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Efficiency of NO Donors in Substituting Impaired Endogenous NO Production: a Functional and Morphological Study

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Summary

Two exogenous NO donors were used to act as substitutes for impaired endogenous nitric oxide (NO) production due to inhibition of NO synthase in rats. Six weeks' lasting inhibition of NO synthase by N^G-nitro-L-arginine methyl ester (L-NAME) induced stabilized hypertension. Simultaneously administered isosorbide-5-mononitrate did not prevent the development of hypertension. Molsidomine, administered concomitantly with L-NAME, significantly attenuated the BP increase. However, BP was still found to be moderately increased compared to the initial values. Remarkable alterations in the geometry of the aorta, carotid and coronary artery found in NO-deficient hypertension were prevented in rats administered L-NAME plus molsidomine at the same time. In spite of 6 weeks' lasting inhibition of NOS, the NOS activators acetylcholine and bradykinin induced BP decrease; the maximum hypotensive value did not differ from the values recorded in the controls or in animals treated with L-NAME plus molsidomine. Notably enough, the hypotension was similar to that found in rats administered L-NAME alone for six weeks. After NO synthase inhibition, Isosorbide-5-mononitrate does not substitute and molsidomine substitute only partially the impaired endogenous NO production.

Key words

Nitric oxide • Hypertension • Conduit arteries • Morphology • Molsidomine • Isosorbide-5-mononitrate • NOS activation

Introduction

Soon after the endogenous production of nitric oxide (NO) has been revealed in living organisms, NO-deficient hypertension extended the hitherto known series of experimental models of hypertension (Ribeiro *et al.* 1992, Bayliss *et al.* 1992, Bernátová and Pecháňová 1994, Kristek *et al.* 1995). Almost concomitantly, studies were performed to ascertain whether the impaired NO production also participates in human hypertension (Panza *et al.* 1993, Dominiczak and Bohr 1995, Preik *et al.* 1996, McAllister *et al.* 1999).

According to the results of numerous experiments, a novel paradigm was constructed, i.e. NO is produced continually during catalysis of arginine to citrulline, triggered by the enzyme NO synthase (Moncada 1992). NO synthase was found to be present constitutively in endothelial cells, neurons and several other cells (Palmer *et al.* 1988, Bredt *et al.* 1990). By binding competitively to NO synthase, exogenous and

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endogenous analogues of arginine were shown to attenuate the above metabolic pathway and NO production (Rees *et al.* 1990, Vallance *et al.* 1992, Matsuoka *et al.* 1997, Gerová *et al.* 1998).

The impaired NO production implies disturbances in cardiovascular regulatory mechanisms, both peripheral and central (Rees *et al.* 1990, Shapoval *et al.* 1991, Gerová *et al.* 1995, Scrogin *et al.* 1998). Actually, conscious animals administered exogenous inhibitors of NO synthase for several weeks were shown to develop hypertension.

On the other hand, beneficial effects of nitrates and nitrites in cardiovascular medicine have been well known since the second half of the 19th century (Murell 1879). It was, however, only recently that nitric oxide was demonstrated to underlie the beneficial effect of nitrates and nitrites, namely the relaxation of vascular smooth muscle (Gruetter *et al.* 1979).

Consequently, an utterly simple question arose, namely whether during declining NO production (after the inhibition of NO synthase) exogenous NO donors would be able to substitute the missing nitric oxide and to prevent the development of hypertension.

Two types of exogenous donors were selected to substitute NO in experimental NO-deficient hypertension: one of a class of nitrates, isosorbide-5-mononitrate, and an other, a recently developed sydnonimine, molsidomine. As far as nitrates are concerned, the NO release requires enzymatic equipment in target cells (Bauer *et al.* 1995). However, this requirement does not apply to molsidomine (Noack and Feelisch 1989).

If any of the two exogenous donors substituted the missing NO, the structure of the vessel wall would prominently come into play. Indeed, NO-deficient hypertension was proved to be accompanied with the remodeling of the vascular wall of both resistance and conduit arteries (Li and Schiffrin 1994, Delacretaz *et al.* 1994, Kristek *et al.* 1996, Tőrők *et al.* 2000). It would thus be justified to expect the influence of the respective donor on the extent of vessel wall remodeling. The geometry, wall thickness and inner diameter of the aorta, coronary and carotid arteries were determined in a group of animals in which NO deficiency was counterbalanced by an exogenous NO donor. The structure of arteries was compared to that of animals with NO-deficient hypertension.

When NO synthase is inhibited for a period of several weeks (and the missing NO is substituted by exogenous donors), the next question concerned the possibility to activate NO synthase. To address this issue, the hypotensive response to activators of NO synthase in anesthetized animals was studied. Two activators, acetylcholine and bradykinin, were selected. The question is even more intriguing since acetylcholine- or bradykinin-induced hypotension was paradoxically found to be amplified in animals with long-term inhibition of NO synthase (Zanchi *et al.* 1995, Gerová 1999).

Methods

The experiments were carried out on Wistar male rats, 10 weeks of age. The procedures followed the Guidelines for the Use of Laboratory Animals (Ethics Committee for Experimental Work, Slovak Academy of Sciences, 1995). The animals were housed in individual cages at a temperature of 22-24 ºC, under a 12 h light:dark cycle and fed a standard pellet diet.

The animals were divided into 4 groups.

Group 1: Eight control animals living under the above standard housing conditions without any medication.

Group 2: Ten animals were administered NO synthase inhibitor N^G-nitro-L-arginine methyl ester (L-NAME, Sigma, Germany) 50 mg/kg daily in drinking water for a period of six weeks.

Group 3: Nine animals were administered L-NAME similarly as in group 2. These animals were concomitantly given isosorbide-5-mononitrate (H. Mack Nachf. Germany) in a dose 5 mg/kg by gavage daily for the first two weeks, except on Saturdays and Sundays, in order to avoid drug tolerance. As blood pressure increased similarly as in animals of group 2, the dose was increased to 10 mg/kg/day from the third week till the end of the experiment.

Group 4: Nine animals were administered L-NAME in the dose, way and time period corresponding to group 2. In addition, they were simultaneously administered molsidomine (Sigma, Germany) 50 mg/kg twice a day by gavage for a period of six weeks, including Saturdays and Sundays.

Blood pressure was measured weekly using the tail-cuff method.

After six weeks of the above regimens, the hypotensive response to the activators of NO synthase (acetylcholine and bradykinin) was studied. The day when the acute experiment was performed, the animals were anesthetized by sodium pentobarbital (50 mg/kg intraperitoneally). The right jugular vein was cannulated for applying the drugs. Immediately after cannulation the

animal was given 25 U of heparin. The right carotid artery was prepared, cannulated and connected to the Stattham pressure transducer. Blood pressure was recorded on a Physioscript Schwarzer. After a period of blood pressure stabilization, the response to acetylcholine (1, 5 and 10 μg dissolved in 0.1 ml of Krebs solution) and bradykinin (100 μg/0.1 ml Krebs solution), administered into the jugular vein in random order, were monitored. The drugs were always administered within 10 s.

At the end of the acute experiment, the chest was opened in the „y" way, the pericardium was opened similarly and the cardiovascular system was perfused with a fixative *via* a cannula placed in the left ventricle. A pressure of 120 mm Hg was used for perfusions lasting 10 min. Glutaraldehyde 3 % in 0.1 M phosphate buffer was used as fixative. The middle part of the thoracic aorta, the middle part of the carotid artery and the upper part of the septal branch of the left descending coronary artery were excised. The arteries were cleaned and divided into approx. 1 mm long segments and immersed into the same fixative for further three hours. After washing in a phosphate buffer, the segments were postfixed with 2 % OsO_4 in 0.1 M phosphate buffer. Then the specimens were stained en block with 2 % uranyl acetate, dehydrated through ascending concentrations of alcohol and embedded in Durcupan ACM. Three randomly selected segments of each artery were cut perpendicularly to the long axis. Both the inner circumference and arterial wall thickness (tunica intima and tunica media) were measured under light microscopy. The arterial wall thickness was measured at about 45º intervals around the vessel circumference. The inner diameter and the cross-sectional area (tunica intima and tunica media) of the arteries were calculated.

The individual parameters were expressed as means ± S.E.M. Statistical significance was evaluated using ANOVA and Bonferroni t-tests. Values of $P<0.05$ were considered significant.

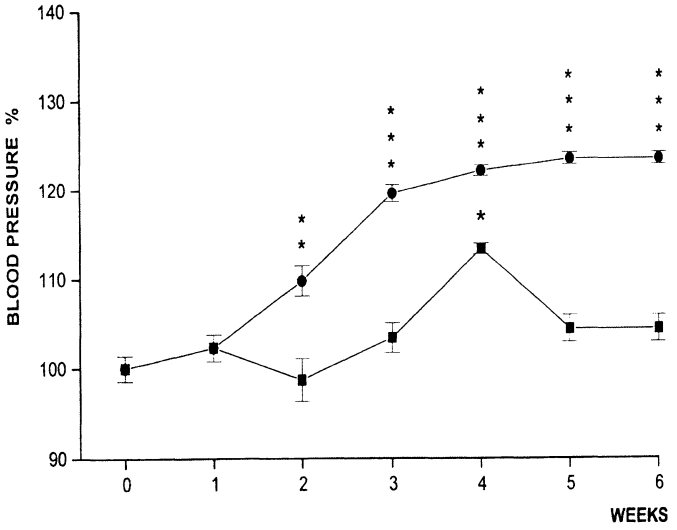

Fig. 1. *Blood pressure of control rats measured weekly during the period of 6 weeks (squares) and BP of rats administered L-NAME 50 mg/kg daily for six weeks (full circles). * P<0.05, ** P<0.01, *** P<0.001 compared to the respective steady-state values.*

Results

The time course of BP changes in control animals and animals treated with L-NAME for six weeks is shown in Figure 1. No significant BP change was found in the control animals during the whole six-week period except for a transient increase in the fourth week. The blood pressure of animals treated with L-NAME increased within two weeks and the increased values were maintained until the sixth week of the experiment (123.5±0.7 %, P<0.001).

Blood pressure of animals in which NO synthase was inhibited for a period of six weeks and blood pressure of animals in which the missing NO was simultaneously substituted by isosorbide-5-mononitrate over the period of six weeks are presented in Figure 2. In both groups, blood pressure increased similarly throughout the period of treatment, irrespective of simultaneous isosorbide-5-mononitrate administration. The elevation of blood pressure in the sixth week of the respective treatment reached 123.5±0.7 % (P<0.001) and 129.3±3.1 % (P<0.001), respectively.

Figure 3 shows the 6-week period of blood pressure development in animals treated either with L-NAME or with L-NAME plus simultaneous molsidomine. In the group treated with

L-NAME plus molsidomine, blood pressure increased slightly and became stabilized from the fourth week, representing 116.0±1.7 % (P<0.05) in the sixth week. BP values in animals treated with L-NAME plus molsidomine were significantly lower than those of animals treated with L-NAME alone (123.5±0.7 %, P<0.001). The former value, however, was still moderately elevated compared with starting steady state value.

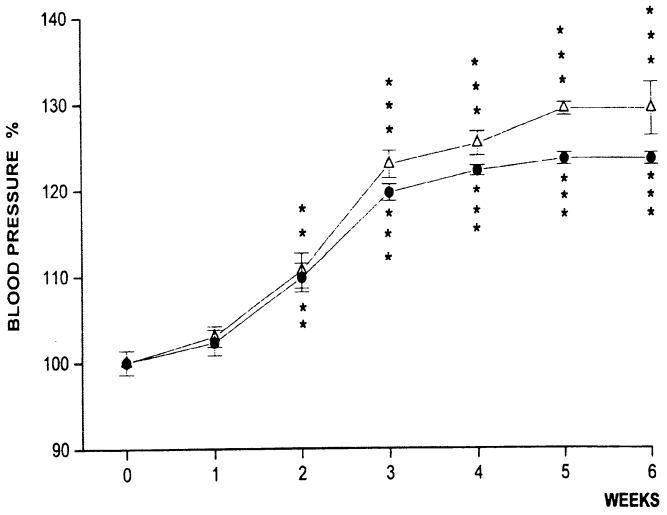

Fig. 2. *Blood pressure of rats administered L-NAME 50 mg/kg daily for six weeks (full circles) and BP of rats administered simultaneously isosorbide-5-mononitrate 5 mg/kg for the first two weeks, then 10 mg/kg till the sixth week (triangles). ** P<0.01, *** P<0.001 compared to respective steady-state values.*

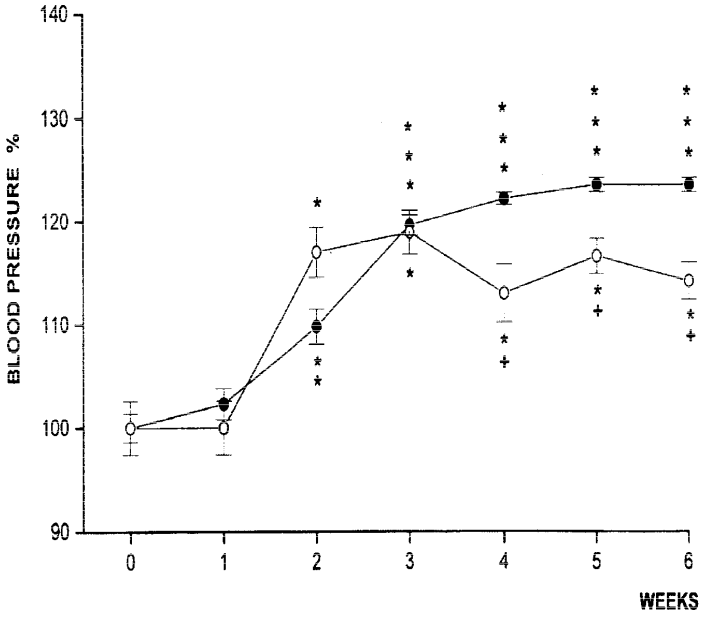

Fig. 3. *Blood pressure of rats administered L-NAME 50 mg/kg daily for six weeks (full circles) and BP of rats administered simultaneously molsidomine 50 mg/kg twice a day (open circles). * P<0.05, ** P<0.01, *** P<0.001 compared to respective steady-state BP values. $^{+}$ P<0.05 compared to corresponding BP values of L-NAME hypertensive rats.*

Morphometric evaluation of the geometry of the three selected vessels yielded the following results. In control animals, the inner diameter was 1824±55.9 μm in the thoracic aorta, 850±39.5 μm in the carotid artery, and 264±19.3 μm in the coronary artery. No significant alterations were found in the inner diameter of all the three vessels after treatment with L-NAME, or L-NAME plus molsidomine (Table 1).

The wall thickness (tunica intima + tunica media) of the thoracic aorta was 69.22±2.52 μm, of the carotid artery 24.99±0.80 μm and that of the coronary artery was 11.96±0.80 μm. In animals treated with

L-NAME, the wall thickness of the above three arteries increased and was 112.70±6.53 μm (P<0.01), 49.46±3.52 μm (P<0.01) and 24.08±1.44 μm (P<0.01), respectively. The wall thickness of arteries treated with L-NAME and simultaneously with molsidomine was very close to the values found in control animals: thoracic aorta 64.00±3.44 μm, carotid artery 24.26±2.28 μm and coronary artery 20.88±2.73 μm (Table 1).

Table 1. Geometry of thoracic aorta, carotid artery and coronary artery (septal branch of the left descending coronary artery).

	ID (μm)	WT (μm)	WT/ID
Thoracic aorta			
Controls	1824±55.90	69.22±2.52	3.83±0.22
L-NAME	1788±48.80	112.70±6.53**	6.40±0.44**
L-NAME + molsidomine	1608±51.90	64.00±3.44^{++}	4.00±0.32^{++}
Carotid artery			
Controls	850.00±39.50	24.99±0.80	3.00±0.20
L-NAME	785.50±24.10	49.46±3.52**	6.32±0.40**
L-NAME + molsidomine	771.20±47.40	24.26±2.28^{++}	3.40±0.44^{++}
Coronary artery			
Controls	264±19.30	11.96±0.80	4.60±0.33
L-NAME	228±14.54	24.08±1.44**	10.90±1.02**
L-NAME + molsidomine	236±15.20	20.88±2.73	8.90±0.98

*Inner diameter – ID, wall thickness – WT, wall thickness/inner diameter ratio – WT/ID. Controls – values of control animals, L-NAME - values of animals administered L-NAME (50 mg/kg/day in tap water), L-NAME + molsidomine – values of animals administered L-NAME as the above group plus molsidomine (50 mg/kg twice a day by gavage). Significant differences: ** p<0.01 vs. controls, $^{++}$ p<0.01 vs. L-NAME.*

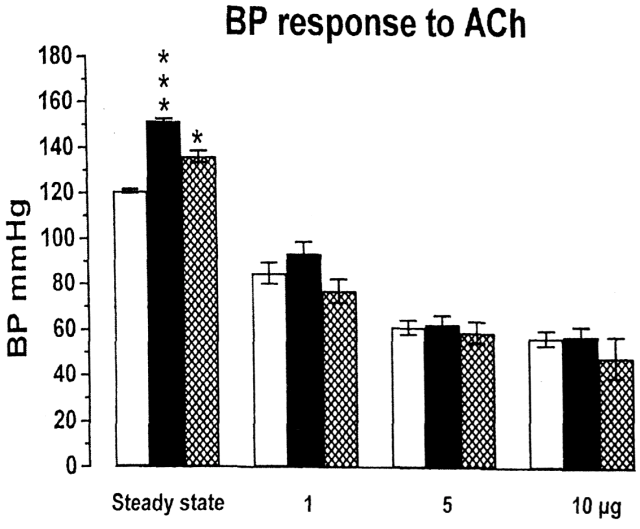

Fig. 4. *BP decrease after i.v. acetylcholine administration (1 μg, 5 μg and 10 μg/0.1ml Krebs solution in 10 s) in rats administered L-NAME for six weeks (black columns), in rats administered L-NAME plus molsidomine for six weeks (cross-hatched columns) and in age-matched control rats (open columns). * P<0.05, *** P<0.001 compared to respective BP value measured in control rats.*

The wall/diameter ratio in the controls was 3.83±0.22 in the thoracic aorta, 3.00±0.21 in the carotid artery and 4.60±0.33 in the coronary artery. In the group of rats administered L-NAME alone, this ratio was significantly increased being 6.40±0.44 ($P<0.01$) in the thoracic aorta, 6.32±0.40 ($P<0.01$) in the carotid artery and 10.90±1.02 ($P<0.01$) in the coronary artery. Long-term administration of molsidomine simultaneously with L-NAME prevented this increase of the wall/diameter ratio. Values of 4.00±0.32 in the thoracic aorta, 3.40±0.44 in the carotid artery and 8.90±0.98 in the coronary artery were close to the control values (Table 1).

The issue whether the hypotensive response to acetylcholine was affected in animals treated by L-NAME plus molsidomine, is documented in Figure 4. After administration of 10 μg acetylcholine, the maximum hypotension values of blood pressure were 56.0±3.0 mm Hg in control animals 58.0±4.6 mm Hg in L-NAME treated animals, and 49.4±3.5 mm Hg in animals treated with L-NAME plus molsidomine.

BP decrease induced by bradykinin (100 μg i.v.) is illustrated in Figure 5. Again, no significant differences were found among the values of reduced blood pressure in control animals, animals treated with L-NAME or L-NAME plus molsidomine: 67.0±5.2 mm Hg, 84.0±7.5 mm Hg and 60.2±3.4 mm Hg, respectively.

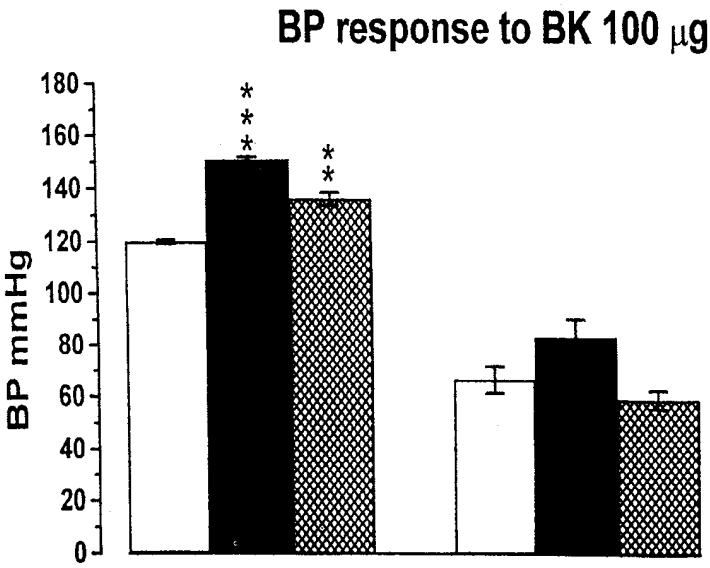

Fig. 5. *BP decrease after i.v. bradykinin administration (100 μg/0.1ml Krebs solution in 10 s) in rats administered L-NAME for six weeks (black columns), in rats administered L-NAME plus molsidomine for six weeks (cross-hatched columns) and in age-matched control rats (open columns). ** $P<0.01$, *** $P<0.001$ compared to respective BP values measured in control rats.*

Discussion

The experiments demonstrated a clear-cut sustained increase in blood pressure after 6 weeks' lasting inhibition of NO synthase, using L-NAME as inhibitor.

Direct NO measurement by means of the Malinski biosensor in the periendothelial area of the femoral artery of anesthetized dogs after inhibition of NO synthase by L-NAME yielded a relatively small decline in NO levels of about 15 % (Gerová *et al.* 1998). It was thus justified to expect that the NO released by exogenous donors would compensate this small deficiency. Our present experiments did not unequivocally confirm the above idea with either of the exogenous NO donors.

The experiments demonstrated that isosorbide-5-mononitrate did not affect the blood pressure increase induced by inhibition of NO synthase, not even after doubling the dose of isosorbide-5-mononitrate from the third week of treatment.

It has repeatedly been shown that nitrates mainly act upon the wall of the capacitance venous system, relaxing smooth muscle cells in this portion of the vascular tree and mainly decreasing the preload (Mülsch *et al.* 1995, Bauer and Fung 1996). Given that this class of NO donors produces a certain amount of NO and

accepting the concept of special attitude to venous smooth muscle, the present experiments indicate that this hemodynamic mechanism is not efficient enough to decline the blood pressure under conditions of impaired NO production induced by inhibition of NO synthase.

A different time course of BP changes was found in animals with inhibited NO synthase in which reduced endogenous NO production was supplemented by molsidomine-generated NO release. The blood pressure of animals of this group was significantly lower than blood pressure of animals with inhibition of NO synthase by L-NAME alone without supplementation of NO from exogenous sources. Nevertheless, the blood pressure was still moderately higher in comparison with BP values before the L-NAME plus molsidomine treatment. It thus appears that molsidomine substitutes, at least partially, the missing NO after NO synthase inhibition.

The main difference between molsidomine and isosorbide-5-mononitrate, which might explain the different effect on NO-deficient hypertension, may involve the bioactivation and metabolism of the above two drugs up to the end-production of nitric oxide. Nitrates are metabolized in various organs such as the liver, kidney, spleen, heart and also in the walls of major veins and probably in the walls of conduit arteries (Bauer *et al.* 1995, Mülsch *et al.* 1995). Feelisch *et al.* (1995) further demonstrated that both smooth muscle cells and endothelial cells of the vessel wall are able to metabolize nitrates. These authors proved that the enzymatic equipment, in particular cytochrome P450, is a prerequisite of nitrate metabolism. Bauer *et al.* (1995) further showed that besides cytochrome P450, another enzyme, glutathione transferase, is involved in the metabolism of nitrates in the vascular wall. Feelisch (1993) suggested that the metabolic pathways nitrates → NO and arginine → NO are parallel and separated. Nevertheless, at least in the first steps of the metabolic cascade it is possible to admit that inhibition of NO synthase in vascular smooth muscle cells and endothelial cells might compromise the enzymatic system necessary for nitrate metabolism. This may account for the inefficiency of isosorbide-5-mononitrate. On the other hand, molsidomine is metabolized to sydnonimine (SIN-1) in the liver and cleaves NO in the vascular wall without enzymatic intervention (Feelisch 1991). This drug has been shown to be more effective in substituting NO in the vascular wall and preventing the rise in blood pressure. Noack and Feelisch (1989) further speculated that metabolites of molsidomine may be more active in stimulating soluble guanylate cyclase than the metabolites of nitrates.

The blood pressure lowering effect of molsidomine in NO-deficient hypertension was consistent with the finding concerning the structural changes of the conduit vessels, namely the aorta, carotid artery and coronary artery. While the wall thickness and wall/diameter ratio were increased in hypertensive animals treated with the NO synthase inhibitor L-NAME, these values were significantly lower in animals treated simultaneously with L-NAME and molsidomine as compared with those of hypertensive animals. However, they were comparable to wall thickness and wall/diameter ratio found in control animals.

The NO synthase activity in animals treated with L-NAME or L-NAME plus molsidomine was inhibited for 6 weeks. It was therefore justified to expect that the activators of NO synthase (acetylcholine, bradykinin) would trigger a lower NO production, manifested by a smaller decrease of blood pressure in response to the NOS activators, administered in the anesthetized animals. This hypothesis could not be proved in the present experiments. The two different activators of NO synthase used, acetylcholine and bradykinin, triggered hypotension, and the bottom blood pressure values were similar in all three groups: (i) in control animals, (ii) in L-NAME and simultaneously molsidomine-treated animals, (iii) and even in animals treated with L-NAME alone. Recently, we described this paradoxical phenomenon of augmented hypotension elicited by acetylcholine or bradykinin in L-NAME treated hypertensive rats and were unable to provide an explanation (Gerová 1999). The experiments in animals treated simultaneously with L-NAME and molsidomine extend the series of puzzling findings which hitherto remain unexplained. These findings indicate that the current concept of NO production and substitution of the missing NO at failing NO production, appears to be oversimplified. Further physiological experiments are badly needed to face the new basic findings and/or to include them into integrated physiological processes.

Hypertension in rats induced by long-term inhibition of NO synthase was not affected by simultaneous isosorbide-5-mononitrate treatment. Another exogenous NO-donor, molsidomine, significantly lowered the high blood pressure in rats with NO synthase inhibition, but it did not return to the control BP levels. Different mechanisms of NO production by exogenous donors in the target cells are considered to underlie the diverse effects observed.

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