Summary: Nitric oxide (NO) is produced by many cells in the body; however, its production by vascular endothelium is particularly important in the regulation of blood flow. Vascular actions of NO include the following: direct vasodilation, indirect vasodilation by inhibiting the vasoconstrictor influences, anti-thrombotic, anti-inflammatory and anti-proliferative effects. Due to its importance in vascular function, abnormal production of NO, occurring in different diseases can adversely affect blood flow and other vascular functions. It has been suggested that alterations in NO generation are a critical cause of injury in the ischemic heart. A biologic link between the endothelial damage and atherosclerotic coronary arterial disease has been presumably related to decreased arterial bioavailability of NO through the increased leucocyte and platelet adhesions, vasoconstriction and smooth muscle cell proliferation. However, the precise mechanism of the impaired NO generation is not known, and there is a considerable controversy regarding whether myocardial ischemia results in increased or decreased NO formation. Asymmetric dimethylarginine (ADMA) is a natural, competitive inhibitor, and one of the primary factors controlling the nitric oxide production. ADMA was found to be elevated and closely correlated with the impaired vasodilator function in conditions associated with the endothelial dysfunction, such as hypercholesterolemia, hypertension, insulin resistance and type 2 diabetes, and renal failure. But ADMA also seems to be involved in myocardial ischemia, since its plasma levels predict future coronary events in patients with the elevated cardiovascular risk. It has been recently reported that the elevated plasma ADMA concentrations in the acute coronary events are an independent car diovascular risk factor.

Keywords: nitric oxide, endothelial dysfunction, coronary heart disease, risk factors

Kratak sadržaj: Azot-monoksid (NO) se sintetiše u mno-gim čelijama organizma ali je njegova pr odukcija u vaskularnom endotelu nar očito važna u r egulaciji protoka krvi. Vaskularni efekti uključuju: dir ektnu vazodilataciju, indirekt- nu vazodilataciju inhibicijom vazokonstriktora, anti-tr ombo-tični efekat, anti-inflamatori i anti-proliferativni efekat. Zbog njegove važnosti u vaskularnoj funkciji, abnor malna produk- cija NO, koja se javlja u različitim bolestima, može imati nepovoljan efekat na protok krvi i druge vaskularne funkcije. Smatra se da je por emećaj sinteze NO kritičan uzor ok ošte- čenja u ishemičnom srcu. Biološka veza između endotelnog oštećenja i aterosklerotske bolesti koronarnih arterija je pr e svega vezivana za smanjenje raspoloživog NO kroz povećane adhezije leukocita i trombocita, vazokonstricije i proliferaci- je glatkomišćih čelija. Međutim, precizni mehanizam pore- mećaja sinteze NO još uvek nije jasan i postoje kontr overze o tome da li miokar dna ishemija dovodi do povećanog ili smanjenog stvaranja NO. Asimetrični dimetilarginin (ADMA) je prirodnji, konpetitivni inhibitor i jedan od primarnih faktora koji kontrolisu produkciju NO. Koncentracija asimetričnog dimetilarginina je povećana i usko korelira sa poremećenom vazodilatacijom u uslovima kada postoji endotelna disfunkcij a kao što su hiperholosterolemija, hipertenzija, resistentna na insulin i dijabet Tip 2 i renalna insuficijencija. Takođe, ADMA je uključen u miokardnu ishemiju i na osnovu njegove vr ed- nosti u plazmi mogu se predvideti budući koronarni događaji kod pacijenata sa povećanim kardiovaskularnim rizikom. Ne-davno je saopšteno da je povećana koncentracija ADMA u akutnim koronarnim događajima nezavisan kardiovaskularni rizik faktor.

Ključne reči: azot-monoksid, endotelna disfunkcija, ishe-mijska bolest srca, faktori rizika

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Introduction

Nitric oxide (NO) is a simple, diatomic gaseous molecule that is a key signaling messenger in the cardiovascular system (1). NO serves many important biological functions in the cardiovascular physiology where it is produced in both endothelial and smooth muscle cells. NO produced in endothelium controls vascular tonus and permeability, maintains vascular integrity by inhibiting the platelet aggregation, leukocyte endothelium adhesion and vascular smooth muscle proliferation. NO produced in cardiac smooth muscle cells regulates cardiac contractility (2). In order for NO to preserve normal vascular physiology, its adequate levels have to be produced. NO is synthesized from amino acid L-arginine and molecular oxygen by one of three nitric oxide synthases (NOS): neuronal NOS (nNOS, NOS1), endothelial NOS (eNOS, NOS3), and inducible NOS (iNOS, NOS2). These isoforms are encoded by the genes located on different chromosomes (12q24.2; 7q35-36; 17cen-q42), and show 50%–60% of homology in their amino acid sequences in the oxidase and reductase domains that bind cofactors FAD, FMN, NADPH and BH4 (3). NOS isoforms exert different characteristics which reflect their various functions in vivo (4).

Endothelial NOS and nNOS are expressed constitutively and their activity is primarily regulated by the levels of intracellular calcium and calmodulin concentrations, while iNOS is expressed during pathological processes, such as heart failure (5), after induction by cytokines and other inflammatory mediators, and produces high levels of nitric oxide (6).

nNOS is predominantly expressed in some neurons and skeletal muscles, eNOS in endothelial cells, and iNOS in macrophages and monocytes. In spite of their names, different types of cells express these isoforms, and numerous tissues express more than one isoform. Endothelial cells express eNOS and iNOS, and cardiomyocytes nNOS. Further on, the inner arterial and vascular structures in all tissues express nNOS and eNOS, while circulating cells may express iNOS.

In order to be activated, NOS proteins have to bind cofactors and dimerize (9). eNOS consists of two identical monomers, and each monomer has two principal domains: C-terminal reductase domain with binding sites for NADPH, FMN, and FAD, and C-terminal oxidase domain which takes away an electron from L-arginine and contains binding sites for (iron) hem, BH4, and L-arginine (9,10). NOS proteins first bind the FAD and FMN cofactors. The addition of L-arginine, BH4, and hem enables the proteins to form dimers. Hem is essential for the process of dimerization. The lack of these cofactors leads to NOS dysfunction (11). Low concentration or lack of L-arginine catalyzes the reduction of oxygen to superoxide or hydrogen peroxide (12), and drop of BH4 level to simultaneous production of NO and superoxide, the products which may interact to form peroxynitrite (13). The formed dimers eNOS and nNOS are inactive till the binding of calmodulin, effectuated via the increased concentration of the intracellular calcium. iNOS is activated even in low calcium concentrations due to high enzyme affinity towards calmodulin (14). This means that the main »switch« for the activa-

![Figure 1](image-url)
tion of nNOS and eNOS is a transient elevation of calcium concentration, and for iNOS, the transcription process.

The process of catalysis of the constitutive NOS involves two oxidation stages: first, L-arginine is hydroxylated to NG-hydroxy-L-arginine, and then the oxidation of this intermediary occurs with the use of one electron of NADPH and formation of L-citrulline and NO (15). In the reaction, 1.5 moles of NADPH and 2 moles of oxygen per a mole of citrulline are consumed (16).

Regulation of eNOS activity and gene expression

eNOS gene is expressed constitutively thus providing the basal concentration of eNOS protein. Stable concentrations of mRNA are maintained by the complex regulatory mechanism of gene expression (17). The eNOS gene promoter has multiple cis-regulatory DNA sequences, including CCAT box, Sp1 sites, GATA motifs, CACCC box, AP-1 and AP-2 sites, p53 binding sites, NF-1 elements, and the sequences responsive to sterol elements and shear stress (18). The positive regulatory domains I and II (PRDI and PRDII) are located in the proximal promoter and involved in the baseline regulation of gene transcription by transcription factors such as Sp-1, Sp-3, Ets-1, Elf-1 YY1 and MY C-associated zinc finger protein (19). Shear stress activation of the promoter is mediated by NF-κB which binds to the responsive element GAGACC located upstream of the transcription site (20). Laminar flow shows a nine-fold increase of mRNA in bovine endothelial cells, and this effect is mediated by the transient increase of gene transcription and prolonged mRNA half-life (21).

The post-transcriptional regulation is accomplished by the dimerization of the protein subunits of NOSs and by the interaction with the caveolin protein and heat shock protein hsp90 (22). The nNOS gene encodes a PDZ domain in exon 2 that is required for membrane association. During nNOS splicing several variants may be formed lacking exon 2 thus resulting in the expression of cytoplasmic nNOS that lacks subcellular localization sequences (2). In the endothelial cells eNOS is localized to caveolae by N-terminal fatty acid modifications as well as interactions with the heat shock protein hsp90 and caveolins (23). eNOS binds to caveolin-1 in the endothelial cells, and in the cardiac muscle to caveolin-3. eNOS activation requires eNOS trafficking from the plasma membrane to the Golgi apparatus and enzyme phosphorylation of the amino acid serine at position 1177 by the Akt, PKA or AMP kinases, which is the main mechanism of eNOS activation and increases in eNOS sensitivity to basal concentrations of calcium/calmodulin (24, 25). Tonic or phasic eNOS activation in response to blood flow is independent of calcium concentration changes and constitutes the shear stress. Although phosphorylation of the serine 1177 residue plays an essential role in eNOS enzyme activation, its regulation is dependent on the phosphorylation of other amino acid residues such as the serine633 residue, which also increases eNOS activity, or the threonine495 residue, which interferes with the calmodulin binding domain, thus down-regulating NO synthesis (22).

Molecular targets of NO

In many cells and for numerous signaling roles of NO, the physiological target is soluble guanylate cyclase (26). NO activates guanylate cyclase by binding to the heme iron, which increases cGMP level in the cell. Via cGMP, NO leads to the relaxation of vascular smooth muscles and vasodilatation. In the brain, NO activates NMDA receptors, and in autonomous nerve system it produces a transmitter which mediates the relaxation of smooth muscles of the gastrointestinal, urinary, and respiratory tract.
The next NO target is sulphydryl groups of proteins, with which it creates nitrosothiols (27). Nitrosylated hemoglobin serves as a natural transporter and pool of NO (28). In the cardiac muscle, NO nitrosylates the SH group of ryanodine receptor in the membrane, thus activating the r receptor (29). Nitrosylation of N-ethylmaleimide-sensitive factor is significant for the regulation of exocytosis. Peroxynitrite anion is created in the reaction with superoxide.

In the case of production of large amounts of NO, which occurs in iNOS activation, this molecule can directly inhibit mitochondrial complexes I and IV. It induces energetic depletion in the cell by way of poly(ADP-ribos)polymerase activation (2). In general, biologic roles of NOS are effectuated via the effects of soluble guanylate cyclase and S-nitrosylation of proteins, while other mechanisms lead to toxic effects of NO.

**Endothelial dysfunction and NO**

Vascular endothelium is an active or gan which in physiologic conditions expresses a number of useful effects, such as vasodilatator y, antoxidative, anti-inflammatory, anticoagulant, and profibrinolytic ones; it inhibits adhesion and migration of leukocytes, inhibits proliferation and migration of smooth muscle cells; inhibits aggregation and adhesion of thrombocytes (30). These atheroprotective effects depend on the balance of substances synthesized and released by healthy endothelium, among which the most important vasoactive component is NO, critical in the pathophysiology of vascular disease and endothelial dysfunction concept. The disorder of endothelium-dependent vasodilation is a systemic disorder recognized as endothelial dysfunction leading to atherosclerosis and its complications due to absence of normal endothelial functions. It occurs in atherosclerosis, hypertension, hypercholesterolemia, and the process of normal aging (2, 31). Endothelial dysfunction in atherosclerotic coronary arteries was described for the first time by Ludmer et al. (32), and its association with the bioavailability of nitric oxide was later described as well. Reduced bioavailability of NO is the most important mechanism in the multifactorial process of the endothelial dysfunction and is involved in most important cardiovascular dysfunctions.

There are several potential mechanisms leading to endothelial dysfunction that can be divided into three categories: reduction of eNOS expression, reduction of eNOS activity, and rapid elimination of NO. First, altered expression of mRNA for eNOS or protein synthesis lead to reduced eNOS activity (33). However, most evidence from the animal models and humans suggest an increased eNOS concentration in diabetes and atheroosclerosis rather than decreased one. Second, the L-arginine substrate may be deficient in the tissues or its transport to the cells may be disturbed. The presence of endogenous competitive inhibitor asymmetric dimethylarginine (ADMA) may reduce the production of NO, even in the presence of physiologic substrate concentrations (34). Third, eNOS requires numerous cofactors for its activity. BH4 is especially important among them, the synthesis of which is controlled by GTP cyclohydrolase, and in the absence of which the transport of electr ons through eNOS becomes uncoupled, and superoxide is created instead of NO (35). Fourth, in order to be activated, eNOS has to be dimerized and adequately localized in the caveolae via caveolin and hsp90 (36). Fifth, eNOS is phosphorylated in the S1179 position via Akt or some other kinase (37). Sixth, with dysfunctional epithelium, there is an increased ROS production, above all via NADPH oxidase activity or uncoupling of eNOS, which may reduce NO levels via several different pathways: direct inactivation by superoxide, with peroxynitrite formation (38), reduction of expression and activity of NOS as the consequence of substrate or cofactor reduction, due to increased ADMA concentration (39), and due to uncoupling of NOS induced by the increased oxidation of tetrahydrobiopterin (24).

The results of recent studies have demonstrated that some drugs, such as antioxidants and r enin-angiotensin system blockers, may reduce the endothelial dysfunction via the mechanism of activation of eNOS by phosphorylation of the amino acid residues in specific positions. Dias et al. (22) have shown that Talmisartan, a blocker of angiotensin II r receptor, reduces endothelial dysfunction by eNOS activation, via phosphorylation of serine residues in the positions 1177 and 635.

Since eNOS plays a significant role in the regulation of blood vessel function, an excessive NO production may contribute to development of atherosclerosis. The source of NO may be iNOS and nNOS expressed in blood vessel smooth muscle cells in the atherosclerotic lesions, as well as iNOS expressed in the activated macrophages and monocytes (40). These isozymes produce NO, which with peroxynitrite, can increase the oxidative stress and oxidative modification of LDL particles (41). The evidence of the presence of peroxynitrite in human atherosclerotic lesions is the finding of nitrotyrosine. NO can affect redox-sensitive transcription of the genes involved in the process of activation of the endothelial cells (42).

**Risk factors for ischemic heart disease and NO**

**Hypertension and NO**

Blood pressure is controlled via the interaction of several homeostatic regulatory mechanisms, including the r enin-angiotensin system, autonomous nervous system, and the local mediators such as NO. The role of NO in the regulation of blood pressure is
very important. NOS inhibition induces blood pressure elevation in many animal species (43). Blood pressure in eNOS knockout mice is 50% higher than in their wild-type counterparts. It is still unclear why other homeostatic mechanisms cannot compensate for eNOS. One of possible explanations is that the renin-angiotensin system and the autonomous norepinephrine system serve primarily to prevent hypertension. Alternatively, there have been infor mation that eNOS is involved in the control of baroreceptors (44). Hypertension is associated with the increased release of vasoconstrictive endothelial mediators, including the angiotensin II as one of the most potent vasoconstrictors which induces the production of endothelin via the MAP kinase pathway (45). It also stimulates ROS production (46) and increases BH4 consumption, inhibiting NO production. The question could be rightfully asked whether endothelial dysfunction in hypertension is a cause or a consequence. On one hand, there are evidence indicating the defective phosphoinositol, NOS-activating pathway, and the suggestion that it is responsible for endothelial dysfunction in the essential hypertension (47). On the other hand, Zizek (48) has demonstrated that there is an endothelial dysfunction in normotensive children of hypertensive patients, and there are evidence of the association of the essential hypertension with the eNOS gene polymorphism (49, 50). Since endothelial dysfunction in hypertension is genetically determined, hypertension could be the cause of endothelial dysfunction. However, the endothelial dysfunction is encountered in patients with secondary hypertension as well, so it could well be the consequence of hypertension. In view of the above facts, the endothelial dysfunction could equally be the cause and consequence of hypertension.

**Dyslipidemia, NO and atherosclerosis**

It has been well known that some of the abnormalities of lipid metabolism, mostly high cholesterol and/or triglyceride levels, are usually encountered in most patients with the ischemic heart disease (IHD). The incidence of IHD in individuals with the disturbed lipoprotein metabolism is 60%–65% (51). Atherogenic lipoproteins are the crucial factors in the initiation and promotion of atherosclerosis, the direct consequence of which is IHD. A cute and chronic manifestations of atherosclerosis are the result of chronic inflammation, partly initiated and maintained by LDL particles penetrating into the subendothelial space from the circulation, where they are oxidatively modified (oxLDL) (52). By the activation of NF-κB, oxLDL induce the expression of the adhesion molecules in endothelial cells, enabling the adhesion of circulating inflammatory cells to the endothelium and their transition into the subendothelial space (53). The elevation of L-selectin, a vascular cellular adhesion molecule-1 and intercellular adhesion molecule-1 in the sera of patients with coronary arterial disease is an indicant of the endothelial activation and dysfunction. Endothelial activation can be induced by triglyceriderich lipoproteins, such as chylomicron remnants and VLDL remnants penetrating the endothelium and reaching the intima. In a prospective cohort study on 7587 women and 6394 men, it has been shown that the increased triglyceride concentration is associated with the increased risk of IHD, myocardial infarction, and mortality in both genders (54).

Highly reactive free oxygen radicals are released in the blood vessel wall from inflammatory and also endothelial cells themselves (55), which oxidatively modify lipoproteins. In the culture of endothelial cells, eNOS may produce large amounts of superoxide after the addition of LDL particles to the medium (56). Increased LDL and decreased HDL concentrations induce disintegration of the caveolae complex, where NOS is bound (57). Vascular smooth muscle cells of rats, in which hypertension is induced by angiotensin II, also produce superoxide by the activation of membrane NADPH oxidase (58). Super oxide and other oxygen radicals may oxidize NO to the metabolites which cannot activate guanylate cyclase, being potentially toxic to the endothelium (such as peroxynitrite). The fact that atherosclerotic rabbit aorta produces more NO supports the notion that dysfunctional endothelium synthesizes more NO compared to normal one. oxLDL particles also stimulate transcription and synthesis of eNOS (59). An increased iNOS expression, producing large amounts of NO, has been demonstrated in human atherosclerotic plaques (60). These findings show that vascular cells in hypercholesterolemia and atherosclerosis synthesize more NO than dormant cells, but NO is rapidly inactivated or converted into toxic oxides due to increased production of free radicals. On the other hand, oxLDL may inhibit NO and consequentially decrease NO production (61). Moreover, an increased production of ADMA, demonstrated in persons with hypercholesterolemia, competitively inhibits NOS (62). The increases of lipoproteins (a), encountered in the impaired coronary endothelium function (63), inhibits NOS with its oxidatively modified components or oxidizes and inactivates NO (64). Reduced NO may stimulate the synthesis and release of the endothelin and proinflammatory cytokines, release of growth factors, hyperplasia, and migration of the smooth muscle cells and thrombocyte adhesion to the endothelium. All these consequences of endothelial dysfunction are significant in the initiation, progression and clinical manifestation of atherosclerosis, i.e. IHD (65).

**Diabetes and NO**

In thin individuals, the insulin stimulates blood flow and reduces vascular resistance in the skeletal muscles (66). Using L-NMMA and by BH4 synthesis inhibition, it has been shown that blood flow stimulation and release of glucose are NO-mediated (67). In
healthy individuals, the insulin increases NOS activity stimulating the phosphatidylinositol-3 kinase and Akt kinase. In insulin resistant patients, the signal transduction is disturbed via phosphatidylinositol-3 kinase pathway, responsible also for glucose uptake by the cells. Due to reduced stimulation of NOS by the insulin, NO production is reduced, and consequently the endothelium-dependent vasodilatation (68). However, the transduction by insulin via MAPK is preserved, resulting in the enhanced production of endothelin and stimulation of the inflammation and thrombosis (70). Ther eupon, hypertension occurs associated with the increased ADMA concentration (71). The occurrence of metabolic disorders (oxidatively modified LDL) leads to downregulation of eNOS expression (72). Clinical studies with ACE inhibitors and statins have shown that these drugs not only reduce coronary disease and mortality from cardiovascular diseases, but also prevent the development of diabetes type 2 (73, 74), confirming the role of endothelial dysfunction in the pathophysiology of the insulin resistance.

Hyperglycemia, accompanying the diabetes, increases superoxide production in the electron transport chain in the mitochondria (73). Super oxide activates protein kinase C, and the kinase activates NADPH oxidase to produce even more superoxide. The reaction of NO and superoxide produces peroxynitrite which oxidizes BH4, uncoupling NOS, which produces superoxide instead of NO. Superoxide increases the production of the advanced glycation end products (74), and they increase the production of superoxide and other ROS, reducing thus NO. The resulting hyperglycemia-induced oxidative stress inhibits dimethylaminohydrolase (DDAH) (75), with consequential ADMA increase and the final result of reduced NO synthesis.

Conflict of interest statement

The authors stated that there were no conflicts of interest regarding the publication of this article.


