The Effects of Nitric Oxide Synthase – *versus* Lipoxygenase Inhibition on Coronary Flow and Nitrite Outflow in Isolated Rat Heart

V. Lj. Jakovljevic[1] and D. M. Djuric[2]

[1] *Department of Physiology, Faculty of Medicine, University of Kragujevac, Serbia and Montenegro*
[2] *Institute of Medical Physiology, University School of Medicine, Belgrade, Serbia and Montenegro*

Abstract. The aim of this study was to assess the changes of coronary flow (CF) and nitrite outflow under inhibition of nitric oxide synthase (NOS) by N^{ω}-nitro-L-arginine monomethyl ester (L-NAME) or lipoxygenase (LOX) induced by nordihydroguaiaretic acid (NDGA) in isolated rat heart. The hearts of male Wistar albino rats ($n = 18$, age 8 weeks, body mass 180–200 g) were retrograde perfused according to the Langendorff's technique at gradually increased constant coronary perfusion pressure (CPP) conditions (40–120 cm H_2O) which induced flow-dependent nitric oxide (NO) release (nitrite outflow). The experiments were performed during control conditions, in the presence of NO synthesis inhibitor L-NAME (30 μmol/l) or nonspecific LOX inhibitor (NDGA, 0.1 mmol/l) which were administered separately or in combination. CF varied in autoregulatory range from 4.12 ± 0.26 ml/min/g wt at 50 cm H_2O to 5.22 ± 0.26 ml/min/g wt at 90 cm H_2O. In autoregulatory range, nitrite outflow varied from 2.05 ± 0.17 nmol/min/g wt at 50 cm H_2O to 2.52 ± 0.21 nmol/min/g wt at 90 cm H_2O and was strictly parallel with CPP/CF curve. The autoregulatory range of CF was significantly extended (40–100 cm H_2O, 2.22 ± 0.12 ml/min/g wt and 2.90 ± 0.25 ml/min/g wt, respectively) under the influence of L-NAME. Hemodynamic effects were accompanied by significant decrease in nitrite outflow after L-NAME administration (0.56 ± 0.11 nmol/min/g wt at 40 cm H_2O to 1.45 ± 0.14 nmol/min/g wt at 100 cm H_2O). NDGA affected CF in the range of CPP 40–70 cm H_2O only (from 42% at 50 cm H_2O to 12% at 90 cm H_2O, respectively) with no significant changes in nitrite outflow. When L-NAME was applied in combination with NDGA *vs.* NDGA only, CF was significantly reduced (from 34% at 50 cm H_2O to 50% at 90 cm H_2O, respectively) with parallel changes in nitrite outflow (from 40% at 50 cm H_2O to 51% at 90 cm H_2O, respectively). The results showed that CF and nitrite outflow could be decreased under L-NAME administration. Nonselective LOX inhibitor (NDGA)

Correspondence to: Vladimir Lj. Jakovljevic, Department of Physiology, Faculty of Medicine, Svetozara Markovića 69, P.O.Box 124, 34000 Kragujevac, Serbia and Montenegro. E-mail: drvladakg@yahoo.com

decreased control values of CF only at lower values of CPP but did not change nitrite outflow indicating antioxidant properties of NDGA. In addition, L-NAME decreased the effects induced by NDGA on CF and nitrite outflow indicating the role of NO.

Key words: Nitric oxide — Nordihydroguaiaretic acid — Coronary flow — Rat

Introduction

Autoregulation of coronary blood flow is the intrinsic ability of the heart to maintain its nutritive supply relatively constant despite the wide range of coronary perfusion pressure (CPP) (Johnson 1986; Dole 1987). It reflects the interaction between myogenic tone with opposite influence of certain endogenous vasodilators and endothelium by producing vasoactive substances in response to various stimuli (ischemia or shear stress) (Jakovljevic et al. 1999; Zink et al. 2001). However, endothelium-dependent coronary vasorelaxation of many species is resistant to nitric oxide synthase (NOS) and cyclo-oxygenase (COX) inhibitors, what suggest the contribution of factors other than nitric oxide (NO) and prostacyclin (PGI_2) (Hecker et al. 1994; Weintraub et al. 1994). These factors produce relaxation by hyperpolarizing vascular smooth muscle and noted as endothelium-derived hyperpolarizing factors (EDHFs) (Nagao and Vanhoutte 1992). EDHFs are responsible for vascular relaxation in some pathological conditions when biological activity of NO is almost completely diminished, such in atherosclerosis (Cohen and Vanhoutte 1995). A number of recent investigations suggest EDHFs might be non-COX metabolites of arachidonic acid or to be lipoxygenase (LOX) products (Imig et al. 1999). Therefore, the interaction between endothelial L-arginine/NO system and LOX products in regulation of coronary circulation is not clear. For this purpose, the aim of this study was to assess the changes of coronary flow (CF) and nitrite outflow under inhibition of NOS by N^{ω}-nitro-L-arginine monomethyl ester (L-NAME) or LOX induced by nordihydroguaiaretic acid (NDGA) in isolated rat heart.

Materials and Methods

Isolated rat heart preparation

The hearts ($n = 18$, six for every experimental protocol) excised from Wistar albino rats, male sex, age 8 weeks, body mass 180–200 g (obtained from Military Technical Institute, Belgrade, Serbia and Montenegro) were perfused with Langendorff apparatus (Hugo Sachs Elektronik, Harvard Aparatus GmbH, March-Hugstetten, Germany). After short-term ether narcosis, animals were killed by cervical dislocation (Schedule 1 of the Animals Scientific Procedures, Act 1986, UK), with heparin premedication as an anticoagulant. After urgent thoracotomy and rapid heart arrest by superfusion with ice-cold isotonic saline, the hearts were rapidly

excised, isolated, the aortas were cannulated and retrograde perfused according to the technique for constant pressure conditions. The composition of the non recirculating Krebs-Henseleit perfusate was as follows (mmol/l): NaCl 118, KCl 4.7, $CaCl_2 \times 2H_2O$ 2.5, $MgSO_4 \times 7H_2O$ 1.7, $NaHCO_3$ 25, KH_2PO_4 1.2, glucose 11, pyruvate 2, equilibrated with 95% O_2 plus 5% CO_2 and warmed to 37℃ (pH 7.4). All hearts were electrically paced (5 V, 320 bpm) by the electric stimulator (Hugo Sachs Elektronik, Harvard Aparatus GmbH) and constant left ventricular draining through the dissected mitral valve was performed.

Physiological assay

After the heart perfusion had been set up, a 30 min period was allowed for stabilization of the preparation. During the stabilization period, all hearts were challenged by short-term occlusions (5–30 s) as well as by bolus injection of 5 mmol/l adenosine (60 μl at a flow rate of 10 ml/min) in order to elicit maximal CF. The hearts were discarded (about 25%) if the flow did not increase by 100% over the control value (for both tests). After the equilibration period, CPP was lowered to 50 and 40 cm H_2O and then gradually increased to 70, 80, 90, 100, 110 and 120 cm H_2O in order to establish coronary autoregulation. When the flow was considered to be stable at each value of perfusion pressure, samples of the coronary effluent were collected. Properly performed control experiments were included into study (i.e., the groups of the hearts in which the CPP/CF relationship is studied twice in the absence of any drug). It was essential to confirm that the used preparation was stable and that the responses to the first and the second run of changes in perfusion pressure did not differ substantially. In the control protocol, stabilization of preparation was performed at basal CPP of 60 cm H_2O for 30 min.

Experimental protocols

In first experimental protocol (designed to estimate the role of NO), the hearts were perfused with an inhibitor of NO synthesis, L-NAME (30 μmol/l, minimum 5 min) (Emery 1995) or in second experimental protocol with nonselective LOX inhibitor, NDGA (0.1 mmol/l, minimum 5 min). In third experimental protocol NDGA perfusion (0.1 mmol/l, minimum 5 min) was performed firstly then the protocol with the sequence of perfusion pressure changes was repeated in the presence of NDGA (0.1 mmol/l, minimum 5 min) plus L-NAME (30 μmol/l, minimum 5 min). The reason for that was to see the effects of NOS inhibition by L-NAME after pre-existed LOX inhibition by NDGA on CF and nitrite outflow.

Biochemical assays

Samples of the coronary venous effluent were collected after the stabilisation of CF at each value of gradually increased perfusion pressure. NO decomposes rapidly to form stable metabolite nitrite/nitrate products. Nitrite was determined and used as an index of NO production by the spectrophotometric method using the Griess's reagent (Green et al. 1982). 0.5 ml of perfusate was precipitated with 200 μl of 30% sulfosalicylic acid, vortexed for 30 min and centrifuged at 3000 $\times$ *g*. Equal

volumes of the supernatant and Griess's reagent, containing 1% sulfanilamide in 5% phosphoric acid / 0.1% napthalene ethylenediamine-dihydrochloride was added and incubated for 10 min in the dark and read at 543 nmol/l. The nitrite levels were calculated by using sodium nitrite as a standard.

Drugs

L-NAME was kindly donated by Slovak Academy of Sciences (Bratislava, Slovakia). NDGA was purchased from Sigma-Aldrich Chemie GmbH (Diesenhofen, Germany). Spectrophotometric assay kit was used for nitrite determination. Napthalene ethylenediamine-dihydrochloride from Sigma-Aldrich Chemie GmbH as well as sulfosalicylic acid, sulfanilamide and phosphoric acid were purchased (Merck KGaA, Darmstadt, Germany).

Statistical analysis

Values are expressed as means ± S.E.M. Statistical analysis was performed by multifactorial analysis of variance for repeated measurements between subject factors as well as Bonferroni's test; p values less than 0.05 were considered to be significant.

Results

Control conditions

In first experimental protocol, CF varied in autoregulatory range from 4.12 ± 0.26 ml/min/g wt at 50 cm H_2O to 5.22 ± 0.26 ml/min/g wt at 90 cm H_2O. In autoregulatory range nitrite outflow varied from 2.05 ± 0.17 nmol/min/g wt at 50 cm H_2O to 2.52 ± 0.21 nmol/min/g wt at 90 cm H_2O and was strictly parallel with CPP/CF curve (Fig. 1).

L-NAME administration, CF and nitrite outflow

L-NAME induced significant reduction of CF from 38% at 40 cm H_2O to 51% at 100 cm H_2O. The autoregulatory range of CF was significantly extended (40–100 cm H_2O, 2.22 ± 0.12 ml/min/g wt and 2.90 ± 0.25 ml/min/g wt, respectively) under the influence of L-NAME. Hemodynamic effects were accompanied by significant decrease in nitrite outflow after L-NAME administration (0.56 ± 0.11 nmol/min/g wt at 40 cm H_2O to 1.45 ± 0.14 nmol/min/g wt at 100 cm H_2O) (Fig. 1).

NDGA administration, CF and nitrite outflow

LOX-inhibition showed completely different effects on coronary autoregulation then L-NAME. NDGA induced statistically significant decreases of CF at lower CPP-values (from 42% at 50 cm H_2O to 12% at 90 cm H_2O, respectively), no significant CF changes at higher CPP-values (80–120 cm H_2O) and no significant changes in nitrite outflow (NDGA group, 0.84 ± 0.14 nmol/min/g at 40 cm H_2O and 5.15 ± 0.14 nmol/min/g at 120 cm H_2O) (Fig. 2).

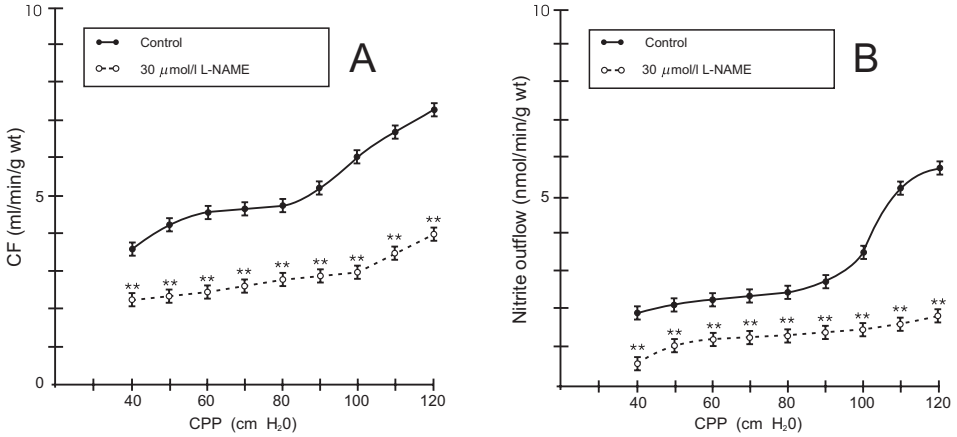

Figure 1. Effects of L-NAME on CF (A) or nitrite outflow (B) in isolated rat hearts compared to control ($n = 6$). The values are expressed as mean ± S.E.M.; L-NAME, N^{ω}-nitro-L-arginine monomethyl ester; CF, coronary flow; CPP, coronary perfusion pressure; ** $p < 0.01$.

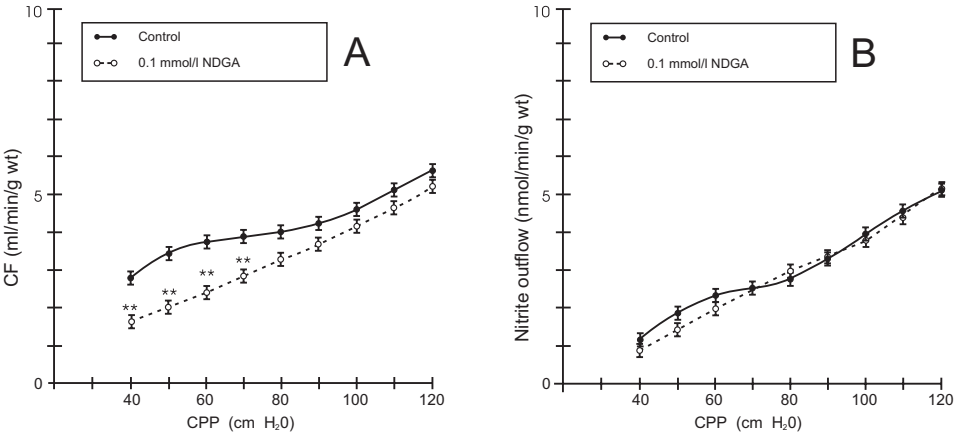

Figure 2. Effects of NDGA on CF (A) or nitrite outflow (B) in isolated rat hearts compared to control ($n = 6$). The values are expressed as mean ± S.E.M.; CF, coronary flow; NDGA, nordihydroguaiaretic acid; CPP, coronary perfusion pressure; ** $p < 0.01$.

NDGA plus L-NAME administration, CF and nitrite outflow

When L-NAME was applied in combination with NDGA *vs.* NDGA only, CF was significantly reduced (from 34% at 50 cm H_2O to 50% at 90 cm H_2O, respectively) with parallel changes in nitrite outflow (from 40% at 50 cm H_2O to 51% at 90 cm H_2O, respectively) (Fig. 3).

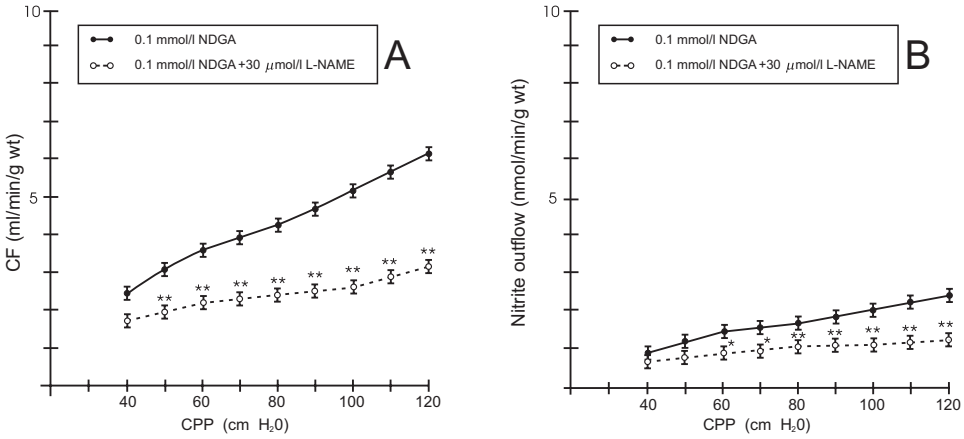

Figure 3. Effects of NDGA or NDGA + L-NAME on CF (A) or nitrite outflow (B) in isolated rat hearts compared to control ($n = 6$). The values are expressed as mean ± S.E.M.; CF, coronary flow; NDGA, nordihydroguaiaretic acid; L-NAME, N^{ω}-nitro-L-arginine monomethyl ester; CPP, coronary perfusion pressure; * $p < 0.05$; ** $p < 0.01$.

Discussion

It is well known that NO produced from L-arginine is an important endogenous regulator of basal coronary vascular tone, as well as in response to reactive hyperemia and shear stress (Kostic and Schrader 1992). Arachidonic acid, released from phospholipids in response to various stimuli, can be converted to prostaglandins and thromboxanes by COX pathway, to epoxyeicosatetraenoic acids by the CYP 450 pathway and finally to 5-, 12- and 15-hydroperoxyeicosatetraenoic acids, leukotriens, hepoxillins and lipoxins by LOX pathway (Chen et al. 1999). Mechanism of action of arachidonic acid in the isolated perfused rat heart was earlier described (Shaffer and Malik 1984). Previous papers showed that the 12-LOX pathway of arachidonic acid metabolism is stimulated in myocardium by hypoxia or ischemia and might be involved in protective effects of ischemic preconditioning (Kuzuya et al. 1993; Murphy et al. 1995). It seems that LOX metabolites have a very important influence on coronary vascular tone and express cardioprotective effects in ischemic preconditioning. Also, it was demonstrated that administration of inhibitors of LOX metabolism, NDGA and eicosatetraynoic acid (ETYA) blocked the protective effects of ischemic preconditioning (Chen et al. 1999). Furthermore, it was found that peptido-leukotrienes participated in the genesis of early ventricular arrhythmias during acute myocardial ischaemia in rats (Chang et al. 1992). Numerous studies suggest that activation of K^+ channels may function as a downstream mechanism in ischemic preconditioning. This is supported by the view that increasing K^+ channel activity can reduce myocardial ishemic injury and inhibitors of K^+ channels can abolish the protective effects of ischemic preconditioning. Certain results indicate

that Ca^{2+}-activated K^+ channel opening by NDGA in human embryonic kidney is due to the direct action on a subunit and also to Ca^{2+} release from sarcoplasmic reticulum, presumably *via* in part the inhibition of mitochondria respiration (Yamamura et al. 2002). Furthermore, activation of protease-activated receptor-2 (PAR-2) has been proposed to be protective in myocardial ischemia/reperfusion (I/R) injury, an effect possibly related to an action on the coronary vasculature. PAR-2 activation causes endothelium-dependent coronary vasodilation that is preserved after I/R injury and is not mediated by NO or prostanoids, but involves the release of an EDHF, possibly a LOX-derived eicosanoid, and activation of VR1 receptors on sensory C-fibers (McLean et al. 2002). This is consistent with previous studies of hypoxia and reoxygenation in isolated cardiac myocytes, that demonstrated accumulation of some LOX products and its regulatory role (Kuzuya et al. 1993) and well documented modulatory role of NO in the regulation of coronary hemodynamics (Ueeda et al. 1992).

The aim of the present study was to assess the changes of CF and nitrite outflow under inhibition of NOS by L-NAME or LOX induced by NDGA in isolated rat heart. Followings are the main findings of the study: i) NO synthesis inhibition by L-NAME significantly decreased control values of CF and nitrite outflow; ii) nonselective LOX inhibition by NDGA changed control values of CF only at lower values of CPP but did not change nitrite outflow; iii) L-NAME significantly decreased CF and nitrite outflow both influenced by NDGA. The former results showed that isolated rat heart autoregulated between 50 and 90 cm H_2O (Jakovljevic et al. 1999), slightly different in comparison with the autoregulatory range of the isolated guinea pig heart (Ueeda et al. 1992). The obtained results clearly show that during control conditions, CF and nitrite outflow could be increased following by gradually increase of CPP and as a consequence of higher shear stress (Figs. 1, 2). Suprisingly, control values of CF and nitrite outflow were different in certain group of animals. One could find possible reason for that in biological diversity only. L-NAME induced strong decrease of CF, with wideness of autoregulatory range, accompanied with significant decrease in nitrite release. LOX inhibition by NDGA affected significantly CF between CPP 40–70 cm H_2O. That means, if i) leukotrienes, or ii) by leukotrienes triggered EDHF, and/or iii) involvement of potassium channel, contribute to CF increase, induced by increase of CPP – then only in limit of 40–70 cm H_2O. Also, NDGA did not affect nitrite production (Fig. 2). The plot of CPP and nitrite outflow after LOX blockade does not differ from controls means not only that NO operates independently of metabolic products of arachidonic acid governed by LOX. This fact was supported by the finding that L-NAME significantly decreased CF and nitrite outflow both influenced by NDGA (Fig. 3). Also, well known antioxidant properties of NDGA probably blocking the formation of nitrite must be taken into consideration (Lundberg et al. 1944; Ramasamy et al. 1999). In general, it is thought that antioxidants improve endothelium-dependent vasodilation by scavenging superoxide and thus increasing the availability of functional NO (Harrison and Ohara 1995). However, it was found that NDGA inhibited not only LOX, but had antioxidant properties

and increased endothelial NOS (eNOS) gene expression in culture of endothelial cells mediated by its phenolic groups (Ramasamy et al. 1999). NDGA-induced increase in eNOS expression is not related to LOS inhibition because commonly used LOS inhibitor ETYA had no effects on either eNOS enzyme activity or mRNA levels on 24 h treatment (Ramasamy et al. 1999). The results obtained here provide an additional mechanism whereby nonselective LOS inhibitor (NDGA) with antioxidant properties affect CF and nitrite outflow in isolated rat heart (i.e., by increasing eNOS expression) and should be useful clinical target.

References

Chang A. C., Dai S., Ogle C. W., Tom W. M. (1992): Role of peptido-leukotrienes in the genesis of early ventricular arrhythmias during acute myocardial ischaemia in rats. Agents Actions **35**, 212—219

Chen W., Glasgow W., Murphy E., Steenbergen C. (1999): Lipoxygenase metabolism of arachidonic acid in ischemic preconditioning and PKC-induced protection in heart. Am. J. Physiol., Heart Circ. Physiol. **276**, H2094—2101

Cohen R. A., Vanhoutte P. M. (1995): Endothelium-dependent hyperpolarization-beyond nitric oxide and cyclic GMP. Circulation **92**, 3337—3349

Dole W. P. (1987): Autoregulation of the coronary circulation. Prog. Cardiovasc. Dis. **29**, 293—323

Emery C. J. (1995): Vasodilator action of the S-nitrosothiol, SNAP, in rat isolated perfused lung. Physiol. Res. **44**, 19—24

Green L. C., Wagner D. A., Glogowski J., Skipper P. I., Wishnok J. S., Tannenbaum S. R. (1982): Analysis of nitrate, nitrite and [^{15}N]nitrate in biological fluids. Anal. Biochem. **126**, 131—138

Harrison D. G., Ohara Y. (1995): Physiologic consequences of increased vascular oxidant stress in hypercholesterolemia and atherosclerosis: implications for impaired vasomotion. Am. J. Cardiol. **75**, B75—81

Hecker M., Bara A. T., Bauersachs J., Busse R. (1994): Characterization of endothelium-derived hyperpolarizing factor as a cytochrome P450-derived arachidonic acid metabolite in mamals. J. Physiol. (London) **481**, 407—414

Imig J. D., Zou A. P., Ortiz de Montellano P. R., Sui Z., Roman R. J. (1999): Cytochrome P-450 inhibitors alter afferent arteriolar responses to elevations in pressure. Am. J. Physiol., Heart Circ. Physiol. **266**, H1879—1885

Jakovljevic V. L., Kostic M. M., Mujovic V. M., Bojic M., Nedeljkovic T. I., Djuric D. M. (1999): Interaction between L-arginine: NO system and cyclooxygenase metabolic products of arachidonic acid in coronary autoregulation. J. Physiol. Pharmacol. **50**, 63—74

Johnson P. C. (1986): Autoregulation of blood flow. Circ. Res. **59**, 483—495

Kostic M. N., Schrader J. (1992): Role of nitric oxide in reactive hyperemia of the guinea pig. Circ. Res. **70**, 208—212

Kuzuya T., Hoshida S., Kim Y. J., Oe H., Hori M., Kamada T., Tada M. (1993): Increased production of arachidonate metabolites in an occlusion-reperfusion model of canine myocardial infarction. Cardiovasc. Res. **27**, 1056—1060

Lundberg W. O., Halvorson H. O., Burr G. O. (1944): The antioxidant properties of nordihydroguaiaretic acid. Oil Soap (Chicago) **21**, 33—35

McLean P. G., Aston D., Sarkar D., Ahluwalia A. (2002): Protease-activated receptor-2 activation causes EDHF-like coronary vasodilation: selective preservation in ischemia/reperfusion injury: involvement of lipoxygenase products, VR1 receptors, and C-fibers. Circ. Res. **90**, 465—472

Murphy E., Glasgow W., Fralix T., Steenbergen C. (1995): Role of lipoxygenase metabolites in ishemic preconditioning. Circ. Res. **76**, 457—467

Nagao T., Vanhoutte P. M. (1992): Hyperpolarization as a mechanism for endothelium-dependent relaxation of the porcine coronary artery. J. Physiol. (London) **445**, 355—367

Ramasamy S., Drummond R. G., Ahn J., Storek M., Pohl J., Parthasarathy S., Harrison G. D. (1999): Modulation of expression of endothelial nitric oxide synthase by nordihydroguaiaretic acid, a phenolic antioxidant in cultured endothelial cells. Mol. Pharmacol. **56**, 116—123

Shaffer J. E., Malik K. U. (1984): Mechanism of action of arachidonic acid in the isolated perfused rat heart. Can. J. Physiol. Pharmacol. **62**, 551—558

Ueeda M., Scott S. K., Olsson R. A. (1992): Nitric oxide modulates coronary autoregulation in the guinea pig. Circ. Res. **70**, 1296—1303

Yamamura H., Sakamoto K., Ohya S., Muraki K., Imaizumi Y. (2002): Mechanisms underlying the activation of large conductance Ca^{2+}-activated K^+ channels by nordihydroguaiaretic acid. Jpn. J. Pharmacol. **89**, 53—63

Weintraub N. L., Joshi S. N., Branch C. A., Stephenson A. H., Sprague R. S., Lonigro A. J. (1994): Relaxation of porcine coronary artery to bradykinin: role of arachidonic acids. Hypertension **23**, 976—981

Zink M. H., Oltman C. L., Lu T., Katakam P. V. G., Kaduce T. L., Lee H. C., Dellsperger K. C., Spector A. A., Myers P. R., Weintraub N. L. (2001): 12-Lipoxygenase in porcine coronary microcirculation: implications for coronary vasoregulation. Am. J. Physiol., Heart Circ. Physiol. **280**, H693—704

Final version accepted: February 9, 2005