Summary

Background: Coronary heart disease is the leading cause of death and disability worldwide. Hypertension is a major independent risk factor for the development of CHD. Abnormalities in NO generation or activity have been proposed as a major mechanism of CHD. The purpose of this article is to determine the activity of eNOS and iNOS in patients with isolated CHD and CHD associated with HT of different age groups.

Methods: Fifty patients with isolated CHD and 42 patients with CHD associated with HT were enrolled in this study. NOS activity was determined by nitrite anion formed in the reaction.

Results: A statistically significant increase in iNOS activity is observed in elderly donors. In patients with isolated coronary heart disease cNOS activity is statistically significantly reduced with respect to the control group. The reduction of enzymatic activity of cNOS is more expressed in elderly patients than in middle-aged patients with coronary heart disease. Alterations in eNOS activity are more expressed in patients with coronary heart disease associated with hypertension than in patients with isolated coronary heart disease. Against the background of cNOS inhibition in the patients, a sharp increase in iNOS activity is observed.

Conclusions: It has been shown that disturbance of endothelial function in patients with coronary heart disease associated with hypertension is characterized by reduced endothelial NO synthesis by cNOS and increased systemic NO synthesis due to increased iNOS activity. It has been found that the lack of endothelial NO and hyperproduction of «harmful» NO by iNOS are more expressed in elderly patients.

Keywords: coronary heart disease, hypertension, nitric oxide, NO-synthase

Kratak sadržaj

Uvod: Širom sveta, koronarna bolest srca (KBS) predstavlja vodeći uzrok smrti i invaliditeta. Hipertenzija je važan nezavisni faktor rizika za KBS. Poremećaj generacije ili aktivnosti NO navodi se kao potencijalno glavni mehanizam u KBS. Srhva ovog članka je da se odrede aktivnosti eNOS i iNOS kod pacijenata sa izolovanom KBS i KBS povezanom sa hipertenzijom (HT) kod različitih starosnih grupa.

Metode: Studija je obuhvatila 50 pacijenata sa izolovanom KBS i 42 pacijenta sa KBS povezanom sa HT. Aktivnost NOS određena je pomoću nitratnog anjona koji se formira u reakciji.

Rezultati: Kod starijih donatora primenjen je statistički značajan porast aktivnosti iNOS. Kod pacijenata sa izolovanom koronarnom bolesti srca aktivnost cNOS je statistički značajno smanjena u odnosu na kontrolnu grupu. Smanjenje enzimske aktivnosti cNOS više je izraženo kod starijih bolesnika nego kod sredovekovnih pacijenata sa koronarnom bolesti srca. Alteracije u aktivnosti eNOS više su izražene kod pacijenata sa koronarnom bolešću srca povezanim sa hipertenzijom nego kod bolesnika sa izolovanom koronarnom bolešću srca. S obzirom na inhibiciju cNOS kod bolesnika, uočen je nagli porast aktivnosti iNOS.

Zaključak: Pokazano je da poremećaj endotelne funkcije kod pacijenata sa koronarnom bolesti srca povezanom sa hipertenzijom karakteriše smanjena sinteza endotelnog NO od strane cNOS i povećana sinteza sistemskog NO usled povišene aktivnosti iNOS. Otkriveno je da su nedostatak endotelnog NO i hiperprodukcija »harmful« NO od strane cNOS izraženiji kod starijih bolesnika.

Ključne reči: koronarna bolest srca, hipertenzija, azotmonoksid, NO-sintaza

Abbreviations: CHD, coronary heart disease; eNOS, endothelial isoform NOS; iNOS, inducible isoform NOS; nNOS, neuronal isoform NOS; HT, hypertension.
Introduction

Coronary artery disease, also known as coronary heart disease (CHD), is the major cause of death and disability worldwide. Despite all the therapeutic advances in the prevention and treatment of CHD, the disease remains the leading cause of death around the world (1, 2). The CHD epidemic has been extremely dynamic over the last fifty years. In many parts of the world, the incidence of CHD is still on the rise, and it is estimated that in the coming years the number of CHD patients will increase substantially, especially in developing and transitional countries (3, 4).

Epidemiological studies have established a strong association between CHD and hypertension (HT). HT is a major independent risk factor for the development of CHD (5). According to modern concepts, endothelial dysfunction is a primary component in the pathophysiology of cardiovascular continuum. The endothelium is an active metabolic system in the regulation of physiological activity where nitric oxide (NO) plays an important role. NO is the key endothelium-derived relaxing factor which plays a pivotal role in maintaining vascular tone and reactivity and is the main determinant of basal vascular smooth muscle tone. Abnormalities in NO generation or activity have been proposed as a major mechanism of endothelial dysfunction, the pathophysiology of CHD and HT (6, 7).

Under physiological conditions, enzymatic NO formation in humans and animals occurs by nitric oxide synthase (NOS, EC 1.14.13.39), which is a cytochrome P-450 type hemoprotein. NOS has been generally considered to be the primary source of NO in biological systems. There are three types of NOS isoenzymes: NOS I and NOS III are each constitutively produced at a low level (nNOS from neurological tissue and eNOS from endothelial cells, respectively) and NOS II (iNOS) is inducibly expressed in macrophages and endothelial cells. The constitutive NOS (cNOS) isoenzymes respond to increases in intracellular Ca$^{2+}$ and produce NO permanently (constitutive) for short periods of time, being induced for example by vasodilators like acetylcholine. In contrast, iNOS is expressed due to the effects of proinflammatory cytokines and can release several times more NO than the constitutive NOS isoenzymes. iNOS is a calcium-independent isoform of NOS and is not expressed constitutively.

All isoforms of NOS are similar in structure, mechanisms of catalytic activity and expressed as products of different genes. They have similar structure with 50%–60% sequence identity. Most cell types of the human body have one or more isoforms of NOS (8, 9). Isoform I of NO synthase has been found mostly in endothelial cells. Under physiological conditions, eNOS predominantly produces NO in the bloodstream. eNOS plays an important role in providing permanent, basal NO, which is physiologically necessary, is involved in local mechanisms of endothelial cytoprotection and is crucial for maintaining vascular homeostasis. eNOS-derived NO serves important functions, including regulation of regional blood flow, suppression of vascular smooth muscle cell proliferation, modulation of leukocyte–endothelial interactions and modulation of thrombosis. Except endothelial cells, eNOS is expressed in erythrocytes, platelets, cardiac and other cells (10).

While relatively small amounts of NO produced by eNOS are important to cardiovascular homeostasis, high NO levels associated with iNOS activity may have detrimental consequences to the cardiovascular system and contribute to hypertension. iNOS inhibits apparently exerts antihypertensive effects, decreases oxidative and nitrosative stress, and improves vascular function. The activity of eNOS expressed in non-vascular tissue might play a role in physiologic blood pressure regulation (11). Also, it was found that iNOS is a potential pharmacological target in hypertension (12).

Thus, elucidating the role of NOS involved in the formation and degradation of NO, a ubiquitous signalling molecule largely responsible for the maintenance of normal endothelial function, seems to be of major significance. Despite a large number of papers devoted to the NOS system under cardiovascular diseases, the age-related changes of the activity of NOS isoenzymes in patients with isolated CHD and CHD associated with HT remain not fully investigated.

The purpose of this article is to determine the activity of constitutive and inducible isoforms of NOS in the blood plasma of patients with isolated CHD and in patients with CHD associated with HT of different age groups.

Materials and Methods

Fifty patients with isolated CHD (32 men, 18 women) aged 45–75 years (mean±SD: 56.8±4.7) and forty-two patients with CHD associated with HT (22 men, 20 women) aged 45–75 years (mean±SD: 54.4±4.6) were enrolled in this study. Inpatients were only included in the study if CHD was verified by instrumental data (ECG, including daily monitoring ECG, echocardiography, bicycle ergometry test). The research included patients who occasionally used nitroglycerin for angina pectoris. The patients of each group were divided into two subgroups with respect to their ages: middle-aged patients (45–60 years, n=44) and older-aged patients (61–75 years, n=48). The patients of both subgroups were matched for sex, disease duration, and number of pain attacks.

Twenty healthy volunteers (divided into two age groups) with no clinical symptoms of cardiovascular
disease, matched by age and sex, were enrolled in this study. Written informed consent was obtained after full explanation of the study procedure. The protocol was approved by the Ethical Committee of Danylo Halytsky Lviv National Medical University.

The total NOS activity was measured by the nitrite concentration in blood plasma using a designed in-house method. Briefly, incubation medium for the determination of total NO-synthase activity contained: 0.1 mol/L Tris-HCl (pH 7.4), 5 mmol/L MgCl₂, 1.0 mmol/L NADPH (Sigma, USA), 1 mmol/L L-arginine and 10 mmol/L CaCl₂. The reaction was initiated by adding 0.2 mL of blood to the incubation mixture (final volume 2.0 mL). The samples were kept for 20 min in a water bath (37 °C) under constant shaking. The enzymatic reaction was stopped by addition of 1.25 mL 85 mmol/L NaOH and 1.25 mL of 75 mmol/L ZnSO₄. Control samples were prepared similarly, but without the substrate in incubation medium. After stopping the enzymatic reaction, the samples were centrifuged at 3000 g for 15 min. The NO₂ concentration was determined in an aliquot of the supernatant using the Griess reaction (13). Total NOS activity was expressed as nmol NO₂/min per 1 mL.

The activity of Ca²⁺-independent isoform (iNOS) was detected similarly, adding Ca²⁺ chelator EGTA to the incubation medium (4 mmol/L) instead of CaCl₂. The activity of Ca²⁺-dependent isoforms of NOS (eNOS) was calculated as the difference between total NOS activity and Ca²⁺-independent activity.

Data are expressed as mean ± standard deviation. The significance of differences in parameters between test groups and between control and test groups was established by Student’s t-test considering the fact that data on NOS activity changes had a normal distribution. Differences were considered statistically significant at a value of \( P \leq 0.05 \).

### Results

It was found that cNOS activity decreases with age, but these changes are not statistically significant. In middle-aged and older-aged healthy donors cNOS activity is 0.2±0.03 and 0.18±0.02 nmol NO₂/min per 1 mL, respectively (Figure 1). iNOS activity in plasma of middle-aged healthy individuals is low compared to the cNOS activity and equals to 0.05±0.009 nmol NO₂/min per 1 mL. A statistically significant increase in iNOS activity is observed in elderly donors. Its value is 0.13±0.014 nmol NO₂/min per 1 mL, which is 2.6-fold greater than the value in middle-aged donors.

In patients with isolated CHD cNOS activity is statistically significantly reduced with respect to the control group. The reduction of enzymatic activity of cNOS is more expressed in elderly patients than in middle-aged patients with CHD. cNOS activity in middle-aged patients with isolated CHD is reduced 1.5-fold compared to healthy individuals of the same age group and equals to 0.13±0.02 nmol NO₂/min per

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**Figure 1** eNOS and iNOS activity in middle-aged (A) and older-aged (B) patients with isolated CHD, patients with CHD associated with HT and healthy individuals.

Values are mean ± SD; *** – \( P < 0.001 \); * – \( P < 0.05 \) compared to healthy individuals; ### – \( P < 0.001 \); ## – \( P < 0.01 \); # – \( P < 0.05 \) compared to middle-aged patients.
In elderly patients cNOS activity is reduced 2.6-fold compared to control group and equals to 0.07±0.015 nmol NO\textsubscript{2}–/min per 1 mL.

Alterations in eNOS activity are more expressed in patients with CHD associated with HT than patients with isolated CHD. The eNOS activities in middle-aged and older-aged patients with CHD associated with HT are 0.10±0.02 and 0.04±0.018 nmol NO\textsubscript{2}–/min per 1 mL, respectively. This is 2.0-fold and 4.5-fold lower than in healthy individuals of the same age group.

Against the background of cNOS inhibition in patients with isolated CHD, a sharp increase in iNOS activity is observed. iNOS activity in middle-aged patients with isolated CHD increases 9.2-fold over individuals of the control group and is 0.46±0.06 nmol NO\textsubscript{2}–/min per 1 mL. In elderly patients with isolated CHD iNOS activity is 0.77±0.04 nmol NO\textsubscript{2}–/min per 1 L i.e. 5.9-fold over donors of the control group.

In middle-aged and older-aged patients with CHD associated with HT iNOS activity is increased 10.4- and 5.2-fold in comparison with healthy individuals of the same age group and equals to 0.52±0.08 and 0.68±0.08 nmol NO\textsubscript{2}–/min per 1 mL, accordingly.

After analyzing the data, it was revealed that the ratio of iNOS/cNOS activity increases 2.9-fold with age in healthy donors (Figure 2). In middle-aged patients with isolated CHD the iNOS/cNOS ratio is increased 14-fold and in elderly patients it is increased 15.3-fold in comparison to healthy individuals of the same age group. In middle-aged and older-aged patients with CHD associated with HT the iNOS/cNOS ratio is increased 20.8-fold and 23.6-fold compared to the control group, respectively. This is due to a decrease in cNOS activity, which produces a basal NO involved in the regulation of vascular tone, and an increase in iNOS activity involved in the synthesis of »harmful« NO.

**Discussion**

The present study was designed to investigate the activity of constitutive and inducible isoforms of NOS in the blood plasma of patients with isolated CHD and in patients with CHD associated with HT of different age groups. We have found a nonsignificant decrease in eNOS activity and significant increase in iNOS activity both in patients with isolated CHD and CHD associated with HT. These findings are inconsistent with the reports (14, 15). They have shown an increase in the eNOS activity and expression in CHD patients which is regarded as a compensatory mechanism against CHD.

It is interesting, however, that human population studies have not demonstrated a link between the variation in the eNOS gene and hypertension (16). But, it was found that lack of eNOS promotes endothelin-induced hypertension (17). eNOS levels in large systemic arteries such as the aorta are generally increased in hypertensive rats (18). Also, *in vivo* inhibition of NO synthase activity by nonhydrolyzable analogs of L-arginine results in a dramatic increase in mean arterial blood pressure (19). In dogs with acute perinephritic hypertension, iNOS protein is undetectable in the heart and no difference in Ca\textsuperscript{2+}-independent NOS activity is apparent between normotensive and hypertensive conditions (20).
NO’s protective effects on the animal endothelium have also been demonstrated in mice with impaired eNOS function giving rise to HT which has a close correlation with CHD (21). Previous studies have reported eNOS polymorphisms as probable risk factors in the CHD pathogenesis (22).

The data regarding age-related changes are consistent with those obtained by other investigators in experiments on rat vascular wall (23). They showed that the content of nitrite anions is reduced by 40% with age due to the lower eNOS activity which decreases with age. iNOS activity increases in older animals, due to the excessive formation and accumulation of lipid peroxidation products in tissues during aging. The researchers also found that NO production in the myocardium of adult and old rats is provided by different mechanisms. The NO production is realized via cNOS in adult rats and via iNOS in old rats (24).

Increased expression of iNOS in the endothelium was previously detected in coronary arteries of aged rats (25). This observation has been confirmed by Santhanam et al. (26).

Inhibition of cNOS in healthy donors with aging, in patients with isolated CHD and patients with CHD associated with HT indicates a decrease in endogenous NO synthesis and endothelial dysfunction. It is known that factors that cause endothelial dysfunction in patients with CHD are dyslipidemia, oxidative stress, high blood pressure etc. It is also known that reduction in eNOS activity leads to a decrease in the endogenous production of NO by endothelialocytes and is a key element in the pathogenesis of CHD (27, 28). eNOS deficiency introduces coronary disease and an array of cardiovascular complications, including spontaneous aortic aneurysm and dissection (29).

iNOS activation leads to overproduction of NO. cNOS is known to produce NO in low concentrations whereas iNOS synthesizes high concentrations of NO (> 300 nm) (30). NO hyperproduction by iNOS may be a compensatory mechanism improving tissue perfusion.

But, in high concentrations, NO is profoundly toxic and is a factor of endogenous intoxication that determines its cytotoxic effect and causes cell death by apoptosis and necrosis. As a result, the activation of apoptotic mechanisms and initiation of destructive processes occur in cardiomyocytes, endothelial cells and other cells, which lead to the progression of dysfunction of the cardiovascular system. iNOS causes production of «harmful» NO and free radical products, which are involved in the activation of lipid peroxidation processes, resulting in cell damage and superoxide anion-radical (O−2) accumulation (31).

Our data indicate the age difference in NOS isoforms activities in patients with CHD associated with HT. It was previously believed that NOS-dependent synthesis of «basal» NO is realized by cNOS and iNOS provides additional amounts of NO in the cell under various pathological conditions. However, present studies revealed constitutive expression of iNOS in some tissues and inducible forms of eNOS and nNOS. In particular, participation of iNOS in the physiological («basal») synthesis of NO was confirmed, and participation of eNOS and nNOS in the overproduction of NO in some pathologies. Thus, the «basal» NO-synthase activity of iNOS activity in the regulation of physiological functions, such as regulation of vascular tone, is critically important (32).

The presence of different NOS isoforms, i.e. their different enzymatic activities demonstrate the complex nature of its regulation. Alteration in the iNOS/cNOS ratio in patients with CHD associated with HT acts as a determining mechanism that leads to disruption of NO-regulatory properties. Our data indicate the disturbance of NO homeostasis and endothelial NO deficiency in patients with CHD associated with HT, which is more expressed in older patients.

As with any clinical study done on patients, limitations exist. The populations used in the present study have a small number of participants that necessitate careful interpretation of results. Future studies should incorporate the investigation of gender, smoking status and other demographic characteristics of the examined patient group that might represent an underlying influence on NOS activity besides age.

Conclusions

It has been shown that disturbance of endothelial function in patients with coronary heart disease associated with hypertension is characterized by reduced endothelial NO synthesis by cNOS and increased systemic NO synthesis due to increased iNOS activity. It has been found that the lack of endothelial NO and hyperproduction of «harmful» NO by iNOS is more expressed in elderly patients. In patients with CHD associated with HT, increase in iNOS, iNOS/cNOS ratio and decrease in eNOS activity are more expressed due to the synergistic effect of both pathologies.

Conflict of interest statement

The authors stated that they have no conflicts of interest regarding the publication of this article.
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