THE EFFECTS OF L-ARGININE AND L-NAME ON CORONARY FLOW AND OXIDATIVE STRESS IN ISOLATED RAT HEARTS

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ABSTRACT

The aim of this experimental study was to assess the effects of the acute administration of L-arginine alone and in combination with L-NAME (a non-selective NO synthase inhibitor) on the coronary flow and oxidative stress markers in isolated rat hearts. The experimental study was performed on hearts isolated from Wistar albino rats (n=12, male, 8 weeks old, body mass of 180-200 g). Retrograde perfusion of the isolated preparations was performed using a modified method according to the Langendorff technique with a gradual increase in the perfusion pressure (40–120 cmH2O). The following values were measured in the collected coronary effluents: coronary flow, released nitrites (NO, as NO2−), and oxidative stress markers (TBARS, index of lipid peroxidation). The results indicated that L-arginine did not significantly increase the coronary flow or the release of NO, TBARS and the index of lipid peroxidation. The effects of L-arginine alone (1 mmol) and L-arginine (1 mmol) + L-NAME (30 μmol) were partially blocked by the joint administration of L-arginine + L-NAME, which indicated their competitive effect. Hence, the results of our study do not demonstrate significant effects of L-arginine administration on the coronary flow and oxidative stress markers in isolated rat hearts.

Key words: L-arginine, L-NAME, redox status, isolated heart, rats

SAŽETAK

Cilj ovog istraživanja je bio procenat efekata akutne administracije L-arginina na koronarni protok i markere oksidacionog stresa, samostalno i/ili u prisustvu L-NAME (neselektivni inhibitorni NOS sintetase), na izolovanim srca pacova. Ovo je eksperimentalna studija, koja je sprovedena na izolovanim srca Vistar albino soja pacova (n = 12, muški, 8 nedelja, telesna masa 180-200g). Retrogradna perfuzija izolovanih organa se sprovodila modificovanom tehnikom prema Langendorfu, sa postepenim povećanjem perfuzijskog pritiska (40–120 cmH2O). Nakon izmjerena koronarnog protoka, u prikupljenim uzorcima koronarnog efluenta mereni su sledeći parametri: nivoi azot monoksid-a (u formi nitritita), superoksid anjon radikala i indeksa lipidne peroksidacije (meren kao TBARS). Eksperimentalni protokol je sproveden pod strogom kontrolom, i podrazumeva administraciju samo L-arginina (1 mmol), i administraciju L-arginina (1 mmol) u kombinaciji sa L-NAME (30 μmol). Rezultati ovog istraživanja ukazuju na to da L-arginin neznačajno povećava koronarni protok, neznačajno povećava nivoe azot monoksida, TBARS-a i superoksid anjon radikala. Ovakav efekat je delimično blokiran u slučaju zajedničke administracije L-arginina i L-NAME što ukazuje na njihovu kompetitivnost. Dakle, rezultati našeg istraživanja ne pokazuju statistički značajne efekte primene L-arginina na koronarni protok i markere oksidacionog stresa izolovanog srca pacova.

Ključne reči: L-arginin, L-NAME, redoks status, izolovano srce, pacovi

INTRODUCTION

L-arginine (2-amino-5-guanidinovaleric acid) is a basic, conditionally essential amino acid that enters an organism via the diet or is obtained by the degradation of body proteins or endogenous de novo synthesis (1). This semi-essential amino acid takes part in numerous key biochemical and physiological activities.

During the last decades of the 20th century, L-arginine was identified as a precursor of nitric oxide synthesis (NO) (2). Specifically, it represents the key source of NO synthesis in many cells of an organism (3). NO is produced during the transformation of L-arginine to L-citrulline in a reaction catalysed by NO synthase (NOS) (4–7).
The L-arginine/NO system is one of the crucial players in the maintenance of microvascular homeostasis. Additionally, NO causes vasodilatation, improves microcirculation by stimulating endothelial proliferation and angiogenesis, and inhibits endothelial apoptosis, the release of endothelin-1, the proliferation of smooth muscular cells and thrombocyte aggregation and adhesion (8).

Endothelial dysfunction is one of the earliest markers of vascular abnormality. It is present in cardiovascular diseases linked to the increased production of reactive oxygen species (ROS) or the state of oxidative stress (9, 10). Cell damage caused by ROS (the most significant among which are the superoxide anion radical and hydroxyl radical) is a significant causal factor of heart diseases, particularly those that present with myocardial ischemia-reperfusion damage (11). Many authors have demonstrated the production and release of free radicals in the ischemic heart, including their intensive release during the reperfusion period (12, 13, 14). The rapid recovery of blood flow increases tissue oxygenation with a consequent secondary production of ROS, leading to reperfusion injury (15). One of the possible mechanisms underlying ROS-mediated cardiovascular diseases is the reduced production of endothelium-dependent vasodilatory substances (16), of which NO is the most significant (17). Moreover, the L-arginine-dependent enzyme arginase is up-regulated in response to the reduction in NO bioavailability during oxidative stress (9).

Because NO is an endothelial-dependent relaxing factor that plays an essential role in the regulation of the vascular tonus and haemodynamics, there has been interest for decades in the application of L-arginine for the prevention and treatment of cardiovascular diseases (18). L-arginine appears to provide “hope” for the treatment of cardiovascular diseases. Based on results obtained to date, oral or parenteral administration of this amino acid seems to recuperate endothelial function and improve coronary microcirculation. L-arginine affects atherosclerotic risk factors (hypercholesterolemia, hypertension, and smoking) by improving endothelial functions in these patients (8).

However, the exact role of the L-arginine/NO system within the coronary circulation is still unknown due to reports of controversial data.

The aim of the present study was to examine the effects of L-arginine alone or in combination with a non-selective NOS inhibitor (N\textsuperscript{G}-nitro-L-arginine monomethyl ester, L-NAME) on the coronary flow, oxidative stress markers and nitrites in hearts isolated from rats.

**MATERIAL AND METHODS**

**Preparation of isolated rat hearts**

Isolated hearts (total number n=12, 6 preparations for each experimental group; rejected hearts did not contribute to the total number) were obtained from Wistar albino rats (male, 8 weeks old, body mass of 180 - 200 g; obtained from the VMA - Military Medical Academy, Belgrade, Serbia) and perfused with a modified apparatus according to the method of Langendorff (Hugo-Sachs Electronik-Harvard Apparatus GmbH, March- Hugstetten, Germany). The animals were euthanised by cervical dislocation following administration of a short ether anaesthetic with the anticoagulant heparin (Schedule 1 of the Animals/Scientific Procedures, Act 1986, United Kingdom). Following the emergency thoracotomy and the induction of heart failure via the superfusion of cold physiological solvent, the heart was quickly prepared and isolated by the removal of all redundant parts (with the exception of ascending aorta, which was cannulated to provide retrograde perfusion under gradually increasing coronary perfusion pressure (CPP)). Krebs-Henseleit buffer was used for retrograde perfusion (in mmol/l: NaCl 118, KCl 4.7, CaCl\textsubscript{2} x 2H\textsubscript{2}O 2.5, MgSO\textsubscript{4} x 7H\textsubscript{2}O 1.7, NaHCO\textsubscript{3} 25, KH\textsubscript{2}PO\textsubscript{4} 1.2, glucose 11, and pyruvate 2). The buffer was balanced with 95% O\textsubscript{2} and 5% CO\textsubscript{2}, with a pH value of 7.4 and temperature of 37°C. In all preparations, an electrostimulator (Hugo-Sachs Electronik-Harvard Apparatus GmbH) ensured the heart rate and its regularity (5 V, 320 bpm) via electrodes set in the atrial region.

**Physiological examination and experimental protocol**

Following the establishment of heart perfusion, the preparations were stabilised within 30 minutes with a basal coronary perfusion pressure of 60 cmH\textsubscript{2}O. During the stabilisation of the preparations, the reactivity of the coronary blood vessels was examined by short occlusion of coronary flow (5-30 s) and bolus injection of 5 mmol/l adenosine (60 μl at a flow rate of 10 ml/min to obtain the maximum flow). The preparations were rejected (approximately 25%) unless an increase in the flow of 100% was achieved compared to the control values for both tests. Following the stabilisation period, the perfusion pressure was reduced to 50 and 40 cmH\textsubscript{2}O and then gradually increased to 70, 80, 90, 100, 110 and 120 cmH\textsubscript{2}O to establish coronary autoregulation. At each given value of coronary perfusion pressure a value of flow was noted for at least 5 minutes. When the flow was determined to be stable, samples of coronary effluent were collected for each value of perfusion pressure. The correctly performed control experiment (control values in each experimental group) included the double examination of coronary perfusion pressure/coronary flow in the absence of any medication. The main goal was to confirm that the preparation was stable and that the response between the first and second series of changes in perfusion pressure were not significantly different. Following the control experimental protocol, the preparations were perfused with L-arginine (1 mmol) and L-arginine (1 mmol) plus an NO synthesis inhibitor (30 μl L-NAME). Testing started immediately after the control experiment to avoid unwanted time-de-
pendent consequences. The administration of medicines lasted until the achievement of a stable flow but not under 5 minutes for each value of perfusion pressure. The results obtained during the experimental protocol (coronary flow, superoxide anion radical concentration, released nitrites and index of lipid peroxidation) were compared to the results obtained after the administration of L-arginine and L-arginine + L-NAME.

**Biochemical analysis**

Samples of coronary venous effluent were collected after the stabilisation of the coronary flow for each value of the gradually increased perfusion pressure. We performed the spectrophotometric determination of nitrites, superoxide anion radicals and the index of lipid peroxidation indirectly via reactive thiobarbituric substances (TBARS) for all samples.

**Determination of nitrites**

Nitric oxide quickly decomposes into stable metabolite nitrites/nitrates. Nitrites are used as an index of NO production via a spectrophotometric method using the Griess reagent. Briefly, 0.5 ml of the perfusate is precipitated with 200 μl of 30% sulfosalicylic acid, mixed for 30 minutes and centrifuged at 3000 x g. Equal volumes of the supernatant and Griess reagent are mixed and stabilised for 10 minutes in the dark, and then the sample is measured spectrophotometrically at a wavelength of 543 nm. The nitrite concentrations are determined using sodium nitrite as the standard (19).

**Determination of superoxide anion radicals**

Superoxide anion radical concentrations are measured using the NTB (Nitro Blue Tetrazolium) reagent in TRIS buffer (assay mixture) with coronary venous effluent. The measurement was performed at a wavelength of 530 nm. The Krebs-Henseleit solvent was used as the blank control (20).

**Determination of the index of lipid peroxidation (TBARS)**

The index of lipid peroxidation was determined indirectly by measuring the products of the reaction of lipid peroxidation with thiobarbituric acid (TBARS or Thiobarbituric Acid Reactive Substances). Briefly, 1% thiobarbituric acid (TBA) in 0.05 M NaOH is incubated with coronary venous effluent at 100°C for 15 minutes and then spectrophotometrically measured at a wavelength of 530 nm. The Krebs-Henseleit solvent was used as the blank control (21).

**Reagents**

The L-arginine and L-NAME solvents were obtained as a gift from the Biomedical Sciences Department of the Academy of Sciences of Slovakia (Bratislava, Republic of Slovakia). A set of reagents for the spectrophotometric determination of nitrites (naphthyl ethylenediamine dihydrochloride and sulfosalicylic acid) were purchased from Sigma-Aldrich Chemie GmbH. Sulfanilamide, phosphorous acid, NTB, TRIS-puffer and TBA were purchased from Merck KGaA Company (Dermstadt, Germany).

**Statistical analysis**

Values were expressed as the arithmetic mean ± S.E.M. A multifactorial analysis of variance with repeated measures was performed. In this model, different values of CPP were given as within-subject factors, whereas the application of a treatment was provided as a measurement of the difference between subjects. A p value less than 0.05 was considered statistically significant.

**RESULTS**

**Coronary flow**

The coronary flow exhibited a significant increase that was proportional to the coronary perfusion pressure over the whole range of perfusion pressure values studied in both the control and study groups. Under the control conditions, the coronary flow varied in the range from 3.00 ± 0.86 ml/min/g of tissue mass (wt) at 40 cmH2O to 8.57 ± 1.77 ml/min/g wt at 120 cmH2O. L-arginine did not induce a significant change in the coronary flow (range from 3.65 ± 1.02 at 40 cmH2O to 10.93 ± 2.80 ml/min/g wt at 120 cmH2O) (Fig. 1).

L-arginine + L-NAME did not induce a significant reduction in the coronary flow compared to the control group. Under the control conditions, the coronary flow varied in the range from 3.15 ± 0.66 ml/min/g wt at 40 cmH2O to 9.93 ± 2.80 ml/min/g wt at 120 cmH2O (Fig. 1).

L-arginine + L-NAME did not induce a significant reduction in the coronary flow compared to the control group. Under the control conditions, the coronary flow varied in the range from 3.15 ± 0.66 ml/min/g wt at 40 cmH2O to 9.93 ± 2.80 ml/min/g wt at 120 cmH2O (Fig. 1).

![Figure 1. Effects of L-arginine (1 mmol) on the coronary flow at different coronary perfusion pressures (CPP). Each value represents the mean ± SE and is expressed relative to the control.](image-url)

A p value < 0.05 was considered to be significant. *p < 0.05.
cmH₂O to 9.40 ± 0.67 ml/min/g wt at 120 cmH₂O. In the treated group, the flow ranged from 2.90 ± 0.72 ml/min/g wt at 40 cmH₂O to 7.85 ± 0.60 ml/min/g wt at 120 cmH₂O (Fig. 2).

**Nitrite outflow**

Under the control conditions, the nitrite outflow varied from 1.04 ± 0.32 nmol/min/g wt at 40 cmH₂O to 2.93 ± 0.90 nmol/min/g wt at 120 cmH₂O. L-arginine did not induce a significant increase in the nitrite outflow (range from 1.28 ± 0.48 nmol/min/g wt at 40 cmH₂O to 3.89 ± 1.23 nmol/min/g wt at 120 cmH₂O) (Fig. 3). Additionally, there was no significant difference between the groups in the dynamics of the increase in the nitrite outflow with increasing CPP.

L-arginine + L-NAME induced a significant decrease in the nitrite outflow compared to the control group. Under the control conditions, the values changed in the range from 1.00 ± 0.13 nmol/min/g wt at 40 cmH₂O to 3.40 ± 1.50 nmol/min/g wt at 120 cmH₂O; in the treated group, the values changed from 0.86 ± 0.21 nmol/min/g wt at 40 cmH₂O to 2.46 ± 0.58 nmol/min/g wt at 120 cmH₂O (Fig. 4). The nitrite concentrations increased as the CPP increased in both groups.

**Superoxide anion production**

L-arginine did not induce significant changes in the superoxide anion radical (O₂⁻) levels. However, a significant increase in O₂⁻ levels was noted in both groups as the CPP increased. Under the control conditions, O₂⁻ production
varied from $8.90 \pm 3.09$ nmol/min/g wt at 40 cmH$_2$O to $33.79 \pm 8.40$ nmol/min/g wt at 120 cmH$_2$O in the treated group. O$_2^-$ production varied from $11.63 \pm 2.67$ nmol/min/g wt at 40 cmH$_2$O to $38.61 \pm 8.67$ nmol/min/g wt at 120 cmH$_2$O (Fig. 5).

L-arginine + L-NAME also did not significantly affect superoxide anion production compared with the control values (Fig. 6).

*Index of lipid peroxidation (TBARS production)*

Under the control conditions, the TBARS production varied from $0.51 \pm 0.40$ μmol/min/g wt at 40 cmH$_2$O to $1.38 \pm 0.69$ μmol/min/g wt at 120 cmH$_2$O. L-arginine did not significantly affect the TBARS production at any CPP value (range from $0.57 \pm 0.16$ μmol/min/g wt at 40 cmH$_2$O to $1.80 \pm 0.88$ μmol/min/g wt at 120 cmH$_2$O) (Fig. 7).

Conversely, L-arginine + L-NAME significantly decreased the TBARS production compared with the control values (decreased from 32.7% at 40 cmH$_2$O to 50.2% at 120 cmH$_2$O) (Fig. 8). Indeed, the difference in TBARS production between the groups increased concomitantly with the CPP values.

**DISCUSSION**

The present study was performed to assess the intracoronary effects of the acute administration of L-arginine and L-arginine in combination with L-NAME (a non-specific NO synthase inhibitor) on isolated rat hearts under different coronary perfusion pressure conditions (40–120 cmH$_2$O). The results obtained under the control conditions were compared with those obtained after the administration of L-arginine and L-arginine + L-NAME. Variations in the tested parameters in different groups of animals under the control conditions were presented for the purpose of biological diversity.

Our results showed that the acute administration of L-arginine (compared to the control group for all values of applied CPP) did not significantly increase the coronary flow or any of the estimated oxidative stress parameters (NO, O$_2^-$, and TBARS) (Figs. 3-8).

The administration of L-arginine + L-NAME did not significantly reduce the coronary flow compared to the control group, although the differences were more evident at the higher CPP values (CPP 90–120 cmH$_2$O, which were out of the autoregulatory range). Moreover, the NO and O$_2^-$ concentrations were not significantly reduced compared to the control group. However, L-arginine + L-NAME significantly reduced the TBARS value, especially at higher CPP values (CPP 90–120 cmH$_2$O).

The L-arginine/NO system plays an important role in the control of the basal tonus of coronary blood vessels and is involved in the coronary autoregulation of the...
isolated rat heart. The isolated rat hearts exhibited autoregulation of the coronary flow between 50 and 80 cmH₂O of coronary perfusion pressure. Below the autoregulatory range the coronary flow slowly went down and above range the value more than doubled (22), which was in line with our results.

Our experimental data showed that the acute exogenous entry of arginine might increase the production of NO and NO-mediated vasodilatation despite the fact that the intracellular concentration of arginine was far beyond the Km (Michaelis-Menten constant) for eNOS. The Km for L-arginine is 2.9 μmol/l (8, 23–27). The intracellular concentration of arginine in endothelial cells is 0.8-2 mmol/l (8, 27), which suggests that the available intracellular L-arginine is more than sufficient for NO production. Based on this observation, intracellular arginine can provide full saturation of eNOS; thus, the endothelial production of NO should not depend on the extracellular concentration of L-arginine. However, the NO production increases in a dose-dependent manner when the concentration of L-arginine increases in endothelial cell cultures (28). Moreover, the increase in the plasmatic value of L-arginine is connected with the increase in vascular NO production. This biochemical phenomenon (or discrepancy) is designated the “arginine paradox”. Theories explaining the arginine paradox include the low basal values of L-arginine in diseased states such as hypertension and hypercholesterolemia, intracellular variations in the concentrations of L-arginine or the potential presence of enzyme inhibitors. Identified competitive inhibitors of NO include N⁵-monomethyl-L-arginine (L-NMMA), N⁵-nitro-L-arginine (L-NNA), N⁵-nitro-L-arginine monomethyl ester (L-NAME) and asymmetric dimethylarginine (ADMA).

Extracellular L-arginine appears to play a significant role in NO synthesis through membrane-linked eNOS. The constitutive transport system that facilitates the entry of arginine into endothelial cells is a cation amino acid transporter (CAT-1). CAT-1 and eNOS are physically connected in the caveolae in endothelial cells (29), which suggests the existence of a direct supply of extracellular arginine to eNOS.

Whether extracellular L-arginine changes the rate of arginine transport to the cell and contributes to the improvement in NO synthesis or whether the intracellular concentration in the microdomains of a cell plays a more important role in the modulation of NO synthesis are unknown (30).

The vascular endothelium plays an important role in vascular physiology. Attention has especially been focused on the endothelial production of NO (endothelial messenger molecules), including the different endothelial-mediated physiological effects in the vascular system. Because endothelial dysfunction is the basis of numerous diseases (atherosclerosis, hypertension, and diabetes mellitus) and is linked with the reduced production of endothelial NO, supplementation with L-arginine (donor NO) could be considered as therapeutic approach to these diseases. Therefore, many researchers are interested in the therapeutic possibilities of L-arginine, including whether supplementation with L-arginine can increase NO production and thereby improve vascular health. The effects of the oral or parenteral administration of L-arginine on vascular health and diseases have been examined in both human and animal models.

Böger et al (31) studied the clinical pharmacology of L-arginine and concluded that the response of the organism to the administration of L-arginine depended on the specific characteristics of the cardiovascular disease, vascular segments and morphology of the arteries of the examinee. Undesired effects of L-arginine administration are rare and are mainly mild and dose-dependent. The results obtained from a number of animal studies (animal models with damaged endothelial-dependent NO biological functions, including hypercholesterolemic rabbits, hypertensive rats, and hyperlipidemic monkeys) suggested that the administration of L-arginine in vivo improved vascular health by increasing NO production. Both acute and chronic administration of L-arginine improved endothelial-dependent vasodilation, whereas chronic administration also modulated other NO-dependent vascular functions, such as the reduction of leukocyte adhesion, inhibition of thrombocyte aggregation and proliferation of smooth muscular cells.

In their review paper, Preli et al (27) summed up the results of studies (animal and human) involving the oral supplementation of L-arginine on the formation of atherosclerotic lesions. The results from hypercholesterolemic animals generally showed beneficial effects. L-arginine appeared to inhibit the progression of atherosclerotic plaques and protect endothelial functions. Moreover, L-arginine affected other mediators of atherosclerosis, including circulating inflammatory cells and thrombocytes. In contrast to the positive results obtained in the animal studies, differences were observed in the human studies.

Some previous experimental and clinical studies indicated that L-arginine could improve the antioxidant status (32–35). L-arginine was reported to act as a free radical scavenger, inhibit the activity of pro-oxidant enzymes and thus act as an antioxidant; these roles of L-arginine were mediated by NO. Tripathi et al (32) indicated that oral supplementation with L-arginine (3 g/day for 7 days) in ischemic patients increased the superoxide dismutase (SOD) level, total thiols (T-SH) and the plasma ascorbate levels, but these increases were not significant. This study demonstrates that L-arginine administration may be beneficial for patients with myocardial ischemic disorders, such as acute myocardial infarction and acute angina. Huang et al (36) suggested that L-arginine supplementation reduced the oxidative damage and inflammatory response of skeletal muscles, liver and kidneys caused by exhaustive exercise in young rats. The rats were fed with 2% L-
arginine diet for 30 days, and this supplementation increased the antioxidant enzyme level although the increase was not significant. In the study by Lucotti et al (33), oral supplementation with L-arginine (8.3 g/day) concurrent with a weight loss diet for 21 days increased the SOD levels in obese, insulin-resistant type 2 diabetic patients. The use of different doses and weight loss diets combined with L-arginine supplementation may explain the different results. Similarly, Jabecka et al (37) reported that the oral administration of L-arginine (2 g/day for 28 days) significantly increased the TAS (total antioxidant status) level in the plasma from patients with mild hypertension. This study confirms the hypothesis that augmented concentrations of L-arginine stimulate NO biosynthesis, which leads to a reduction in oxidative stress.

The results of our study clearly show a non-significant effect of L-arginine on the coronary flow and oxidative stress markers in isolated rat hearts. However, research interested in the application of L-arginine for the treatment of cardiovascular diseases should be continued. Long-term random clinical studies are necessary (27, 31) to obtain “broad and clear” scientific knowledge in the field.

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Conflicts of interest

The authors declare no conflict of interest.

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