toxic molecules which help in elimination of bacteria and thus improve the host defence system but when the capacity of the antioxidant system is in disbalance with the amount of ROS produced, oxidative stress occurs (25,26). Under these pathologically increased quantities, ROS reacts with Fe^{2+} and Cu^{2+} metal cations, in order to produce hydroxyl radicals that are highly reactive. Hydroxyl radical reacts with cellular constituents like DNA, proteins, lipids which induces cellular malfunction, cell death and ultimately organ failure (27). Increased concentrations of ROS in DMT2 is thought to be the main event, as it is able to induce many other pathways and pathological states that are hallmark of DMT2, thus leading towards appearance and worsening of complications.

3.2 NOS uncoupling

As ability to release vasoactive factors and regulation of blood flow is the main role of healthy endothelium, inadequate availability of NO is one of the main features that is at the same time main mechanism of certain endothelial features that alltogether lead towards endothelial dysfunction (28). Mismatch in availability of NO and activity of vasoconstrictory factors is of crucial importance towards vasoconstriction in ED.

Main mechanisms of reduced NO concentrations in ED can be divided into two groups : decreased production by endothelium and consumptive processes that divert available NO towards other reactions, transforming it into other substances (29). There are 3 isoenzymes of NOS: endothelial (eNOS), inducible (iNOS), neuronal (nNOS). NOS is an enzyme which catalyses the process of production of the NO and citrulline from L-arginine and oxygen as substrates (25,30). Synthesis of NO requires tetrahydrobiopterin as a cofactor - lack of it and/or substrates leads to superoxide formation by the eNOS (31). Metabolic impairment caused by DMT2 increases mitochondrial superoxide and other ROS production in endothelial cells in both micro- and macrovasculature and heart itself. and thus cancels NO effect on target molecules (32,33). Superoxide is able to reduce the amount of tetrahydrobiopterin (BH4) which causes loss of interaction with eNOS. Loss of interaction leads to eNOS uncoupling and leads to additional increase in production of superoxide (34,35). In addition to eNOS being able to produce superoxide instead of NO under circumstances of lacking cofactor or substrates, NO is capable of reacting with superoxide anion, which marks the appearance of endothelial dysfunction. In the reaction with NO, superoxide anion reacts and forms peroxynitrite, an powerful oxidizing agent (36,37) Consequently, peroxynitrite promotes protein nitration, lipid peroxidation and contributes to dysfunction and death of EC (38,39).

3.3 Leukocyte adhesion and inflammation

The production of ROS has a wide variety of effects on the endothelium. Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) is a protein complex that is omnipresent in the human body, controls many processes, in particular cytokine production, DNA transcription and cell survival (40). It can be activated by various inducers but the focus will be to those of importance in DMT2, which would be ROS and reduced bioavailability of NO. In each of these ways, various genes are activated which are involved in fostering inflammation (12,41,42). NF-κB is found to be chronically active in plenty of inflammatory procesess, including atherosclerosis. Elevation of some activators of NF-kB, like osteoprotegrin, is connected with higher mortality, especially from cardiovascular disease CVD. E-selectins, VCAM, ICAM-1 have also drawn much attention, as their function is to transport leukocytes and other inflammatory cells into the arterial wall. Normal shear forces exerted on endothelium by blood, reduce inflammatory response by inactivation of these adhesion molecules, and further on have anti-atherogenic properties. As biomechanics of blood flow are highly altered in DMT2, adhesion molecules are constantly under some degree of activation, and thus produce constant inflammation. Their activation is, aside from NF-kB activation, considered as the main characteristic of atherosclerosis (43–45). Additionally, C-reactive protein(CRP) is moderately increased in states of DM and atherosclerosis which is thought to be a consequence of constant inflammatory reaction in the vascular system (46,47). Additionally, increase of soluble forms of VCAM-1 and ICAM-1 has been markedly increased in diabetic patients which may reflect ongoing formation of atherosclerotic lesions and is related to increased risk of development of CVD (48,49).

3.4 Polyol pathway

Pathophysiology of endothelial dysfunction in DMT2 seems to be very complex and consists of many interconnected reactions and metabolic pathways. Most often multiple of these imbalances are present at the same time. As already mentioned, hyperglycemia seems to be the main factor in development and progression of the complications. It leads to activation of many biochemical pathways that are at the same time among most important causative agents of ED, and consequently microvascular degeneration as one of chronic complications of diabetes (50). In between hyperglycemia and activation of aforementioned pathways, are reactive oxygen species. Superoxide inhibits glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and thus causes accumulation of glycolitic intermediates (51). As glucose cant be broken down through glycolysis, it is shunted sideways into other metabolic routes, which can facilitate glucose breakdown. Major pathways that are activated by glycolytic intermediates are polyol pathway, hexosamine pathway, protein kinase C (PKC) pathway, in addition to advanced glycation end products (AGEs) formation.

The polyol pathway consists of two reactions, that are catalysed by 2 different enzymes. First reaction is catalyzed by aldose reductase (AR) and reduces glucose into sorbitol. Under physiological conditions, AR has low affinity towards glucose, but in hyperglycemia , AR and polyol pathway become the main pathway into which glucose is disposed and equals to approx. 30% of glucose being shunted from glycolysis. In ^{1st} reaction nicotinamide adenine dinucleotide phosphate (NADPH) is being consumed and converted into $NADP^+$ which reduces NADPH/$NADP^+$ ratio. As NADPH is involved in biosynthesis of nitric oxide and fatty acids, lack of it can cause decrease in NO availability and deleterious effects on anabolic pathways (52). In 2^{nd} reaction sorbitol is converted into fructose, which is thought to produce major redox imbalance in diabetes due to NADH being produced from NAD^+. Increased ratio od NADH/NAD^+ leads to ROS production and consequently increased oxidative stress. In

addition, excess NADH can inhibit glycolitic pathway, Krebs cycle and pyruvate dehydrogenase complex, leading to more glucose being diverted through polyol pathway. In certain tissues such as kidney, retina and nerves, concentration of sorbitol dehydrogenase is low, which can contribute to sorbitol being accumulated intracellularly (53–55). Sorbitol accumulation causes osmotic stress, and ultimately leads to diabetic nephropathy, retinopathy and neuropathy which constitute microvascular complications of DMT2.

3.5 Advanced glycation end products

AGEs normally form during aging, but their production and accumulation is known to be increased in some pathologic conditions including DMT2. Otherwise their effect is not of major importance, but in DMT2 AGEs contribute to pathophysiologic alterations (56) . Prolonged hyperglycemia is the cause of AGE formation, which can be endogenous and exogenous. Exogenously they are produced while food is termically processed, and endogenously in reaction to reducing sugar with either protein, nucleic acid or lipids (57,58). Activation of AGE – RAGE(AGE receptor) contributes to a variety of microvascular and macrovascular complications. The AGE receptor is called RAGE and activation of this axis results in increased oxidative stress, protein crosslinking, inflammation, and apoptosis autophagy (59). AGEs reduce eNOS activity, which may lead to additional production of ROS through NOS uncoupling mechanism (60). In the cardiovascular system, RAGE is found mainly in vascular endothelium and capillaries, which explains AGE deposits in DMT2, that are found mainly in atherosclerotic plaques, vascular smooth muscle, and in myocardial tissues (61,62).

4. TREATMENT

Althought there is no specific pharmacological therapy for endothelial dysfunction, multiple drugs on trials have proved to cause improvement of it.

Glucagon-like peptide-1 (GLP-1) agonists, also called incretin mimetics, are antidiabetic drugs, which work in such a manner that they mimic actions of endogenously produced hormones, incretins. These functions include stimulation of insulin release from pancreas, inhibition of glucagon release from pancreas. slowing down emptying of the stomach and thus preventing hyperglycemia from occurring (63). Apart from their effect on glucose metabolism, GLP-1 agonists, for instance liraglutide, induce eNOS expression and increase NO in serum, thus improving ED present in DMT2 (64,65). Aside from incretin mimetics, incretin enhancers are also available for improvement of endothelial dysfunction. They inhibit enzyme dipeptidyl peptidase-4 (DPP-4) which is responsible for cleavage of GLP-1, and in such a way increase GLP-1 bioavailability. Sitagliptine, which is a highly selective DPP-4 inhibitor, induces expression of eNOS, increases NO synthesis, and reduces mean arterial pressure (66).

Calcium channel blockers (CCB) are antihypertensive drugs, which are divided into dihydropyridines (DPH) and nondyhydropyridines. Dihydropyridines have potent effects on vessel tone, by binding to L-type calcium channels and thus preventing Ca^{2+} influx into the cell which is needed to vasoconstrict. CCBs, mainly DHP, increase endothelium dependent relaxation, and improve endothelial function in many vascular beds (67,68). But it seems that endotelial improvement from DHP administration stems from their antioxidant effect rather than from Ca^{2+} channel antagonism , since EC doesn't possess voltage gated Ca^{2+} channels (69). In such manner DHPs protect EC from ROS, which are among the most detrimental substances contributing to ED, thus providing an increase in available NO.

Angiotensin converting enzyme (ACE) inhibitors is another group of drugs that improves endothelial function, and they do so by several mechanisms. They increase levels of bradykinin, an endothelium dependent vasodilator, by inhibiting its degradation. Moreover, by depleting angiotensin II, ACE inhibitors are of particular interest due to the range of actions angiotensin II has on ED. Angiotensin II can induce ED by inhibiting NO-synthase activity or by activation of NADH-oxidase. NADH-oxidase is an enzyme system that promotes ROS generation and raises the possibility that these pathways, activated by angiotensin II function as second messengers for long-term responses such as hyperplasia or hypertrophy (70).

Statins are cholesterol lowering drugs, but recent numerous studies have shown that they have a pleiotropic effect in the human body. Hypercholesterolemia also impairs endothelial function and by obstructing the conversion of hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) to mevalonate, statins inhibit an early step in cholesterol synthesis. This leads to an increase in the number of hepatic LDL receptors and thus to improvement in uptake of cholesterol by the liver, which reduces the level of serum cholesterol (74,75). Except for the liver, one of the major target organs of statins is vascular endothelium on which they have direct, cholesterol level - independent effects. They can restore or improve endothelial function by increasing NO availability, promoting re-endothelialization after damage to vessel wall, and by reducing adhesion of inflammatory cells thus inhibiting inflammatory responses within the vasculature (71). An early step in atherogenesis includes monocyte adhesion to the endothelium and penetration into the subendothelial space where they differentiate into macrophages (72). Inflammatory cytokines secreted by macrophages alter endothelial function, collagen degradation, smooth muscle cell (SMC) proliferation, and thrombosis (73).

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7. BIOGRAPHY

PERSONAL DATA

Name and surname: Karlo Ružić

Date of birth: 17.03.1995

Place of birth: Zagreb, Croatia

EDUCATION

Faculty: School of medicine, University of Zagreb (2013-2021)

High school: IV gymnasium, Zagreb, Croatia

Primary school: I. OŠ. Dugave, Zagreb, Croatia

SPECIAL KNOWLEDGE AND SKILLS

Foreign languages: English, German