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Long-Term Effect of Molsidomine and Pentaerythrityl Tetranitrate on Cardiovascular System of Spontaneously Hypertensive Rats

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Summary

We studied the effects of long-term administration of molsidomine and pentaerythrityl tetranitrate (PETN) on the cardiovascular system of spontaneously hypertensive rats (SHR). One control and three experimental groups of 10-week-old animals were used: 1) control Wistar rats, 2) SHR, 3) SHR treated with molsidomine in tap water (100 mg/kg/day, by gavage), and 4) SHR treated with PETN in tap water (200 mg/kg/day, by gavage). After six weeks, the content of cGMP in platelets and NO synthase (NOS) activity in aortas were evaluated in the experimental groups. For morphological evaluation the rats were perfused at 120 mm Hg with a glutaraldehyde fixative and the arteries were processed for electron microscopy. Blood pressure and heart weight/body weight ratio (HW/BW) were increased in all experimental groups with respect to the controls. HW/BW was lower in the molsidomine group in comparison to both SHR and PETN-treated group. The platelet content of cGMP was increased and the activity of NOS in the aortas was decreased in the molsidomine and PETN-treated groups. Wall thickness and cross-sectional area of thoracic aorta, carotid artery and coronary artery were increased similarly in all experimental groups compared to the controls, but there were no differences among the experimental groups. We summarize that long-term administration of exogenous NO donors did not improve pathological changes of the cardiovascular system in SHR.

Key words

Spontaneously hypertensive rat • Nitric oxide donors • Cyclic GMP • Nitric oxide synthase activity • Morphometry

Introduction

Several experimental models have been developed to clarify the pathophysiological background of hypertension. It is supposed that a defect in endothelial NO production may play a significant role and that declined NO production could be responsible for both physiological and morphological alterations in the

cardiovascular system of different types of hypertension. Unfortunately, no reliable simple methods are available for *in vitro* and particularly for *in vivo* NO measurements. Thus the majority of data about NO levels in the cardiovascular system have to be based on indirect evaluation.

The status of NO in spontaneously hypertensive rats (SHR) is unclear, as conflicting data have been

published on NO production in this type of hypertension. Decreased endothelium-dependent relaxation was observed in coronary arteries (Pourageaud and Freslon 1995), aorta (Lüscher 1990, Soloviev *et al.* 1998), cerebral arteries (Mayhan 1990) and mesenteric resistance arteries (DeMey and Gray 1985, Tesfamariam and Halpern 1988, Watt and Thurson 1989). Using direct measurements of NO by a porphyrinic sensor, Malinski *et al.* (1993) reported decreased NO production in cultured smooth muscle cells harvested from SHR. Contrary to these findings, Tschudi *et al.* (1994) observed increased endothelium-dependent relaxation in the coronary artery, Papapetropoulos *et al.* (1994) in the aorta and Mourlon-LeGrand *et al.* (1992) in the carotid artery of SHR. Nava *et al.* (1995) found the upregulation of constitutive NO synthase in cardiac endothelial cells of SHR. Akiba *et al.* (1995), Gil-Longo *et al.* (1996), and Wu *et al.* (1996) observed enhanced activity of the NO system in SHR. Further authors did not find any changes in comparison to control animals. Arnal *et al.* (1993) found similar values of cyclic guanosine monophosphate (cGMP) in the aorta of Wistar-Kyoto rats and SHR. The observations on conduit arteries of SHR *in vivo* (Minami *et al.* 1995) and *in vitro* (Török and Kristek 2001) did not reveal any difference in response to acetylcholine compared to arteries from control Wistar rats. A long-term administration of L-arginine, as the rate limiting step for NO production, did not influence blood pressure and geometry of conduit arteries in SHR (Chen and Sanders 1991, Kristek 1998).

Blood pressure elevation in hypertension is accompanied by an increase of arterial wall thickness in both conduit and resistance arteries. It can be speculated that the increased NO levels due to exogenous NO supplementation could not only stimulate vasorelaxation but also exert an antiproliferative effect on smooth muscle cells. If this holds, a retardation of hypertension-evoked changes in the geometry of arteries could have a beneficial effect on the whole cardiovascular system (Folkow 1995).

Both pentaerythrityl tetranitrate (PETN) and molsidomine represent effective and tolerance-devoid NO donors. In comparison to other NO donors, PETN was found to be the most active drug in cGMP activation (Fink and Bassenge 1997, Hinz *et al.* 1998). It does not induce oxidative stress even after a long-term administration (Dücker and Richard 1990) and it may have a protective effect against atherosclerosis (Kojda *et al.* 1995, Müllenheim *et al.* 2001). Molsidomine belongs to the group of sydnonimines. It is metabolized in the liver

to SIN-1 which does not require enzymatic bioactivation and NO is released spontaneously in the arterial wall (Bohn and Schonafinger 1989, Noack and Feilish 1989, Hinz and Schröder 1999). Similarly as PETN, molsidomine evokes endothelium-independent dilatation of the arterial wall (Megson 2000). However, no morphological data are currently available on the long-term *in vivo* effect of either PETN or molsidomine on the cardiovascular system of SHR.

The aim of this study was to clarify whether long-term administration of the exogenous NO donors PETN and molsidomine in SHR will affect 1) morphological parameters of the thoracic aorta, carotid artery and septal branch of the left descending coronary artery, 2) NOS activity in the aorta and 3) the cGMP content in platelets.

Methods

All procedures followed the guidelines laid down by the Guide for the Use of Laboratory Animals (Ethics Committee for Experimental Work, Slovak Academy of Sciences, 1995).

The animals were housed at a temperature of 22-24 °C, in individual cages under a 12 h light:dark cycle and fed a regular diet. Ten-week-old male Wistar rats and spontaneously hypertensive rats (Charles River) (285-300 g) were studied. The experiments lasted six weeks. The animals were divided randomly into four groups of 20 animals each (10 animals for biochemical procedures and 10 animals for morphological evaluation). The first group (control Wistar rats) and animals of the second group (SHR) were given tap drinking water *ad libitum*. The third group (SHR) received molsidomine (Hoechst, Germany) p.o. by gavage in a concentration of 50 mg/kg twice daily (in the morning and in the afternoon) in a total daily dose of 100 mg/kg. The fourth group (SHR) received pentaerythrityl tetranitrate (Dipharma, Italy) at the concentration of 100 mg/kg twice daily (in the morning and afternoon) p.o. by gavage, in a total daily dose of 200 mg/kg. Both substances and prepared aqueous solutions for administration were stored in dark vials.

Systolic blood pressure and heart rate were measured weekly by the indirect tail plethysmographic method in all groups.

NOS activity was determined in crude homogenates of aortas by measuring the formation of ³H-L-citrulline from [³H]-L-arginine (Amersham, Little Chalfont, UK), as previously described by Brendt and

Snyder (1990), with some modifications. Briefly, 50 μ l of 10% homogenates were incubated in the presence of 50 mmol/l Tris/HCl, pH 7.4, 10 μ mol/l [3 H]-L-arginine (specific activity 5 GBq/mmol, about 100 000 d/min), 30 nmol/l calmodulin, 1 mmol/l β NADPH, 3 μ mol/l BH_4 and 2 mmol/l Ca^{2+} in a total volume of 100 μ l. After 20 min incubation at 37 °C, the reaction was stopped by addition of 1 ml of 20 mmol/l HEPES buffer (pH 5.5), containing 2 mmol/l EDTA, 2 mmol/l EGTA and 1 mmol/l L-citrulline. The samples were applied to 1 ml Dowex 50WX-8 columns (Na^+ form). [3 H]-L-citrulline was eluted by 1 ml of water and measured by liquid scintillation counting.

cGMP was determined in platelets as described by Fink and Bassenge (1997), with some modification. Briefly, blood was drawn into a solution consisting of 8.5 mM sodium citrate, 10 mM glucose monohydrate, 20 mM citric acid, 0.1 mM acetylsalicylic acid, 0.1 mM 3-isobutyl-1-methylxanthine. Platelet-rich plasma was obtained by centrifugation at 160 x g for 10 min. This fraction was centrifuged at 450 x g for 15 min and cells were washed in HEPES buffer (pH 7.4). Platelets were counted microscopically and cGMP concentration was determined using a RIA-kit (Amersham, UK). cGMP radioimmunoassay was based on the competition between succinylated cGMP of the sample and [125 I]-labeled tracer for binding to polyclonal antibody coated onto tubes. To determine the cGMP concentration, the vials were assessed using a gamma counter. The cGMP concentrations in the samples were calculated from the standard curve.

For morphological evaluation, the animals were sacrificed by an overdose of anesthesia. The chest was opened and the cardiovascular system was perfused *via* the left ventricle with glutaraldehyde fixative (300 mmol/l glutaraldehyde in 100 mmol/l phosphate buffer, pH 7.2-7.4) at a constant perfusion pressure of 120 mm Hg for 10 min. After perfusion, the upper half of the septal branch of the left descending coronary artery, the middle part of the carotid artery and the middle part of the thoracic aorta were excised, cleaned, divided into about 1 mm long segments and fixed in the same fixative overnight at 4 °C in a refrigerator. After fixation the segments were postfixated with 40 mmol/l OsO_4 in 100 mmol/l phosphate buffer, washed in 100 mmol/l phosphate buffer, and stained *en block* with uranylacetate. The samples were dehydrated through ascending concentrations of alcohol, washed in propyleneoxide and embedded in Durcupan ACM.

Two randomly selected blocks of each artery were cut perpendicularly to the longitudinal axis. The inner circumference and arterial wall thickness (tunica intima + tunica media) of the individual vessels were measured in semithin sections under a light microscope. From these data the cross sectional area (tunica intima + tunica media) of the arterial wall, the inner diameter, and wall thickness/inner diameter ratio of the corresponding vessel were calculated.

The obtained values were expressed as means \pm S.E.M. ANOVA and the Bonferroni test for unpaired variables were used for statistical evaluation. The results were considered to be significantly different when $P < 0.05$.

Results

General hemodynamic parameters

The systolic blood pressure of SHR was markedly increased ($p < 0.01$) in all experimental groups in comparison to control Wistar rats. Six-week administration of NO donors to SHR did not decrease blood pressure in either the molsidomine or PETN group. At the end of the experiments, the value of the heart rate did not significantly differ among the groups (Table 1).

The body weight (measured before sacrificing the animals) was significantly ($p < 0.01$) lower in SHR than in the Wistar rats. In the molsidomine group, the body weight of rats was significantly lower compared to all other groups: control, SHR and PETN ($p < 0.01$). In the PETN group, the body weight was lower in comparison to controls ($p < 0.01$) and SHR ($p < 0.05$), but higher ($p < 0.01$) in comparison with the molsidomine group (Table 1).

After 10-min whole body perfusion with the fixative, the heart weight was higher in SHR than in the control rats ($p < 0.01$). In the molsidomine group, the heart weight was significantly ($p < 0.01$) lower in comparison to Wistar rats, SHR and rats of the PETN group. In the group with PETN, the heart weight was lower than in SHR ($p < 0.05$), but higher ($p < 0.01$) than in the molsidomine group (Table 1). The heart weight/body weight ratio was significantly higher in all three experimental groups in comparison to control Wistar rats ($p < 0.01$), but it was lower in the molsidomine group than in SHR and the PETN group ($p < 0.01$). No differences were observed between SHR and PETN group (Table 1).

Table 1. Systolic blood pressure (BP), heart rate (HR), body weight (BW), heart weight (HW), and heart weight/body weight ratio (HW/BW) in age-matched Wistar rats, SHR and SHR treated for six weeks with NO donors - molsidomine (SHR+MOLS) and pentaerythrityl tetranitrate (SHR+PETN).

	BP (mmHg)	HR (beat/min)	BW (g)	HW (g)	HW/BW $\times 10^{-3}$	n
Wistar rats	127±1.4	374±11.6	423±3.8	1.35±0.03	3.13±0.03	10
SHR	214±7.3**	394±15.9	324±5.7**	1.59±0.04**	4.91±0.18**	10
SHR+MOLS	224±5.1**	439±15.9	274±2.3***	1.04±0.02***	3.77±0.08***	10
SHR+PETN	220±2.5**	433±13.9	307±3.1***	1.36±0.03***	4.45±0.03***	10

Values are means \pm S.E.M. * $P < 0.05$, ** $P < 0.01$ vs. control Wistar rats, + $P < 0.05$, ++ $P < 0.01$ vs. SHR, ### $P < 0.01$ vs. SHR + MOLS

Biochemical parameters

Activity of NOS in the aorta of SHR was 53.26 ± 1.43 dpm/ μ g/min. NOS activity was significantly decreased in molsidomine (47.12 ± 1.55 dpm/ μ g/min, $p < 0.05$) and PETN-treated rats (45.88 ± 1.37 dpm/ μ g/min, $p < 0.01$). Values of NOS activity did not significantly differ between the molsidomine and PETN group (Fig. 1).

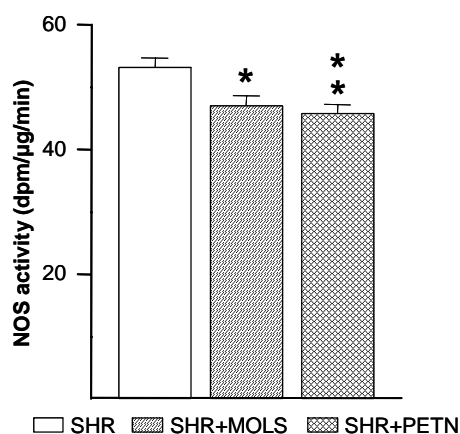


Fig. 1. Effect of long-term administration of exogenous NO donors on NOS activity in aortas from the SHR, SHR treated with molsidomine (SHR+MOLS) and SHR treated with pentaerythrityl tetranitrate (SHR+PETN). * $P < 0.05$, ** $P < 0.01$ with respect to SHR.

Morphological evaluation

The morphological evaluation of the conduit arteries - thoracic aorta, carotid artery and septal branch of the left descending coronary artery - revealed that their arterial wall thickness (tunica intima plus tunica

media) was significantly lower in the control groups than in the corresponding arteries of all three experimental groups. No differences in this respect were found among the arteries in the experimental groups (Tables 2, 3 and 4).

The value of cGMP in platelets was 0.105 ± 0.071 pmol/ 10^9 in SHR. In the group administered molsidomine, this value was significantly increased to 0.641 ± 0.103 pmol/ 10^9 ($p < 0.01$). In the group with PETN administration, the value was significantly higher than in SHR, representing 0.345 ± 0.071 pmol/ 10^9 ($p < 0.05$). No differences in this respect were observed between the molsidomine and PETN groups (Fig. 2).

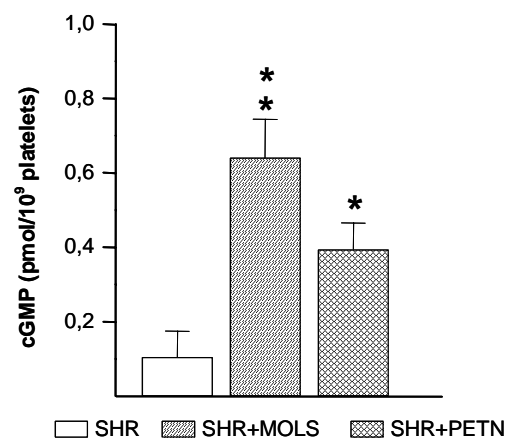


Fig. 2. Effect of long-term administration of exogenous NO donors on cGMP concentration in platelets of the SHR, SHR treated with molsidomine (SHR+MOLS) and SHR treated with pentaerythrityl tetranitrate (SHR+PETN). * $P < 0.05$, ** $P < 0.01$ with respect to SHR.

Because the inconstant perfusion pressure during fixation could influence the arterial wall thickness, we also calculated the cross sectional areas (tunica intima plus tunica media) of the corresponding vessels as the cross-sectional area is independent of perfusion pressure. The values of cross sectional areas were significantly higher in all experimental groups ($p < 0.01$) than in the control group. Among the experimental groups, no

significant differences were found in the cross sectional areas of the conduit arteries (Tables 2, 3 and 4).

The inner diameter of all control arteries calculated from the inner circumference did not significantly differ from that of the corresponding arteries in the experimental groups. We did not find any differences in this respect among matching experimental arteries (Tables 2, 3 and 4).

Table 2. Thoracic aorta: wall thickness (WT), cross sectional area (CSA), inner diameter (ID), and wall thickness/inner diameter ratio in Wistar rats, SHR and SHR treated for six weeks with NO donors – molsidomine (SHR+MOLS) and pentaerythryl tetranitrate (SHR+PETN).

	WT (μm)	CSA (μm^2) $\times 10^3$	ID (μm)	WT/ID $\times 10^{-2}$	n
<i>Wistar rats</i>	60.74 \pm 2.37	332 \pm 12	1683 \pm 40	3.56 \pm 0.19	10
<i>SHR</i>	81.37 \pm 2.27**	424 \pm 17**	1571 \pm 29	5.20 \pm 0.18**	10
<i>SHR+MOLS</i>	76.18 \pm 3.79**	386 \pm 12**	1569 \pm 44	4.50 \pm 0.37**	10
<i>SHR+PETN</i>	84.41 \pm 1.92**	427 \pm 12**	1525 \pm 22	5.55 \pm 0.15**	10

Values are means \pm S.E.M. ** $P < 0.01$ vs control Wistar rats.

Table 3. Carotid artery: wall thickness (WT), cross-sectional area (CSA), inner diameter (ID), and wall thickness/inner diameter ratio in Wistar rats, SHR and SHR treated for six weeks with NO donors – molsidomine (SHR+MOLS) and pentaerythryl tetranitrate (SHR+PETN).

	WT (μm)	CSA (μm^2) $\times 10^3$	ID (μm)	WT/ID $\times 10^{-2}$	n
<i>Wistar rats</i>	23.95 \pm 1.76	59.5 \pm 3.1	778 \pm 34	3.16 \pm 0.33	10
<i>SHR</i>	41.31 \pm 2.43**	109.8 \pm 8.2**	801 \pm 22	5.15 \pm 0.25**	10
<i>SHR+MOLS</i>	38.91 \pm 1.09**	96.1 \pm 2.8**	755 \pm 30	5.34 \pm 0.35**	10
<i>SHR+PETN</i>	44.24 \pm 1.60**	110.1 \pm 2.8**	748 \pm 13	5.94 \pm 0.25**	10

Values are means \pm S.E.M. ** $P < 0.01$ vs control Wistar rats.

Table 4. Septal branch of the left descending coronary artery: wall thickness (WT), cross sectional area (CSA), inner diameter (ID) and wall thickness/inner diameter ratio in Wistar rats, SHR, and SHR treated for six weeks with NO donors – molsidomine (SHR+MOLS) and pentaerythryl tetranitrate (SHR+PETN).

	WT (μm)	CSA (μm^2) $\times 10^3$	ID (μm)	WT/ID $\times 10^{-2}$	n
<i>Wistar rats</i>	9.33 \pm 0.67	7.8 \pm 0.8	250 \pm 12	3.70 \pm 0.23	10
<i>SHR</i>	20.11 \pm 1.25**	21.7 \pm 2.4**	321 \pm 26	6.75 \pm 0.71**	10
<i>SHR+MOLS</i>	18.11 \pm 1.89**	20.0 \pm 2.8**	323 \pm 23	5.57 \pm 0.50**	10
<i>SHR+PETN</i>	19.29 \pm 0.88**	19.4 \pm 1.3**	292 \pm 19	6.64 \pm 0.29**	10

Values are the mean \pm S.E.M. ** $P < 0.01$ vs control Wistar rats.

The arterial wall thickness/inner diameter ratio showed significant differences ($p < 0.01$) between arteries from the control group and corresponding arteries from the experimental groups. Differences in the values among the experimental groups were not significant (Tables 2, 3 and 4).

Discussion

It is generally accepted that in comparison to control normotensive Wistar rats, the cardiovascular system of SHR exhibits important physiological and morphological changes. Our present data (enhanced blood pressure, hypertrophy of the myocardium and hypertrophy of arterial wall of conduit arteries without changes in their diameter) are fully in agreement with the data of Lee *et al.* (1983). In spite of many experiments on SHR, the exact pathogenesis of these alterations remains unknown. It has been suggested that NO, a powerful vasodilator agent, could be involved in this process. Nevertheless, contradictory results reported in SHR imply that the theories on the role of NO in the cardiovascular system of SHR remain somewhat speculative.

Our present data have shown that long-term administration of exogenous NO donors molsidomine and PETN did not exert any effect on either blood pressure or heart rate in SHR. On the other hand, a decrease in heart weight was observed in both experimental groups. In the group of rats administered molsidomine, this decrease was significant not only in comparison with SHR but also in comparison with the group of rats receiving PETN. Despite the assumption that the decrease of heart weight may be explained by a concomitant decrease in body weight, the heart weight/body weight ratio showed that this is true in the PETN group only. The finding in the molsidomine group appears to be rather interesting because the decrease of the heart weight/body weight ratio was not accompanied by a decrease of blood pressure. It seems that high blood pressure may not be necessarily associated with cardiac hypertrophy. A similar finding was reported after long-term administration of low doses of ACE inhibitors (Saleh and Jurjus 2001).

The question remains open why the presumed increase in NO levels derived from two different exogenous NO donors fails to affect the geometry of conduit arteries in SHR. Moreover, in spite of the general view that besides its vasorelaxant action, NO also exhibits an antiproliferative effect on the smooth muscle

of the arterial wall, we did not observe any decrease of arterial wall thickness or cross sectional area. These findings could not be explained by the development of nitrate tolerance after long-term administration, namely the declined efficacy of either drug. Molsidomine and PETN have been shown to be tolerance-devoid exogenous NO donors (Bohn and Schonafinger 1989, Noack and Feilish 1989, Dück and Richard 1990, Kojda *et al.* 1995, Fink and Bassenge 1997, Hinz *et al.* 1998, Hinz and Schröder 1999, Megson 2000). The significant increase of cGMP content in platelets, which we observed in both molsidomine and PETN groups, also supports these findings. Moreover, the long-term ability of the body to bioconvert molsidomine and PETN to NO without nitrate tolerance was observed in NO-deficient hypertension (using the same doses and duration) (Kristek 2000, Gerová and Kristek 2001). The difference in the effect of molsidomine and PETN in SHR and in NO-deficient hypertension may be related to the different conditions for their action. In NO-deficient hypertension exogenous NO donors probably supplement the deficiency in endogenous NO. On the contrary, the deficiency of NO in genetically hypertensive rats is still an open question of debate. Furthermore, NO in SHR may represent an important counter-regulatory mechanism against stimuli leading to cardiovascular alterations, mainly against increased blood pressure. Intact NO production in SHR was indirectly documented in a number of experiments and our present findings are in good agreement with these indications. Tucker *et al.* (2000) did not observe any differences in NO-synthase activity between SHR and Wistar rats. Arnal *et al.* (1993) emphasized that there was no evidence for an alteration in the endothelial NO pathway as a primary cause of hypertension in SHR. Maintenance of both endothelial functions and the L-arginine/NO system was also observed in the New Zealand genetically hypertensive rat strain (Orange *et al.* 2000). These observations were indirectly confirmed in experiments where blockade of NO synthase enhanced blood pressure in SHR (Akiba *et al.* 1995, Minami *et al.* 1995, Gil-Longo *et al.* 1996, Rees *et al.* 1996, Orange *et al.* 2000, Tucker *et al.* 2000). Fouyas *et al.* (1997) reported that the administration of L-NAME to SHR increased blood pressure and this increase in blood pressure did not differ from that in normotensive Wistar rats after the administration of L-NAME. Moreover, subsequent administration of sin-1 evoked a similar decrease of blood pressure in both groups. These findings are in good agreement with our

earlier observations that a shortage of L-arginine did not induce changes in the cardiovascular system of SHR (Kristek 1998). In this study, we did not observe any significant changes in either general physiological or morphological parameters of conduit arteries after long-term administration of L-arginine to SHR (using the same parameters as in the present report).

We suggest that the failure of molsidomine and PETN action in SHR could at least partially be explained by decreased activity of NO synthase in vascular wall after exogenous donor administration. It is therefore possible that overproduction of NO may act as a negative feedback modulator and that the total NO availability may thus remain at approximately the same level. This suggestion is in good agreement with the observations of Rogers and Ignarro (1992), Bult *et al.* (1991), and Rengasamy and Johns (1993). The latter observed that NO and exogenous NO donors S-nitroso-N-acetylpenicillamine, sodium nitropruside and glyceryl trinitrate inhibited NO synthase activity in the bovine cerebellum. This effect was concentration-dependent. Bult *et al.* (1991) observed depressed biosynthesis of NO in aortal segments of rabbits treated with molsidomine.

Furthermore, excess superoxide production found in SHR (Huie and Padmaja 1993, Kojda *et al.* 1998, Haj-Yehia *et al.* 2000) could also decrease the effectiveness of exogenous NO in SHR. Superoxide close to the site of NO generation forms peroxynitrite with NO and thus inhibits the biological activity of NO. Administration of superoxide dismutase (scavenger of superoxide) decreases the level of superoxide and normalizes blood pressure in SHR (Schnackenberg *et al.*

1998, Inoue *et al.* 2000). Moreover, overproduction of superoxide in SHR may evoke desensitization of vascular soluble guanylate cyclase (Kojda *et al.* 1998). Increased shear stress on endothelial cells may also lead to desensitization of soluble guanylate cyclase (Moncada *et al.* 1991) and could thus be responsible for decreased sensitivity of the arterial wall to NO.

In summary, long-term administration of exogenous NO donors to SHR increased cGMP content in platelets, decreased NO synthase activity in the aorta, but it failed to evoke significant changes in either general parameters of the cardiovascular system, such as blood pressure, heart rate, heart weight/body weight ratio (except in the molsidomine group) or in the geometry of conduit arteries, i.e. the inner diameter, arterial wall thickness, cross-sectional area and wall/diameter ratio. Taking into account these results as well as literary data, we conclude that increased NO levels (due to NO donors) apparently do not exert a beneficial effect on the cardiovascular system of SHR. Pathological changes in the cardiovascular system of SHR are thus presumably not evoked by NO deficiency.

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