Today it is well known that NO is one of the key physiological mediators. The ensuing studies have shown that Nitric oxide (NO) is an endogenous mediator of vasorelaxation property and was originally named endothelium-derived relaxing factor (EDRF) [1]. The ensuing studies have shown that nitric oxide is an important physiological mediator and potent modulator of many biological functions. Today it is well known that NO is one of the key signaling molecules in cardiovascular and nervous system and that it plays a significant role in body’s defense mechanism. NO has a prominent signaling function in macrophages, neurons, endocrine cells, skeletal muscle fibers and numerous other cell types. The most recent studies of cardiomyocytes have identified the function of NO in the regulation of cardiac growth and remodeling, contractile performance, rhythmicity and metabolic rates.
Nitric oxide is a small non-polar molecule with one unpaired electron which makes it a weak free-radical [2]. It is a mediator acting as though it has opposite biological effects. The complexity of NO activity is manifested through the diversity of chemical reactions in which it participates and the characteristics of the tissue in which it operates. The level of synthesized NO is the key that determines the biological outcome of precise cellular responses to its various concentrations. In general, lower concentrations of NO promote the cell survival and proliferation, while higher levels favor the degradation of the cell, apoptosis and/or senescence. Interactions with free radicals affect the NO signaling pathways and reduce its bioavailability.

There are a number of regulatory mechanisms mediated by NO both under the physiological conditions in the healthy and pathological heart. The NO effect on the intracellular milieu and cell function is determined by its concentration, the time factor and by the kinetic determinants. Synthesis, diffusion and consumption rates, interaction with target tissues, free radicals and oxygen concentration contribute to the cellular and tissue-specific response to NO.

**Biosynthesis of Nitric Oxide and its Control**

NO can be synthesized from L-arginine using three different nitric oxide synthase (NOS) isoforms, two of which are constitutive, endothelial (eNOS, NOSIII) and neuronal (nNOS, NOSI) isoforms, manifested under physiological conditions, while the third isoform, inducible nitric oxide synthase (iNOS, NOSII), is biosynthesised only after the stimulation by a variety of stressors and cytokines [3]. Constitutive enzymes generate a small amount of NO, while the activity of iNOS is approximately one thousand times higher [2]. Endothelial NOS is associated with the caveolin in caveolae, specialized microstructures of the plasma membrane (Figure 1). The interaction between eNOS and caveolin is reversible and the release of eNOS from caveolin activates the enzyme. Neuronal and inducible NOS are more commonly found in their soluble form than the membrane-bound one. Constitutive enzymes are activated by Ca\(^{2+}\)-calmodulin and the control is carried out through the endothelium-dependent agonists, acetylcholine, bradykinin and substance P, which increase the concentration of intracellular calcium, thus increasing the Ca\(^{2+}\)-calmodulin. In contrast, iNOS is independent from Ca\(^{2+}\). As iNOS has a Ca\(^{2+}\)-calmodulin binding domain, a high affinity of this ligand-binding domain means that iNOS is activated even at low calcium levels, which are present under basal conditions [2, 4].

**The Distribution of NO Synthase in the Heart is Characterized by the Pronounced Non-uniformity**

Endothelial NOS is mainly present in the coronary vascular and endocardial endothelium and to a lesser extent in cardiomyocytes. Neuronal NOS is present only in the subpopulation of intracardiac ganglia and nerve fibers in the atrial tissue, as well as in some of the perivascular nerve fibers of the ventricular myocardium. The manifestation of nNOS in cardiomyocytes and its physiological role are still being researched. A healthy heart does not usually exhibit iNOS under physiological conditions [5].

Despite the fact that eNOS is distributed in all cardiac endothelial cells, there is a significant disparity of eNOS activity between the endocardial endothelial, arterial, venous, and endothelial cells of myocardial capillaries with an exceptionally intense activity in the endocardial endothelial (EE) cells and endothelial cells of coronary arteries. EE cells have a more distinct Golgi complex than other endothelial cells in blood vessels of the heart. The size of the Golgi complex is most likely a marker of the eNOS synthetic activity; thus, these data indicate that the endothelial cells of the coronary arteries and EE cells have a higher synthetic

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**Abbreviations**

- NO – nitric oxide
- EDRF – endothelium-derived relaxing factor
- NOS – nitric oxide synthase
- eNOS – endothelial nitric oxide synthase
- iNOS – inducible nitric oxide synthase
- nNOS – neuronal nitric oxide synthase
- MVE – microvascular endothelial
- TGF – transforming growth factor
- cGMP – cyclic guanosine monophosphate
- ANG – angiotensin
- EE – endocardial endothelium
- ET-1 – endothelin-1
- PGI\(_2\) – prostaglandin I\(_2\)

**Figure 1. Biosynthesis of nitric oxide. Opposing roles for eNOS and nNOS in regulating heart contraction**

Slika 1. Biosinteza azot-oksida. Suprotne uloge endoteljalne i neuronske sintaze azot-oksida u regulaciji srčane kontraktilnosti
activity than microvascular endothelial (MVE) and venous endothelial cells. Immunohistochemical staining of caveolin-1 have shown that the peripheral boundaries of EE cells are almost completely devoid of caveolin features. This indicates that the enzymatically active eNOS in EE cells, contrary to cardiomyocytes, can be connected to membrane components other than caveolin or to the cytoskeletal parts.

Immunohistochemical staining of the complete myocardial tissue has shown weak cytoplasmic activity of eNOS in MVE cells with a poorly developed Golgi complex. However, the staining of caveolin-1 is more pronounced in MVE than in EE or arterial endothelium. The increased levels of caveolin-1 in MVE has a cardioprotective effect in ischemia/reperfusion-induced damage, probably due to the increased endothelial NO release [5].

The reasons for the differences in eNOS distribution are still unknown. Experiments in the cultured endothelial cells showed that eNOS activity could be modified by a number of modulators, including the friction force, transforming growth factor (TGF-β), protein kinase C, tumor necrosis factor TNF-α, oxygen and proliferation. The differences in friction forces in the heart can explain the differences of the eNOS activity in the arterial, capillary and venous endothelial cells and the EE cells since different friction forces affect the endothelium. Friction forces in the laminar blood flow are not strong along the surface of the EE cells [6]. However, the EE cells exhibit eNOS activity almost as strong as that of the arterial endothelial cells. Endocardial endothelium may be exposed to the turbulent blood flow, but this type of flow does not increase eNOS activity or NO release. eNOS activity may be affected by the mechanical strain of the endocardium due to the three-dimensional changes of the inner wall during the cardiac cycle. Endothelial cells cultured on flexible substrates, which had been subjected to a cyclic tensile strain, displayed an enhanced production of NO. Some parts of the endocardium are subject to specific differences in the mechanical deformation during the cardiac cycle, such as tendon endings of ventricular papillary muscle and the atrioventricular valves. EE cells covering these elastic structures are smaller, and have a cytoskeletal organization which differs from the organizations of other endocardial fields, but they have not shown any consistent differences in eNOS characteristics or the size of the Golgi complex [6].

Endothelial NOS in cardiomyocytes is associated with caveolin-3, a muscle-specific isoform in caveolae protein layers [7–9]. However, the major physiological source of NO in normal, adult, and unstrained cardiac tissue is most likely eNOS originating from EE and MVE cells, while NO originating from cardiomyocytes is probably negligible if they are not activated. Cardiomyocytes do not release NO in direct response to bradykinin or α2 receptor agonists [10], which further indicates that NO originating from the cardiomyocytes is actually negligible in its basal, unstrained physiological condition. In contrast, the exposure of cardiomyocytes to β-adrenergic agonists increases the production of endogenous NO almost five times, indicating the regulation of eNOS in cardiomyocytes via β-adrenergic stimulation [11]. In a similar manner, cyclic guanosine monophosphate (cGMP), as a measure of NO activities, is almost 10 times increased in cardiomyocytes after the stimulation either by bradykinin, in contrast to previous studies [10], or by acetylcholine [12]. Myocardial stretching may participate in the activation of eNOS [13].

Accordingly, there is a significant non-uniformity in the distribution of eNOS in the cardiac endothelium, followed by a more intensive activity in EE and the endothelial cells of coronary arteries than in MVE and coronary venous endothelium. eNOS activity is only partially manifested in normal cardiomyocytes under the basal conditions and it can be affected by a number of mediators. Therefore, despite its role in many heart diseases, NO originating from cardiomyocytes is very likely to have an insignificant role in controlling the overall structure and function of the normal adult heart under unstrained physiological conditions. NO plays a role in the modulation of cardiac function in response to a specific stimulus, or in a state of myocardial stress, the release of parasympathetic and adrenergic neurotransmitters, muscle stretching, and the like.

Effects of NO on Cardiac Contractility

There is an enormous difference between the frequently conflicting effects of NO on myocardial contractility, depending on the animal species and experimental conditions, but most importantly on the experimental hierarchical level of research, no matter whether the individually isolated cardiomyocytes, multicellular preparations or in vivo intact heart are studied. Individual cardiomyocytes were extensively studied during the last 30 years. Despite the theoretical advantages of this experimental model, such as in the application of sophisticated molecular techniques for determining the specific signal molecules in cardiomyocytes, many uncontrolled artifacts induced by experimental isolating procedures can provide a distorted image of reality. This shows the limitations of such an approach in cardiac research, not only because of the isolation of cardiomyocytes from the inherent endothelial cells, but also of the neighboring cardiomyocytes [14, 15].

The positive inotropic response to NO on isolated cardiomyocytes has been obtained in several studies [16, 17]; however, there are also data on NO causing a negative inotropic effect but at higher concentrations [18]. Nevertheless, it remains unclear whether any response obtained in the indi-
Data that are more relevant come from multicellular cardiac preparations. They show a typical dose-dependent biphasic inotropic response to NO. Low values of NO, which correspond to endogenously generated NO, cause a positive inotropic response, whereas higher concentrations cause a consistent negative inotropic response. The response to the increased concentrations of cGMP is similarly biphasic [19]. When the concentration of cGMP-inhibited phosphodiesterase is low, it increases the intracellular level of cyclic adenosine monophosphate (cAMP), which can be considered a positive inotropy (Figure 2). Cyclic GMP at higher concentrations activates the cGMP-dependent protein kinase, which inhibits adenosine triphosphate (ATP) synthesis, and closes the voltage-dependent calcium channels, which can also be considered a negative inotropic response. Alternative interpretations, including the cGMP independent mechanisms, are possible due to the complexity of many NO signaling pathways and interactions [20, 21]. Although the modular interactions of NO with an autonomic nervous or other cardiomodulatory systems cannot be excluded in the human ventricle, no direct NO influence has been detected. The advantage of multicellular cardiac preparations is that they allow the complete tracking of both systolic (contraction and relaxation) and diastolic (tension at rest) phases of the cycle, both of which should not be ignored during the full assessment of the contractile function. For example, higher concentrations of NO cause a premature onset of relaxation during isometric contraction [19, 22]. NO-dependent effects on maximum rate of tension and peak tension can manifest themselves as decreased, unchanged or even increased.

It is very difficult to confirm the NO inotropic effects in the heart directly, in vivo, because of the confounding effects of varying loading conditions, coronary flow and neurohormonal control and interaction with β-adrenergic or cholinergic pathways as well as with the atrial natriuretic peptide. Several studies have provided indirect evidence of the less positive inotropic effects [23, 24]. The biphasic effect of NO, positive at low and negative at high concentrations of NO, is explained in transgenic mice in which the activity of eNOS was exaggerated. It is important to note that NO induces an earlier onset of ventricular relaxation in vivo, thus improving the ventricular relaxation, early rapid filling and diastolic compliance. This effect may be accompanied by a small decrease in the peak systolic pressure despite the frequently unchanged rate of pressure development and unchanged ejection fraction [25]. This effect is partially attributed to the NO-induced reduction of preload and afterload [24].

The negative inotropic effect can be considered potentially harmful. In fact, it should be considered potentially useful for the cardiac function since it acts as a compensatory feedback loop when the physiological effects on the duration of the contraction in the increased previous and/or subsequent ventricular load are superior to the pathologically prolonged duration of contraction in the ventricular hypertrophy. Pinsky et al. [26] have shown that there is a cyclic release of NO in the heart during heart rate, mainly in subendocardial zone, which reaches its peak during ventricular relaxation and early rapid chamber filling phase. This time convenient, short-term bursts of NO contribute to the important modulation of the chamber relaxation phases, especially in early filling and coronary perfusion during diastole. Subendocardial localization, as the principal area with the highest concentration of NO, indicates that EE cells are the main source of NO.

Accordingly, NO has a double effect on cardiac contractile performance. In response to the lower concentrations of the endogenously synthesized NO through eNOS activation, a positive inotropic effect is achieved, which contributes to the maintenance of cardiac contractility under basal physiological conditions. At higher NO levels, there is a negative response to peak contractile performance resulting from the iNOS activity or the pharmacological application of NO donors. Its impact on the onset of chamber relaxation, through the heart rate optimization and coronary perfusion is certainly more important, especially as a compensatory mechanism in the ventricular filling phase, when the latter is disrupted by disease.
NO has a significant effect on the time sequence of events and the very onset of the ventricular relaxation and modulation of cardiac systolic function. By delaying the onset of relaxation, the heart prolongs and maintains systole, as a part of heterometric autoregulation (Starling law) under the conditions of increased capacity and load pressure. In contrast, the earlier onset of ventricular relaxation resulting from NO release, favors the ventricular relaxation, early rapid filling, diastolic compliance, and coronary perfusion during diastole, which all together represent a compensatory mechanism against the ventricular load occurring under the conditions of severe maladaptive hypertrophy and/or tachycardia. When considering the impact of NO on contractility, we must take into account its interaction with endothelin-1 (ET-1), prostaglandin I2 (PGI2), angiotensin II (ANG II), β-adrenergic and cholinergic innervation, atrial natriuretic peptide (ANP) and aldosterone [25–28].

**Effect of NO on Cardiac Metabolism**

Endogenous and exogenous NO reduces the oxygen consumption of the myocardial tissue in both a healthy and diseased human heart [29, 30]. Similarly, a reduced production of NO leads to an increase in myocardial oxygen consumption in conscious dogs [31]. The inhibition of oxygen consumption by NO is also observed in the non-contractile cardiac muscle cells [32]. NO produced in the endothelial cells, and its inhibition of the myocardial oxygen consumption, is increased by angiotensin-converting-enzyme (ACE) inhibition, most probably by increasing the levels of bradykinin. Bradykinin-induced reduction in the myocardial oxygen consumption is reduced in eNOS of knock-out mice [33]. The ability of NO to reduce the myocardial oxygen consumption indicates potential cardio-protection effects of NO as it partially reduces the heart rate during systole and leads to the increased myocardial metabolic efficiency. It seems to be achieved through a better use of the energy substrate (free fatty acids, as opposed to glucose), or by the regulation of the mitochondrial metabolism. NO originating from MVE and EE cells regulates the local myocardial metabolism directly. NO has a tendency to compete reversibly with oxygen for a common binding site on cytochrome-c oxidase, thus inhibiting the transfer of electrons to oxygen. It has recently been shown in cultured cardiomyocytes not normally producing NO in situations where they are not strained that cytokine-induced NO production by iNOS activity or exogenous NO provided by NO donors reduces the energy consumption, i.e. ATP production and its consumption by myocardial contraction. This is accomplished by the inhibition of the mitochondrial iron-sulfite reductase [34].

Nitric oxide contributes to cellular respiration and affects cell function due to its ability to bond the heme groups of the important biological proteins (such as cytochrome - c oxidase) in which it competes with oxygen [2].

**Importance of NO in Embryonic Development of Heart**

A congenital heart disease is the most common malformation in children at birth. Endothelial NOS is essential during the heart development. Lack of eNOS results in congenital septal defects, cardiac hypertrophy and postnatal heart failure. In addition, eNOS is essential for the morphogenesis of major coronary arteries and the development of myocardial capillaries. The effects of NO are mediated by the induction of the transcription of growth factors that are essential in angiogenesis. Insufficient eNOS results in a high incidence of bicuspid aortic valve, complicated by stenosis or regurgitation, endocarditis, aortic aneurysm and aortic dissection. Thus, NO produced by eNOS plays a critical role in the embryonic development of the heart, in the morphogenesis of the coronary arteries and the aortic valve [35].

As result of eNOS activity, NO contributes to the embryonic development of the heart as shown in the experimental model of mice lacking eNOS. These animals without eNOS survive until they reach maturity; however, they exhibit frequent malformations (bicuspid aortic valve) [36]. A study involving mice without eNOS reported a large volume of aortic and ventricular septal defect, and a pillow deformity, resulting in death shortly after birth [37]. Pharmacological inhibition of NOS leads to slowing down or stopping cardiomyocytes differentiation, which suggests that cardiomyocyte-originated NO together with the endothelial NO may also play an important role in cardiomyogenesis at some stage, which probably corresponds to the later stage of the pillow formation and early myocardial compactness. Brutsaert et al. [38] have come to the conclusion that eNOS is present in EE cells in the developmental stage of the heart, in the morphogenesis of the coronary arteries and the aortic valve [35]. Endothelial mediators such as NO, ET-1, PGI, and ANG II may affect the myocardial growth and remodeling, since they affect the growth of vascular smooth muscles [40]. Regardless of their origin, which may be myocardial or endothelial, NO and ET-1 can take part theoretically in reactions of the adult heart growth. Cardiac endothelium dysfunction is used to explain a maladaptive growth during the progression of heart failure [5].
Many experimental and clinical observations confirm the effect of NO on the growth and remodeling of the heart. Bradykinin-induced NO synthesis contributes to the negative effects on the growth and is an example of the intersection of endocardial-myocardial signaling pathways of the heart. Bradykinin directly stimulates the growth of cardiomyocytes in culture, and the anti-growth effect depends largely on the presence of endothelial cells in co-culture, and the release of their mediators NO and PGI, [40, 41]. In the experiment with mice, the dysfunction of bradykinin B receptors leads to cardiac hypertrophy followed by the chamber dilation and reparative fibrosis, through the effects that can be prevented by ANG I receptor antagonists [40]. Kinins normally stimulate NO release through the activation of bradykinin B receptors. Kallikrein/kinin system has a cardioprotective effect as it eases the process of remodeling by activating the cardiac endothelial/myocardial signaling pathways [42, 43].

NO may act as a molecular switch in the promotion and/or inhibition of the growth factors effects, such as basic fibroblast growth (bFG), vascular endothelial growth (VEGF) in TGF-β in an adult heart. The levels of eNOS activity in cardiac endothelial cells may, therefore, be valuable in controlling the growth and remodeling of the heart [44, 45].

**NO and Heart Failure**

Over the last couple of decades, we have witnessed an impressive progress in demystifying the pathogenesis of heart failure, which has emphasized the importance of a number of compensatory mechanisms, such as cardiac dilatation or hypertrophy, the participation of neurohumoral factors, synthesis of cytokines and the activation of endothelial cells. Compensatory mechanisms, either cardiac or non-cardiac ones, may be insufficient to adapt to the new conditions, with the resulting clinical manifestation of heart failure [46].

Endothelium, that is the endothelial activation and dysfunction, hold an important place in the pathogenesis of heart failure. Endothelial dysfunction in clinical practice is mainly related to the reduced production of endothelial NO and its bioavailability. It is believed that endothelial dysfunction occurs when vasodilatation is expected, mediated by endothelial NO in response to acetylcholine, bradykinin, substance P and/or serotonin deficient. The concomitant use of a NO donor, e.g. nitroprusside, would lead to the expected vasodilatation (Figure 3).

Measuring of the NOS levels during heart failure has given disappointing inconsistent data, which may have resulted from the different etiology of heart failure (dilated, idiopathic, ischemic, hypertrophic). Thus, at the last stage of human heart failure, the level of eNOS is increased in the sub-endocardial cardiomyocytes, but it is decreased in the myocardial capillary endothelial cells [47]. In heart failure caused by septic shock, the activity of iNOS is increased, which results in the increased synthesis of NO with the consequent decreased strength of the heart contractions. Cytokines, such as TNF-α, whose plasma values are increased during the septic shock, are also considered strong inducers of iNOS [48].

There is a significant positive correlation between the activities of the endocardial and sub-endocardial iNOS and eNOS with ventricular contractile performance which suggests a beneficial effect of NO released from the endothelial cells in patients with dilated cardiomyopathy. Endogenous NO also has a cardioprotective effect in patients with idiopathic dilated cardiomyopathy by lowering the contractile response to β-adrenergic stimulation, thus saving myocardial oxygen [49].

Drugs used in the treatment of heart failure and angina pectoris, which are based on organic nitrates, act through NO in which they are metabolized. NO activates the soluble guanylate cyclase, increases the synthesis of cGMP, activates protein kinase A, and leads to the cascades of effects in the smooth muscle, ending with the dephosphorylation of light myosin chains, and the removal of Ca²⁺, and muscle relaxation. NO donors cause a distinct venodilation with a reduction of central venous pressure and afterload. Its direct effect on coronary arteries decreases the spasm and increases the coronary flow. Moreover, drugs such as glyceryl trinitrate redirect the blood from normal to ischemic areas of the myocardium through the collaterals. In addition to their effect on smooth muscle, NO donors lead to the relaxation of the heart muscle.

**Conclusion**

The following conclusion can be made according to the aforementioned:
- nitric oxide synthesis in the heart is one of the factors that provides a normal cardiac activity under physiological conditions
- there is a significant non-uniformity in the distribution of endothelial nitric oxide synthase in heart enlarge...
the cardiac endothelium and cardiomyocytes with the activity that is more intense in the endocardial endothelial cells
- endothelial nitrous oxide synthase activity is only partially expressed under the basal conditions in normal cardiomyocytes and can be affected by a number of mediators
  - nitric oxide plays a significant role in modulating cardiac function in response to a specific stimulus, or in a state of myocardial stress, sympathetic or parasympathetic neurotransmitter releases, muscle stretching, and similar.
  - endothelial nitrous oxide synthase-derived nitric oxide has a vasodilatation and atheroprotective effect, while nitric oxide synthesized by inducible nitrous oxide synthase in macrophages, which has a much higher capacity, has oxidative and anti-atherogenic function.

References