Cardiovascular Effects of Chronic Inhibition of Nitric Oxide Synthesis and Dietary Salt in Spontaneously Hypertensive Rats


The cardiovascular effects of chronic inhibition of nitric oxide synthesis and dietary salt were studied in 9-wk-old spontaneously hypertensive rats (SHR). Nω-nitro-L-arginine methyl ester (L-NAME, 0.025% in food, about 20 mg/kg/d) was given to rats receiving diets containing low, moderate, and high salt levels (NaCl 0.2%, 1.1%, and 6.0% of the dry weight of the chow) for 3 wk. L-NAME increased systolic blood pressure by 50 to 60 mmHg in all treated groups, as compared with an average rise of 10 to 20 mmHg in the control SHR. The high-salt diet did not further increase blood pressure. L-NAME also induced cardiac and renal hypertrophy, and these changes were aggravated by the high-salt diet. In addition, 19 of the 30 rats treated with L-NAME suffered strokes and all of them had several myocardial infarctions and renal damage, while the rats not treated with L-NAME had no evidence of stroke or myocardial or renal injury. Responses of mesenteric arterial rings in vitro were studied at the end of the experiment. The vascular contractile responses to noradrenaline were increased, and the relaxation responses to acetylcholine were inhibited in the L-NAME treated groups. In addition, the high-salt diet alone tended to inhibit the response to acetylcholine. Plasma renin activity was markedly increased by L-NAME treatment and decreased by the high-salt diet. The 24-h urine protein excretion was increased both by the L-NAME treatment and by the high-salt diet. The combination of L-NAME and the high-salt diet markedly raised the serum creatinine concentration. Our findings show that the coronary and renal functions are particularly vulnerable in SHR during impaired nitric oxide synthesis, and that the end-organ damage is worsened by an increased intake of dietary salt. We suggest that dysfunction of the endothelium is the primary cause of the effects observed in this study. (Hypertens Res 1997; 20: 183-192)

Key Words: SHR, L-NAME, sodium, blood pressure, heart infarction, kidney damage, endothelium

Nitric oxide (NO) is one of the most important regulators of cardiovascular hemodynamics (1). It mediates the vasorelaxing effect of acetylcholine and bradykinin as well as that of the exogenous nitrovasodilators. NO is synthetized in endothelial cells from L-arginine by nitric oxide synthases (NOS), which can be inhibited by L-arginine analogs such as Nω-nitro-L-arginine methyl ester (L-NAME) (2). Both acute and chronic inhibition of NOS induces an increase in blood pressure in different rat strains (3-6) and other experimental animals (7, 8). In humans, vasodilatation by acetylcholine and bradykinin can be attenuated by infusion of an NOS-inhibitor (9).

High intake of dietary salt (sodium chloride) accelerates hypertension and left ventricular hypertrophy in spontaneously hypertensive rats (10-15) and is an important contributory factor in human arterial hypertension (16-19). Moreover, sodium chloride loading increases kidney weight, even without a rise in blood pressure (12, 20, 21).

Increased intake of salt enhances the formation of NO in normotensive rats (22, 23), which apparently improves the ability of the kidneys to excrete sodium. Deficient renal production of NO, resulting in impaired sodium handling, has been suggested as one possible mechanism in experimental and human essential hypertension (24).

This study was designed to evaluate the role of NO in the development of the harmful cardiovascular effects of salt. The urinary excretion of protein and electrolytes, plasma renin activity, serum creatinine concentration, and responses of mesenteric arterial rings in vitro were examined to gain some insight into the pathophysiology of the observed effects.

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Methods

Experimental Animals and Diets

Sixty inbred male spontaneously hypertensive rats (SHR) were purchased from Harlan Sprague Dawley Inc. (Indiana, IN, USA). The rats were housed, five animals per cage, in an animal laboratory (illuminated from 6:30 a.m. to 6:30 p.m., room temperature 22–24°C). At the beginning of the study, the 9-wk-old rats were divided into six subgroups assigned to receive different diet and drug regimens for 3 wk. The low-salt diet consisted of a commercially available low-salt rat chow (Teklad LM486, Harlan Teklad, Madison, WI, USA) with a 0.2% content of NaCl. Other minerals in the chow included calcium 0.8%, potassium 0.9%, magnesium 0.2%, and phosphorus 0.7%.

The moderate-salt diet was produced by adding 9 g of NaCl (JOZOTM, Akzo Salt Europe, Amersfoort, Holland) to 991 g of the low-salt chow, thus achieving a 1.1% concentration of NaCl, which approximates the level of most standard rat chows. The high-salt diet was produced by adding 58 g of NaCl to 942 g of the low-salt chow to obtain a 6.0% level of NaCl. Nω-nitro-L-arginine methyl ester (L-NAME, Sigma Chemical Co., St Louis, MO, USA) was added to the diets at the level of 250 mg/kg of the dry weight of the chow to produce an average daily dose of approximately 20 mg/kg body weight. The rats had free access to tap water and chow during the experiment. Body weight was measured once a week. During the third week of the study, the rats were housed individually in metabolic cages. Food intake was recorded and urine collected over a 24-h period. The rats were examined daily for survival and signs of stroke during the study. Assessment of stroke was based on the presence of evident and stable hemiplegia, akinesia, lethargy, and hyporesponsiveness according to the symptomatic classification described by Yamori et al. (25). The procedures and protocols of the study were in accordance with our institutional guidelines and were approved by the Animal Experimentation Committee of the Institute of Biomedicine, University of Helsinki, Finland.

Measurement of Blood Pressure and Sample Preparation

Systolic blood pressure and heart rate of the pretrained rats were measured weekly with the use of a tail-cuff analyzer (Apollo-2AB Blood Pressure Analyzer, Model 179-2AB, IITC Life Science, Woodland Hills, CA, USA). The digital values for systolic blood pressure and heart rate were evaluated automatically from the analog data by a microprocessor. Before the measurements the rats were warmed for 10 to 20 min at 28°C to make the pulsations of the tail artery detectable. Values for systolic blood pressure and heart rate were obtained by averaging readings from three measurements.

To minimize stress-induced fluctuations in blood pressure, all measurements were taken by the same person in the same quiet environment between 2 and 6 p.m. At the beginning of the experiment, the systolic blood pressure values in the different groups were 139 ± 5 mmHg in the low-salt group, 139 ± 5 mmHg in the low-salt with L-NAME group, 139 ± 5 mmHg in the moderate-salt group, 138 ± 5 mmHg in the moderate-salt with L-NAME group, 137 ± 6 mmHg in the high-salt group, and 138 ± 5 mmHg in the high-salt with L-NAME group.

The rats were killed during the third experimental week; thus, the average duration of the experiment was 18 days. After decapitation, blood samples were taken into two tubes chilled on ice, using EDTA (4.5 mM) as anticoagulant, and one tube without anticoagulant, used to obtain serum. The heart was excised, the great vessels, atria, and the free wall of the right ventricle were dissected, and the left ventricular mass was measured. The kidneys were excised, washed with ice-cold saline, and weighed. Left-ventricular-weight-to-body-weight and kidney-weight-to-body-weight ratios were calculated and used as indices to estimate hypertrophy in these organs. The brain was excised and observed for evidence of hemorrhage. The superior mesenteric artery was carefully excised and cleaned of adherent connective tissue.

Mesenteric Arterial Responses in vitro

A modification of the method described by Pörsti et al. (26) was applied. Briefly, two 3-mm-long standard sections of the mesenteric artery, 3 mm distal from the artery-aorta junction, were cut. In one ring, the endothelium was left intact, while from the other ring it was removed by rubbing with a flattened needle. The rings were placed between stainless steel hooks and mounted in an organ bath chamber in physiological salt solution (PSS) (pH 7.4) of the following composition (mM): NaCl 119.0, NaHCO3 25.0, glucose 11.1, CaCl2 1.6, KCl 4.7, KH2PO4 1.2, and MgSO4 1.2, aerated with 95% O2 and 5% CO2. The ring was equilibrated for 20 min at +37°C with a resting tension of 1.0 g. The force of contraction was measured with an isometric force-displacement transducer and registered on a polygraph (FT03C transducer, Model 7C8 Polygraph, Grass Instrument Co., Quincy, MA, USA). The presence or absence of intact endothelium in the vascular preparations was confirmed by observing the relaxation response to 1 μM acetylcholine (ACh) in rings precontracted with 1 μM noradrenaline (NA). The contractile-concentration curves to NA and to KCl, and the relaxation-concentration curves to cumulative ACh and sodium nitroprusside (SNP) were determined as described by Kähönen (27). The EC50 values for NA and KCl were the concentrations producing 50% maximal contraction, and those for ACh and SNP the concentrations producing 50% relaxation of 1 μM NA-induced precontraction. All EC50 values are presented as the negative logarithms of the concentrations of agonists (pD2).

Morphological Studies

From two animals in each study group, heart muscle and kidney tissue were prepared for light mi-
After excision, the tissue samples were fixed in phosphate buffered formalin and stored at +4°C. The samples were then prepared according to standard laboratory procedures, embedded in paraffin, cut into 3-μm sections, stained by the Vaigert van Gieson method, and examined and photographed under light microscopy.

**Biochemical Determinations**

Plasma renin activity was determined by using a commercial radioimmunoassay for angiotensin I (Medix Biochemica, Kauniainen, Finland). Serum creatinine concentration was determined with an enzymatic analyzer (Kone Specific, Kone Corp., Espoo, Finland). Total protein concentration in urine was determined by using the method of Lowry et al. (28) after precipitation with 10% trichloroacetic acid. The concentrations of the elements sodium, potassium, phosphorus, magnesium, and calcium in urine were determined by using a Baird PS-4 inductively coupled plasma emission spectrometer (Baird Co, Bedford, MA, USA).

**Statistical Analysis**

Statistical analysis was carried out by one-way analysis of variance (ANOVA) supported by Tukey’s test. Data for multiple observations over time were analyzed by two-way ANOVA with repeated measures for overall treatment effect, and Tukey’s test was used for multiple pairwise comparisons of treatment groups at different times. Survival analysis was supported by the Mantel-Cox test. Differences between means that had p < 0.05 were considered significant. The data were analyzed with SYSTAT Statistical Software (SYSTAT Inc., Evanston, IL, USA). The results are expressed as
Results

Stroke Rate, Heart Infarctions, and Kidney Damage

Nineteen of the 30 rats receiving L-NAME suffered a clinically diagnosed stroke before the end of the experiment, and they were sacrificed soon after the diagnosis was made. In contrast, no signs of stroke were observed in the rats receiving diets without L-NAME. The cumulative stroke rate of each group is presented in Fig. 1. The low-salt group tended to have fewer and later strokes than did the other L-NAME treated groups, but the difference was not significant. All rats that received L-NAME were observed to have macroscopic white patches in cardiac and renal tissues. These findings were interpreted as infarctions. In fact, multiple myocardial infarctions were confirmed under light microscopy in all the samples taken from the L-NAME-treated rats (Fig. 2), whereas the heart muscle was normal in the groups without L-NAME. Gradation of lesion severity and comparison between different dietary salt levels was not possible because of the small number of the samples.

In the L-NAME-treated rats, the arteries of the heart appeared normal, but medial degeneration, fibrinoid necrosis of the media, and intimal thickening were observed in the small arterioles (Fig. 2).

In the kidneys (Fig. 3), necrosis and sclerosis of the glomeruli were observed, associated with similar arteriolar changes as seen in the heart.

Blood Pressure, Left Ventricular Hypertrophy, and Renal Hypertrophy

In all six treatment groups, the systolic blood pressure increased steadily during the experiment. Treatment with L-NAME produced a marked increase in systolic blood pressure, as compared with the groups receiving diets without L-NAME (Fig. 4). This effect was independent of the salt content of the diets. After the first week, the rats in the L-
NAME-treated groups who later suffered strokes had higher blood pressure than those who survived until the end of the study (180 ± 5 vs. 165 ± 4 mmHg, \( p = 0.037, n = 31 \)). There were no significant differences in heart rate among the groups (data not shown). Both the left-ventricular-weight-to-body-weight and kidney-weight-to-body-weight ratios were increased by the high-salt diet and treatment with \( \text{L-NAME} \). The biggest increase in the organ-weight-to-body-weight ratios was seen in the group receiving both the high-salt diet and \( \text{L-NAME} \) (Fig. 5). The organ wet weights are given in Table 1.

**Weight Gain and Food Intake**

After the first week, the rats in the \( \text{L-NAME} \)-treated groups started to lose weight, most markedly in the high-salt group, while the groups receiving diets without \( \text{L-NAME} \) gained weight steadily during the experiment (Fig. 6).

Because of their poor condition during the last week of the experiment, the rats in the group receiving both the high-salt diet and \( \text{L-NAME} \) were not taken to the metabolic cages. The \( \text{L-NAME} \)-treated rats ate significantly less than did the non-treated ones (Table 2), and the decrease in the intake of food was more pronounced in the moderate-salt group than in the low-salt group.

**Urine Electrolytes and Protein**

The 24-h urinary excretion of sodium was increased in the moderate- and high-salt groups (Table 2). The excretion of sodium was increased about five-fold by \( \text{L-NAME} \) treatment in the low-salt group. Treatment with \( \text{L-NAME} \) decreased the excretion of potassium to the same extent as it decreased the intake. The high-salt diet increased the excretion of magnesium, calcium, and phosphorus. Neither the moderate-salt diet nor treatment with \( \text{L-NAME} \) had any effect on the excretion of magnesium or phosphorus, but the excretion of calcium was slightly increased in the low-salt \( \text{L-NAME} \)-treated group. The urinary excretion of protein was higher in the \( \text{L-NAME} \)-treated groups than in the corresponding groups without \( \text{L-NAME} \) treatment.

**Plasma Renin Activity and Serum Creatinine**

Treatment with \( \text{L-NAME} \) increased plasma renin activity (PRA) about 5-fold in the low-salt and moderate-salt groups (Table 1). In the high-salt group, the increase in PRA was as high as 11-fold. The high-salt diet decreased PRA in the rats, irrespective of whether or not they received \( \text{L-NAME} \).
NAME.

Treatment with L-NAME increased serum creatinine concentration in the moderate- and high-salt groups (Table 1). The high-salt diet alone did not increase the serum creatinine concentration.

Mesenteric Arterial Responses in vitro

Because of technical reasons, these data could not be obtained from the high-salt L-NAME-treated group. With intact endothelium, the vascular contractile responses to noradrenaline were increased in the low- and moderate-salt L-NAME-treated groups (Fig. 7). In these groups, the first significant
contractile response was found at 10 nM noradrenaline, while in the other groups about 10-fold higher concentrations were needed for equal contractions. Also, the concentration needed for half of the maximal response (EC50) was decreased in the L-NAME treated groups (Table 3). The vascular relaxation responses to acetylcholine were impaired by L-NAME treatment as well as by the high-salt diet alone, although the differences between the groups in the maximal relaxation responses to acetylcholine did not quite reach statistical significance (p = 0.08). No differences between the groups were found in the vascular contractile responses to potassium chloride or in the relaxation responses to sodium nitroprusside.

When the endothelium was removed from the vascular preparations, no statistically significant differences between the groups were found in the vascular contractile responses to noradrenaline or potassium chloride or in the relaxation responses to acetylcholine or sodium nitroprusside (Table 4).

Table 4. Variables of Contractile and Relaxation Responses of Isolated Endothelium-Denuded Mesenteric Arterial Rings from Spontaneously Hypertensive Rats after 3 wk on the Different Diet and Drug Regimens

<table>
<thead>
<tr>
<th></th>
<th>LS (NaCl 0.2%)</th>
<th>MS (NaCl 1.1%)</th>
<th>HS (NaCl 6.0%)</th>
<th>ANOVA EC50</th>
<th>L-NAME treated</th>
<th>L-NAME not treated</th>
<th>t-test</th>
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<tbody>
<tr>
<td>Noradrenaline</td>
<td></td>
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<tr>
<td>pD2 (−log M)</td>
<td>6.99±0.07</td>
<td>6.76±0.12</td>
<td>7.00±0.14</td>
<td>0.191</td>
<td>7.00±0.14</td>
<td>6.91±0.15</td>
<td>0.640</td>
</tr>
<tr>
<td>Maximal contraction force (g)</td>
<td>1.74±0.15</td>
<td>1.59±0.17</td>
<td>2.19±0.23</td>
<td>0.099</td>
<td>2.03±0.09</td>
<td>1.81±0.22</td>
<td>0.365</td>
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<tr>
<td>Potassium chloride</td>
<td></td>
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</tr>
<tr>
<td>pD2 (−log M)</td>
<td>42.1±1.9</td>
<td>42.5±2.1</td>
<td>38.3±1.4</td>
<td>0.249</td>
<td>38.9±1.6</td>
<td>43.1±2.7</td>
<td>0.201</td>
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<tr>
<td>Maximal contraction force (g)</td>
<td>1.48±0.12</td>
<td>1.54±0.16</td>
<td>1.97±0.20</td>
<td>0.114</td>
<td>1.59±0.06</td>
<td>1.44±0.14</td>
<td>0.328</td>
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<tr>
<td>Sodium nitroprusside</td>
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<tr>
<td>pD2 (−log M)</td>
<td>7.08±0.11</td>
<td>6.99±0.12</td>
<td>6.85±0.18</td>
<td>0.531</td>
<td>7.11±0.13</td>
<td>6.89±0.30</td>
<td>0.474</td>
</tr>
<tr>
<td>Maximal relaxation (%)</td>
<td>91.5±0.5</td>
<td>87.1±4.0</td>
<td>85.6±4.5</td>
<td>0.485</td>
<td>88.6±4.4</td>
<td>91.7±5.0</td>
<td>0.653</td>
</tr>
</tbody>
</table>

LS, low-salt (NaCl 0.2%) diet; MS, moderate-salt (NaCl 1.1%) diet; HS, high-salt (NaCl 6.0%) diet; n=5 in each group. PD2 is the concentration of the agonist producing 50% of the maximal response. The maximal relaxation responses are expressed as percent of 1 µM noradrenaline-induced precontraction. ANOVA, p-values from one-way analysis of variance. Means±SEM.

Discussion

The 100% rate of heart muscle and kidney damage in the L-NAME treated rats was quite unexpected, although some recent studies (29, 30) indicate that myocardial ischemia and structural lesions in the kidneys can occur in rats treated with L-NAME. It is possible that the dose of about 20 mg/kg used in this study was too high for the SHR. Normotensive Sprague-Dawley and Wistar rats have been given doses up to 100 mg/kg/d of L-NAME for 4 to 7 wk, resulting in a marked rise in blood pressure but no infarctions or strokes (31, 32). Arnal et al. (31) showed a dose-dependent increase in blood pressure and decrease in aortic cGMP in Wistar rats; at least 10 mg/kg was required for maximal effects. In another study by Arnal et al. (4), 25 mg/kg of L-NAME was given by gavage to SHR, and 55% of them died by 2 wk; the causes of death were not re-

Fig. 6. Body weight gain in spontaneously hypertensive rats during the different diet and drug regimens (means±SEM). Open circles, low-salt diet (NaCl 0.2%); solid circles, low-salt diet with L-NAME; open squares, moderate-salt diet (NaCl 1.1%); solid squares, moderate-salt diet with L-NAME; open triangles, high-salt diet (NaCl 6.0%); solid triangles, high-salt diet with L-NAME; n=10 in each group. Repeated analysis of variance: between-subjects effects, p<0.001; within-subjects effects, p<0.001; time-group interaction, p<0.001. L-NAME-treated groups vs. groups without L-NAME: **p<0.01; high-salt group with L-NAME vs. all other groups: ***p<0.001.
ported. On the other hand, Kobayashi et al. (33) gave about 15 mg/kg of Nω-nitro-L-arginine in food to stroke-prone SHR (SHRSP), and no strokes or infarctions were reported after 1 wk. These reports and the results of the present study suggest that the coronary and renal circulation is more dependent on NO in SHR and SHRSP than in other rat strains.

L-NAME markedly increased blood pressure. This is consistent with previous findings in different rat strains (3-6). The high-salt diet alone did not significantly increase the blood pressure during this short follow-up period. This is in agreement with the results of previous studies in SHR and SHRSP, which show that the hypertensive effect of salt usually takes place only after 2 to 4 wk (10-14, 34). The high-salt diet did not exacerbate the effect of L-NAME on blood pressure. This may be due to the high dose of L-NAME, which rapidly caused severe toxic systemic effects and possibly masked the effects of salt. In previous studies, rats given high-salt diets have shown a greater increase in blood pressure in response to L-NAME than rats on standard-salt diets, both in long-term experiments (23, 35) and in an acute study in anesthetized rats (36).

Plasma renin activity was markedly increased by L-NAME treatment. This is in agreement with the findings of recent studies in SHRSP (37) and in Wistar rats (5). Arnal et al. (4, 31) found increased PRA in L-NAME-treated SHR but not in Wistar or Wistar-Kyoto rats. On the other hand, Gardes et al. (38) and Johnson and Freeman (39) showed that in isolated perfused rat kidney, NO synthesis blockade by L-NAME resulted in vasoconstriction and a decrease in renin release. However, the hyperreninemia may also be a consequence of uncontrolled renin release due to renal arteriolar and tubular damage, as suggested by Nagaoka et al. (40). Volpe et al. (34) have demonstrated that high PRA correlates with increases in mortality, strokes, and renal and cerebrovascular lesions in SHRSP fed a high-salt diet. In the present study, proteinuria and increased serum creatinine in the L-NAME-treated groups indicate renal failure, and thus the high PRA is apparently a consequence of kidney damage. In any case, activation of the renin-angiotensin system is likely to worsen both hypertension and kidney damage, since L-NAME-induced kidney damage can be prevented by angiotensin converting enzyme (ACE) inhibitors (41) and the angiotensin II inhibitor losartan (35). However, myocardial ischemia during L-NAME treatment cannot be prevented by the ACE inhibitor enalapril (42), so other mechanisms must be involved. Increased intake of salt decreased renin release, as expected, which indicates that some control mechanisms were still working in spite of the severe kidney damage.

Considering the lowered food intake and, thus, lowered intake of sodium, L-NAME increased sodium
excretion. This is in agreement with some previous studies in isolated perfused rat kidneys (38) and anesthetized rats (43), but contrasts with other studies in which infusions of L-NAME decreased urine volume and sodium excretion (44). This apparent discrepancy might be explained by “pressure-natriuresis” as suggested by Johnson and Freeman (39) and Shultz and Tolins (22). They found that NOS inhibition resulted in natriuresis only if the renal perfusion pressure was allowed to rise, and when it was kept constant there was a decrease in urinary sodium excretion. In the present study, renal perfusion pressure was not controlled, but if it did rise along with the systemic pressure, such pressure-natriuresis may have occurred.

Previous studies have shown that in experimental hypertension the endothelium-dependent relaxation of vascular smooth muscle is impaired (45, 46). In the present study, the relaxation responses to acetylcholine were reduced in the arteries from the L-NAME-treated rats. The high-salt diet alone also reduced these relaxation responses. This might be explained either by direct NO synthesis inhibition by L-NAME or by structural changes in the endothelium caused by prolonged treatment. An endothelium-dependent mechanism is suggested by the fact that there were no differences between the groups in the relaxation responses to sodium nitroprusside or in any of the responses in the endothelium-denuded vascular rings. Another important finding in the present study was that L-NAME increased the vascular contractile responses to noradrenaline. Deng et al. (32) have recently demonstrated that this enhanced sensitivity to noradrenaline in L-NAME-treated rats is related to structural changes in resistance arteries. This effect, together with impaired endothelial function, could cause permanent vasoconstriction, which would then lead to hypertension, strokes, and infarctions.

Vascular relaxation to acetylcholine was also impaired by the high-salt diet alone but the responses to noradrenaline were not increased. Interestingly, there was no hypertension yet either. This may indicate that endothelial damage is a very early change in salt-induced hypertension, occurring before the rise in blood pressure, and that enhanced sensitivity to noradrenaline is also essential in the development of hypertension. In previous studies, salt stress alone has been able to increase the blood pressure by 50 to 60 mmHg and cause left ventricular hypertrophy, very much like in the present study with L-NAME (10–15), but no strokes or infarctions have been observed. Thus, profound dysfunction of the vascular endothelium induced by L-NAME is probably the most important factor for the effects observed in the present study.

In conclusion, treatment of SHR with L-NAME, a NO synthesis inhibitor, resulted in a rapid rise in blood pressure, impairment of growth, strokes, myocardial infarctions, and renal damage. The detrimental effects of L-NAME were exacerbated by a high dietary salt intake.

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