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Early Changes of Protein Synthesis in Myocardium and Coronary Arteries Induced by NO Synthase Inhibition

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Summary

The question was addressed whether short-term (4 hour) NO deficiency, inducing an increase in blood pressure in anaesthetized dogs, does influence proteosynthesis in the myocardium and coronary arteries. A potentially positive answer was to be followed by the study of the supporting role of ornithine decarboxylase for the polyamines pathway. N^G-nitro-L-arginine-methyl ester (L-NAME) (50 mg/kg per hour) was administered i.v. to inhibit NO synthase. After the first L-NAME dose diastolic blood pressure increased from 131.8±2.0 to 149.4±3.9 mm Hg ($p<0.001$) and was maintained at about this level till the end of the experiment. Systolic blood pressure only increased after the first dose (from 150.8±1.1 to 175.0±5.8 mm Hg, $p<0.01$), returning thereafter to the control level. Similarly, the heart rate declined only after the first dose (from 190.4±5.3 to 147.6±4.5 beats/min, $p<0.01$). Total RNA concentrations increased in the left cardiac ventricle (LV), the left anterior descending coronary artery (LADCA) and left circumflex coronary artery (LCCA) by 15.9±0.7, 29.7±1.3 and 17.6±1.0 %, $p<0.05$, respectively. The same applied to [^{14}C]leucine incorporation (by 86.5±5.0, 33.5±2.6, 29.3±4.1 %, $p<0.05$, respectively). The above parameters indicated an increase of proteosynthesis in the LV myocardium and both coronary arteries LADCA and LCCA after short-term NO deficiency. Surprisingly, the ornithine decarboxylase activity in the LV myocardium decreased significantly by 40.2±1.6 % ($p<0.01$) but the changes were not significant in the coronary arteries. This unexpected finding makes the role of polyamines in increasing proteosynthesis during a pressure overload due to NO deficiency questionable.

Key word

Nitric oxide deficiency – Pressure overload – Proteosynthesis – Ornithine decarboxylase – Polyamines – NO synthase inhibition

Introduction

NO-deficient hypertension, a novel model of cardiovascular loading, has been developed after the role of nitric oxide in cardiovascular control had been established (Ribeiro *et al.* 1992, Arnal *et al.* 1993, Dananberg *et al.* 1993, Bernátová and Pecháňová 1994). The inhibition of NO synthase, the enzyme catalyzing the reaction of the arginine to citrulline with nitric oxide as the paramountly important by-product, has been supposed to underlie the above type of hypertension. The resulting involvement of nitric oxide (Gerová *et al.* 1998) increases basal vascular tone and amplifies vasoconstriction to endogenous and exogenous constrictor agents (Wo *et al.* 1991, Gerová *et al.* 1998, Zanchi *et al.* 1995). Both factors might be partially responsible for high blood pressure.

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The data reported on the effect of nitric oxide deficiency on cardiac function are, however, somewhat controversial (Weyrich *et al.* 1994, Klabunde *et al.* 1992, Manning *et al.* 1993, Kirstein *et al.* 1995, Preckel *et al.* 1997) and so are the findings concerning cardiac hypertrophy in hypertension after long-lasting NO synthase inhibition. Arnal *et al.* (1993) and Banting *et al.* (1997) were unable to find cardiac hypertrophy in NO-deficient hypertension, while Morton *et al.* (1993) and Kristek and Gerová (1996) did find cardiac hypertrophy in this type of pressure overload lasting several weeks.

In our previous experiments focused on the early metabolic processes in the myocardium and coronary arteries in pressure overload, we demonstrated in anaesthetised dogs that (i) 4-hour aortic stenosis or (ii) 4-hour noradrenaline infusion suffice to enhance proteosynthesis in the myocardium as well as in epicardial conduit coronary arteries (Gerová *et al.* 1995, 1996a). Thus, the completely unknown early metabolic processes in the myocardium and coronary arteries in NO-deficient hypertension were addressed first in this study.

The polyamines proved to precede and support proteosynthesis and cell proliferation (Flamigni *et al.* 1985). The role of polyamines in the model of pressure overload due to NO deficiency is of particular interest, since the precursor of polyamines – ornithine – directly originates from arginine. Moreover, a very close functional relation was revealed to exist between polyamines and nitric oxide (Morgan 1994). The crucial enzyme, arginase, involved in ornithine production was found to operate not only in hepatocytes and macrophages (Boucher *et al.* 1994) but also in endothelial cells (Buga *et al.* 1996). The second question addressed followed from the above hypothesis: By blocking one metabolic pathway of arginine to citrulline by inhibition of NO synthase, the other pathway of arginine to ornithine should probably be facilitated, thus producing more substrate for the synthesis of polyamines. The supporting role of polyamines, along with the increase of vascular tone due to NO deficiency, might be involved in the expected increase of proteosynthesis in the myocardium and coronary arteries.

Thus an experimental model of a 4-hour pressure overload induced by NO synthase inhibition has been developed. Proteosynthesis in the myocardium and coronary arteries was estimated by determining the RNA as well as DNA levels and [^{14}C]leucine incorporation. The suggested involvement of polyamines was assessed from the activity of ornithine decarboxylase.

Materials and Methods

The experiments were performed on 10 mongrel dogs of either sex, weighing 8–13 kg. The procedures and protocols used in this study were approved by the Animal Care and Use of Experimental Animals Committee of the Institute and conformed with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publ. No 8523, revised 1985).

All animals were anaesthetised with thiopental sodium in a dose of 10–15 mg/kg i.p. at the beginning of the experiment; supplementary doses of 4–7 mg/kg were administered at approximately hourly intervals. The control group comprised 5 animals which lay quietly under the above anaesthesia for a period of 4 hours. In the experimental group (5 animals) under the same anaesthesia, NO synthase was inhibited by N^G-nitro-L-arginine-methyl ester (L-NAME), administered intravenously each hour in a dose of 50 mg/kg, amounting to a total of 200 mg/kg in the course of the 4-hour period. Blood pressure and heart rate were monitored *via* a cannula introduced into the brachial artery by a Statham Manometer and registered on a Physioscript Schwarzer.

At the end of the 4-hour period of NO-deficient pressor response and of the 4-h lasting anaesthesia in controls, the animals were sacrificed by an overdose of thiopental sodium. The chest and then the pericardium were immediately opened. The left descending coronary artery and the left circumflex coronary artery were prepared, and samples consisting of all three layers of each vessel (intima, media, adventitia) were put into ice-cold Krebs solution for measuring DNA, total RNA and [^{14}C]leucine incorporation. The samples for the determination of ornithine decarboxylase activity were immediately immersed in liquid nitrogen.

Samples of the anterior wall of the left ventricle, consisting of both epi- and endocardial layers, were processed similarly as the coronary arteries for assessment of DNA, total RNA, [^{14}C]leucine incorporation and ornithine decarboxylase activity.

Determination of nucleic acid concentration

The spectrophotometric method according to Canev and Markov (1960) was used to determine the concentration of nucleic acids. Briefly, the tissue was homogenised in 0.3 mol/l perchloric acid, rehomogenised three times, delipidated, and alkaline hydrolysis was performed for 18 h at room temperature. After reextraction by 1 mol/l perchloric acid, the concentration of total RNA was determined by absorbance measurements at 260 and 286 nm, using a spectrophotometer GBC 911A.

The concentration of total DNA was determined after sediment hydrolysation in 1 mol/l perchloric acid at 85 °C for 30 min by absorbance measurement at 268 and 284 nm.

Determination of [^{14}C]leucine incorporation

Proteosynthesis was determined by [^{14}C]leucine incorporation into proteins, as previously described by Gerová *et al.* (1996a). Briefly, the samples (about 25 mm^3) from the wall of the left ventricle and segments of coronary arteries (5 mm) were incubated separately in 3 ml of Krebs-Ringer solution containing (in mol/l) 100.0 NaCl, 2.5 KCl, 1.5 KH_2PO_4, 1.0 $MgSO_4$, 35.0 $NaHCO_3$, 60.0 Na-acetate. 1.0 $CaCl_2$, and 1.0 glucose and bubbled with 95 % O_2/5 % CO_2 for 10 min at 37 °C. The samples were placed in 5 ml of Krebs-Ringer solution containing [^{14}C]leucine (0.25 mmol/l final concentration, 32 kBq/mmol specific radioactivity; Institute for Research, Production, and Application of Radioisotopes, Prague, CR). Each sample was allowed to incorporate [^{14}C]leucine for 30 min. The solution contained 0.25 mmol/l unlabelled leucine (final concentration) to ensure equalized specific activities of intracellular and extracellular leucine. In preliminary studies, the incorporation of [^{14}C]leucine into proteins was linear for a period of one hour. The samples were homogenised in ice-cold 1 mmol/l perchloric acid to denature all proteins and to extract unincorporated [^{14}C]leucine. The samples were reprecipitated twice and the sediment was dissolved in 1 mmol/l NaOH. A portion of this solution was assayed for protein content and the remainder was used to determine [^{14}C]leucine by liquid scintillation counting, using a Hewlett-Packard spectrophotometer. The protein concentration was determined by Lowry's method (1951).

Determination of ornithine decarboxylase (ODC) activity

The activity of ODC was assayed as $^{14}CO_2$ released from [^{14}C]ornithine hydrochloride using the method of Slotkin and Bartolome (1983). [^{14}C]ornithine hydrochloride (Institute of Isotopes of Hungarian Academy of Sciences, Budapest, Hungary) was purified immediately prior to use by thin-layer chromatography on silica gel plates. After developing with a mixture of chloroform: methanol: 17 % ammonium hydroxide (4:4:2), a spot corresponding to [^{14}C]ornithine hydrochloride was eluted with an aqueous solution of 2 % ethanol. Tissue samples of the myocardium and coronary arteries were homogenised in a twenty-fold quantity of 10 mmol/l Tris-HCl buffer (pH 7.2, at 2 °C). After centrifugation at 15 000 x *g* (10 min) the ODC activity was determined in the supernatant. The activity of ODC was expressed as the amount of $^{14}CO_2$ in picomoles produced by the catalysis of [^{14}C]ornithine hydrochloride per mg protein per hour. The amount of proteins in individual samples was determined according to Lowry's method (1951).

Evaluation of experiments and statistical analysis

Diastolic and systolic blood pressure as well as heart rate were monitored continuously. The resting values and the values recorded one hour after each administration of L-NAME were evaluated. In control animals, the above parameters were evaluated at corresponding time intervals. The values of blood pressure and heart rate, tissue nucleic acid content, [^{14}C]leucine incorporation and $^{14}CO_2$ as an index of the activity of ornithine decarboxylase were expressed as means ± S.E.M. Analysis of variance, Bonferroni test and Student's t-test for unpaired variables were used. Values were considered significant at $p<0.05$.

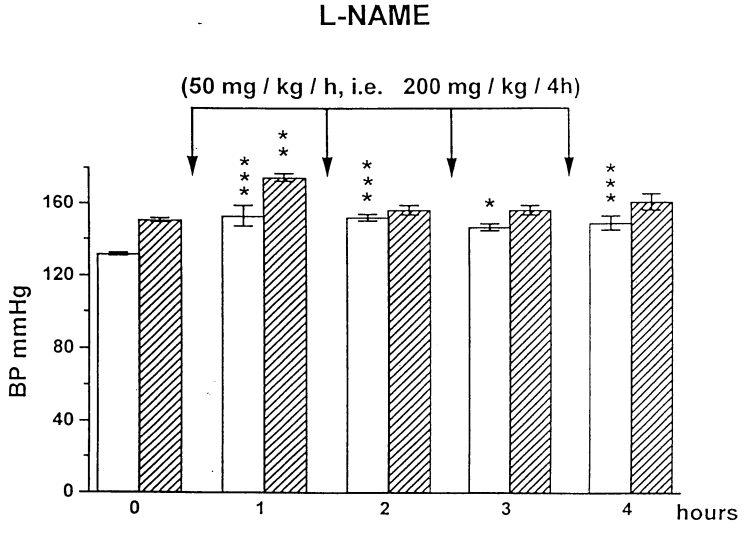

Fig. 1. *Blood pressure in brachial artery (diastolic – open columns, systolic – hatched columns) before and one hour after the first, second, third and fourth dose of L-NAME (*p<0.05, **p<0.01, ***p<0.001).*

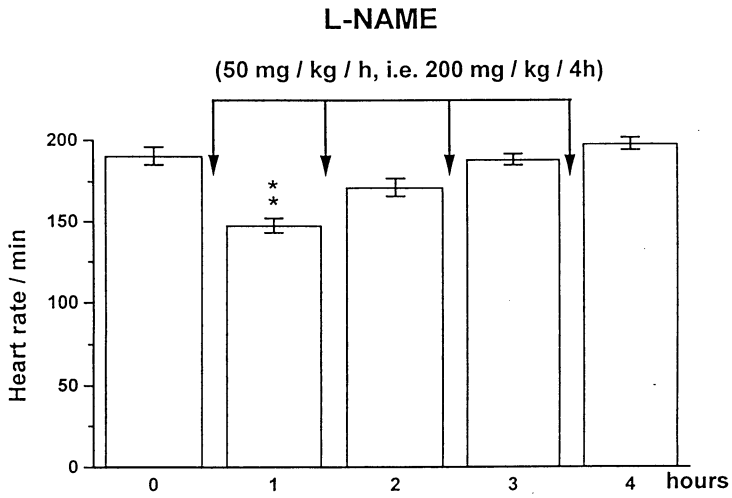

Fig. 2. *Heart rate before and one hour after the first, second, third and fourth dose of L-NAME (**p<0.01).*

Results

Blood pressure and heart rate

Figure 1 demonstrates the blood pressure in the course of the 4-hour inhibition of NO synthase. The steady-state diastolic blood pressure of 131.8±2.0 mm Hg was increasing and reached 153.2±2.2 mm Hg ($p<0.001$) one hour after the first dose of L-NAME. The diastolic blood pressure was maintained at about this level one hour after the second, third as well as the fourth dose, and at the end of the experiment it was 149.4±3.9 mm Hg ($p<0.001$). The steady-state systolic blood pressure of 150.8±1.1 mm Hg increased one hour after the first dose of L-NAME to 175.0±5.8 mm Hg ($p<0.001$). Thereafter it returned approximately to the control level and remained at this value till the end of the experiment.

The heart rate decreased significantly one hour after the first dose of L-NAME from 190.4±5.3/min to 147.6±4.5/min (Fig. 2). One hour after the second, third and fourth dose of L-NAME, no further significant change in heart rate was found in comparison with the steady-state value.

Nucleic acids concentration

The concentration of DNA was 0.87±0.08 mg/g protein in the myocardium, 0.97±0.07 mg/g protein in the LADCA and 0.86±0.03 mg/g protein in the LCCA of the control group. There were no significant changes in DNA concentration after 4 hours of L-NAME treatment: 0.84±0.03, 1.30±0.12 and 1.10±0.07 mg/g protein, respectively.

Figure 3 demonstrates the RNA values. The concentration of RNA was 2.35±0.05 mg/g protein in the myocardium, 2.32±0.06 mg/g protein in the LADCA and 2.05±0.13 mg/g protein in the LCCA of the control group. After 4 hours of L-NAME treatment RNA concentration increased to 3.0±0.09 mg/protein (by 27.6 %, $p<0.05$), to 2.69±0.13 mg/protein (by 15.9 %, $p<0.05$) and to 2.66±0.12 mg/protein (by 29.7 %, $p<0.05$), respectively.

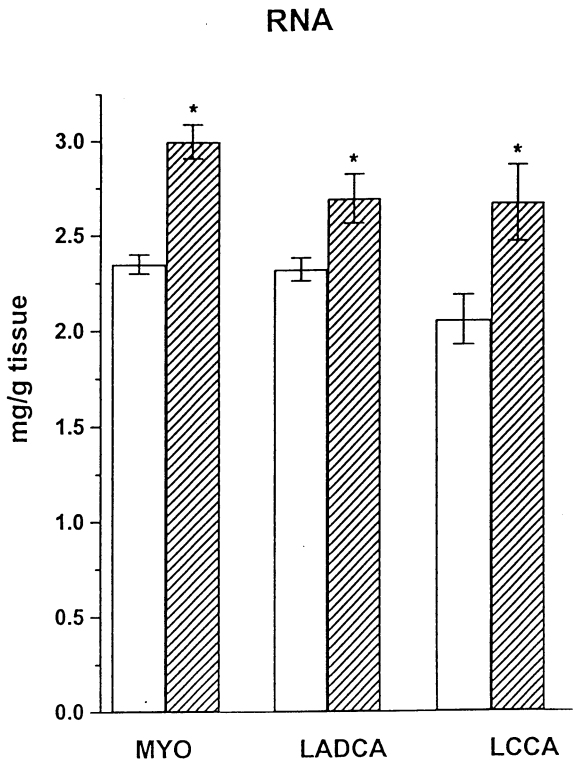

Fig. 3. *Total RNA content in left ventricle myocardium (MYO), left descending coronary artery (LADCA) and left circumflex coronary artery (LCCA). Open columns: samples from control animals, hatched columns: samples from animals administered L-NAME (in a total 200 mg/kg b. w.) for a period of 4 hours (*p<0.05).*

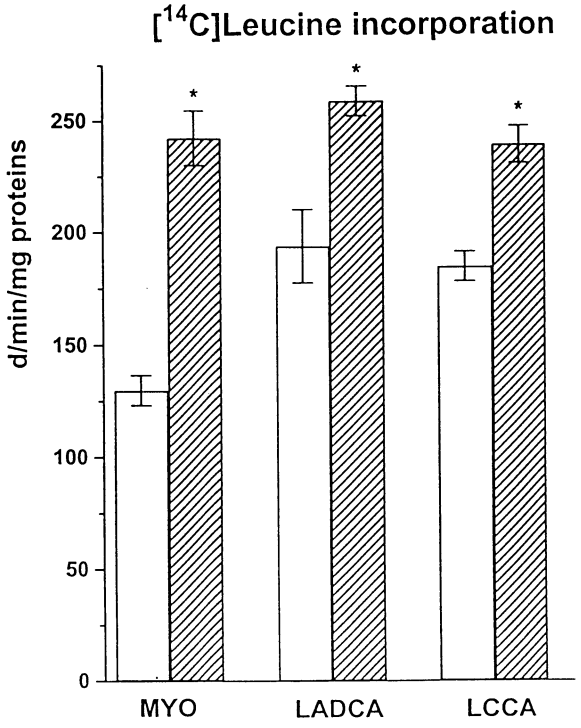

Fig. 4. *[^{14}C]leucine incorporation in left ventricle myocardium, LADCA and LCCA. Open columns: samples from control animals, hatched columns: samples from animals administered L-NAME (in a total 200 mg/kg b. w.) for a period of 4 hours (*p<0.05).*

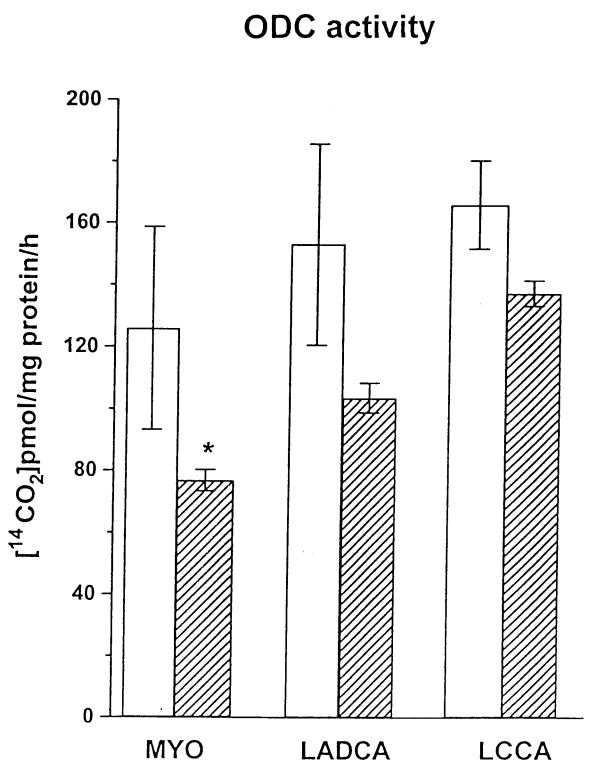

Fig. 5. *Ornithine decarboxylase activity in LV myocardium, LADCA and LCCA. Open columns: samples from control animals, hatched columns: samples from animals administered L-NAME (in a total 200 mg/kg b w) for a period of 4 hours (**p<0.01).*

[^{14}C]leucine incorporation

The incorporation of [^{14}C]leucine represented 129.9±6.7 d/min/mg proteins in the myocardium, 193.9±16.3 d/min/mg proteins in the LADCA and 184.8±6.6 d/min/mg proteins in the LCCA of the control group. After 4 hours of L-NAME treatment [^{14}C]leucine incorporation increased to 242.3±12.3 d/min/mg proteins (by 86.5 %, $p<0.05$), to 258.8±6.7 d/min/mg proteins (by 33.5 %, $p<0.05$) and to 239.0±8.3 d/min/mg proteins (by 29.3 %, $p<0.05$), respectively (Fig. 4).

Ornithine decarboxylase activity

Figure 5 presents the ODC activity in the LV myocardium and coronary arteries. In the myocardium the value 125.97±32.66 pmol/mg protein/h decreased to 76.82±3.45 pmol/mg protein/h in L-NAME administered animals (by 40.2 %, $p<0.01$). The steady-state value of ODC activity of 153.15±32.59 pmol/mg protein/h in the left descending coronary artery, and of 165.78±14.35 pmol/mg protein/h in the left anterior circumflex coronary artery declined to 103.50±4.75 pmol/mg protein/h and 137±4.12 pmol/mg protein/h, respectively, in L-NAME-treated animals. However, these differences were not significant.

Discussion

The inhibition of NO synthase by L-NAME lasting 4 hours induced a continuous increase in diastolic blood pressure which persisted during the whole period studied. Systolic blood pressure increased significantly after administration of the first dose of L-NAME (50 mg/kg). After the second, third and fourth dose of L-NAME, systolic blood pressure returned close to control values. Consequently, the pulse pressure amplitude declined after the second, third and fourth dose of L-NAME, despite the administration of a total of 200 mg/kg L-NAME. Most studies on the effect of NO deficiency on the cardiovascular system, induced by NO synthase inhibition, used rats as experimental objects and did not obtain data on diastolic and systolic pressure and/or pulse pressure amplitude. Unfortunately, the authors who used dogs as experimental animals, provided only mean blood pressure values. These authors did not mention the unexpected relationship between systolic blood pressure and pulse pressure amplitude (Lechevalier *et al.* 1992, Manning *et al.* 1993).

As far as the surprising findings on systolic blood pressure and/or the decline in pulse pressure amplitude in NO deficiency are concerned, the following explanations appear to be plausible: (i) They may be the consequence of alterations in biomechanical properties of large elastic arteries. However, since NO deficiency increases in smooth muscle tone and vessel wall stiffness (Pourageaud and Freslon 1996), this factor could hardly contribute to the decline in pulse pressure amplitude. (ii) A decrease in stroke volume might underlie and contribute to the small pulse pressure amplitude. Lechevalier *et al.* (1992) provided evidence that the cardiac output in dogs declined significantly even at lower doses of L-NAME than were used in our experiments. Thus the declining cardiac output along with small or no changes in heart rate indicate a decline in stroke volume and might be assumed to be the probable cause of small changes in systolic blood pressure. The above data support the opinion that very probably NO deficiency, at least in the early period, may decrease the contractility of cardiac muscle.

A significant increase in RNA concentrations was detected in the myocardium, in the left descending coronary artery and left circumflex coronary artery. A similar elevation was observed in the incorporation of [^{14}C]leucine into the LV myocardium and both main branches of the left coronary artery. Both parameters justify the assumption that proteosynthesis is increased in the early phase of NO deficiency in the myocardium and the left descending and left circumflex coronary arteries. The mild increase in diastolic pressure could be a contributing factor to the increase in proteosynthesis in cardiac muscle and both branches of the left coronary artery in NO deficiency.

On the other hand, no changes in DNA concentrations were found indicating that no hyperplastic processes and/or tendency to polyploidy had occurred in the myocardium and coronary arteries.

When the response of the coronary arteries (LADCA and LCCA) in NO deficiency was compared with the blood pressure response of the same vessels due to 4-hour lasting aortic stenosis (Gerová *et al.* 1996a), a substantial difference emerged. In the aortic stenosis model, in agreement with the idea that cell deformation is the main stimulus for proteosynthesis (Mann *et al.* 1989), a significant increase of proteosynthesis was found in the myocardium and LADCA but not in LCCA, reflecting the quantitative difference in radial and longitudinal deformation of the respective vessels in this type of pressure overload (Gerová *et al.* 1992). It should be considered (i) the mild increase in diastolic pressure, (ii) the very short transient increase in systolic pressure followed by a return to the steady-state value, and (iii) the increased proteosynthesis in the myocardium and both main branches of the left coronary artery, strongly suggest that NO deficiency itself is closely involved in initiating the increase in proteosynthesis.

As far as the supporting role of polyamines in the increase of proteosynthesis is concerned, our findings were rather unexpected. The activity of ornithine decarboxylase, used as a putative index of polyamine levels, (Flamigni *et al.* 1985, Majesky *et al.* 1985), was previously found to be increased even in a similar experimental model of a 4-hour blood pressure increase induced by noradrenaline infusion (Gerová *et al.* 1995). However, completely different findings, namely a significant decrease in ODC activity in the left cardiac ventricle and a tendency to decreased values in LADCA and LCCA were found during inhibition of NO synthase lasting 4 hours. Yet, the finding of decreased ODC activity failed to substantiate the hypothesis mentioned in the introduction. Namely, that the inhibition of NO synthase and a decline in NO production might be expected to facilitate the production of ornithine from arginine, thus making more ornithine available for ODC and polyamine production. However, the reason why ornithine decarboxylase activity was decreased is still not clear.

The only study devoted to the role of polyamines in hypertension induced by NO synthase inhibition in rats (Banting *et al.* 1997) described a short transient increase in ODC activity in the myocardium and aorta. Proteosynthesis was not assessed in those experiments, yet strangely enough, the authors did not find cardiac hypertrophy even after 12-day treatment of rats with L-NAME, accompanied by an increase of blood pressure.

Polyamines were reported to be involved in cell growth and proliferation namely in cardiovascular overload connected with increased sympatho-adrenergic activity (Majesky *et al.* 1985). We have demonstrated, however, that long-term NO deficiency inducing high blood pressure and cardiac hypertrophy downregulated the cardiac sympathetic system (Gerová *et al.* 1996b, Sládek *et al.* 1996). Indeed, a nearly 50 % decrease in density of adrenergic nerve terminals was found in the hypertrophied left ventricle and septal myocardium of rats after 6 weeks of NO synthase inhibition. Having these findings in mind, new approach is opening for further studies of the puzzling role of NO in these physiological mechanisms.

Finally, it should be mentioned that, in the light of recent studies, a feedback mechanism between ODC activity and the level of polyamines in these tissues might operate (Nillson *et al.* 1997). However, if this view holds true, then ODC activity need not be directly responsible for the level of polyamines in various tissues. Future analysis will have to focus attention on the estimation of polyamine levels.

It can thus be concluded that 4-hour lasting inhibition of NO synthase due to L-NAME administration elevates diastolic blood pressure during the whole 4-hour period; no increase in systolic blood

pressure was found in the second, third and fourth hour, and a significant decrease was recorded in heart rate only one hour after the first L-NAME dose. The relatively mild cardiovascular overload increased the total RNA concentration as well as [^{14}C]leucine incorporation in the LV myocardium and in the left descending and left circumflex coronary arteries, indicating enhanced proteosynthesis. Besides the mild pressure increase, the NO deficiency itself was considered to contribute to the increase in proteosynthesis. The significant decrease in ODC activity in the myocardium still remains unexplained. It is possible the metabolic pathway from ornithine to polyamines is inhibited.

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