Hypertension and Impairment of Endothelium-Dependent Relaxation of Arteries from Spontaneously Hypertensive and L-NAME-Treated Wistar Rats

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Abstract

Effects of chronic treatment of normotensive Wistar rats with Nω-nitro-L-arginine methyl ester (L-NAME) on blood pressure and on endothelium-dependent relaxation of the aorta, carotid and iliac arteries were studied. The endothelium-dependent relaxation was compared in arteries from normotensive Wistar Kyoto rats (WKY) and genetically hypertensive rats (stroke-prone spontaneously hypertensive rats, SHRSP). Chronic treatment of normotensive Wistar rats with L-NAME caused an elevation of blood pressure. The elevated blood pressure at 15 weeks of age was significantly higher in these animals than that of untreated Wistar rats, but lower than that of SHRSP. Endothelium-dependent relaxation of the arteries induced by acetylcholine (ACh) was almost abolished by chronic treatment with L-NAME. The remaining small relaxation in arteries from L-NAME-treated rats was completely inhibited by application of L-NAME (10^-4 M). In such preparations, higher concentrations of ACh induced a contraction, which was abolished by removal of the endothelium or by an application of indomethacin (10^-5 M). Endothelium-independent relaxation induced by sodium nitroprusside was similar between preparations from untreated and L-NAME-treated Wistar rats. Endothelium-dependent relaxation was significantly impaired in preparations from SHRSP, when compared with that in those from WKY. However, the impairment was less prominent in preparations from SHRSP than in those from L-NAME-treated rats. These results suggest that the impairment of endothelium-dependent relaxation in the arteries from L-NAME-treated rats is not due to the elevated blood pressure resulting from the chronic treatment, and that impairment of NO synthesis by the endothelium does not play a major role in the initiation of hypertension in SHRSP.

Key words: arteries, L-NAME chronic treatment, stroke-prone spontaneously hypertensive rats, acetylcholine-induced relaxation, blood pressure
Introduction

It has been known that the endothelium regulates the contraction of vascular smooth muscles by releasing various endothelium-derived factors (see Furchgott and Vanhoutte, 1989; Pearson and Vanhoutte, 1993). Although the factors released from the endothelium vary in different types of blood vessels (Nagao et al., 1992; Zygmunt et al., 1995), the most common factor is thought to be endothelium-derived relaxing factor (EDRF, Furchgott and Vanhoutte, 1989; Pearson and Vanhoutte, 1993). This EDRF has been identified as nitric oxide (NO), which is synthesized from L-arginine by NO synthase in endothelial cells (Palmer et al., 1988; Moncada et al., 1988).

The synthesis of NO can be inhibited by L-arginine analogues such as N^G-monomethyl-L-arginine (L-NMMA, Palmer et al., 1988, Aisaka et al., 1989), N^ω-nitro-L-arginine methyl ester (L-NAME, Rees et al., 1990) or N^ω-nitro-L-arginine (L-NNA, Ishii et al., 1990; Mulsch and Busse, 1990). These compounds enhance the vascular contraction due to an inhibition of NO production in the endothelium (Matsuda et al., 1995; Dowell et al., 1996), which leads to an increase in total vascular resistance (Humphries et al., 1991). Thus, these compounds have been reported to elevate the blood pressure of animals when they are infused (Rees et al., 1989; Aisaka et al., 1989).

It has also been reported that chronic application of these compounds induced hypertension of animals (Takahashi et al., 1995; Manning Jr., et al., 1993; Ribeiro et al., 1992; Baylis et al., 1992; Arnal et al., 1992; Vargas et al., 1996). This may indicate that the chronic inhibition of NO production in vivo brings about hypertension of animals. However, we have observed that endothelium-dependent relaxation of blood vessels from spontaneously hypertensive rats (SHR) is impaired and that the impairment is proportionally dependent on the degree of hypertension (Sunano et al., 1989; see Vanhoutte and Boulanger, 1995). In addition, the impaired endothelium-dependent relaxation can be improved by antihypertensive treatment (Sunano et al., 1993; Shimamura et al., 1991), indicating that hypertension itself is, in part, a cause of the impairment, but not that an impairment of endothelial function is a cause of hypertension.

In the present experiment, the effects of chronic treatment with L-NAME on blood pressure and endothelium-dependent relaxation of blood vessels of Wistar rats were studied. In addition, changes in endothelial function by the treatment were compared with those in sustained hypertension in stroke-prone SHR (SHRSP), so that the contribution of the changes in endothelial function to the initiation of hypertension in this genetically hypertensive rats could be compared.

Methods

Animals and measurement of blood pressure

Animals were cared for and treated according to the principles for the care and use of laboratory animals approved by The Japanese Pharmacological Society.

Wistar rats, Wistar Kyoto rats (WKY) and SHRSP were used in the present experiment. These rats were obtained at 5 weeks of age and fed in our animal facility on normal chow (SP,
Funabashi, Japan) and provided with tap water freely under conditions of constant temperature (22°C), humidity (50%) and light-dark cycle (12 h).

Systolic blood pressure of these rats was measured by the tail-cuff method. Prior to the measurement, rats were warmed at 40°C for 10 min. This procedure was required to obtain accurate and constant values of blood pressure.

**Chronic treatment of Wistar rats with L-NAME**

L-NAME was given to rats dissolving in the drinking water in the concentrations between 15 and 20 mg/100 ml. The concentrations of the drug were determined while measuring the blood pressure at systolic phase being approximately 200 mm Hg. The dose of L-NAME, which was calculated from the amount of water intake, was between 20 and 30 mg/kg/day in the present experiment. Studies on the effects of L-NAME at higher concentrations in an attempt to obtain an even higher blood pressure that was closer to that of SHRSP were not successful because of high mortality.

**Preparations and solutions**

At 15 weeks of age, rats were killed by bleeding from the vena cava under anesthesia with carbon dioxide (CO₂). The thoracic aorta, carotid and iliac arteries were excised and immediately immersed in a modified Tyrode’s solution with the following composition (mM): NaCl, 137; KCl, 5.4; CaCl₂, 2.0; MgCl₂, 1.0; NaHCO₃, 11.9; NaH₂PO₄, 0.4; glucose, 5.6; ethylenediaminetetraacetate (EDTA), 0.026, equilibrated with a gas mixture of 95% O₂ and 5% CO₂. K+-Tyrode’s solution was made by replacing all of the NaCl with KCl and high-K⁺ Tyrode’s solution containing 80 mM K⁺ was made by mixing appropriate proportions of Tyrode’s solution and K⁺-Tyrode’s solution. Connective tissue and fat surrounding the excised blood vessels were removed and 1 mm wide rings prepared in modified Tyrode’s solution under a microscope. Care was taken not to damage the endothelium. In 10 preparations from each blood vessel, the endothelium was removed by rubbing the inner surface of the lumen with a soft rubber band.

**Mechanical recordings**

Two tungsten wires (30 µm in diameter) were inserted in the lumen of the preparation, and one of them was tied to a plastic holder. The holder was then mounted in an organ bath (10 ml) filled with modified Tyrode’s solution kept at a constant temperature of 37°C. The other tungsten wire was connected to a force transducer (Shin-koh, Nagano, Japan), so that tension changes could be measured isometrically. The basal tension was fixed to 8 mN, 4 mN and 6 mN, respectively in preparations from the aorta, carotid and iliac arteries. The preparations were subjected to two successive high-K⁺-induced contractions with a 20 min interval to obtain control contractions for the following experiments.

Endothelium-dependent relaxation was observed by the application of acetylcholine (ACh) cumulatively to preparations that had been precontracted with noradrenaline (NA). The concentration used to initiate the precontraction was determined from preliminary concentration-response curves for NA to initiate a contraction that was just submaximal and varied as stated in the figure legends. Relaxation by sodium nitroprusside (SNP) was also
observed by applying SNP cumulatively to preparations precontracted with the concentration of NA used in ACh-induced relaxation studies.

After these experiments, the preparations were relaxed completely by adding verapamil \((10^{-5}\text{ M})\) and papaverine \((10^{-4}\text{ M})\) and this relaxed level used to determine the amplitude of contraction or relaxation.

**Drugs**

Drugs used in the present experiments were: N⁶-nitro-L-arginine methyl ester (L-NAME, Sigma, St. Louis, USA), indomethacin (Sigma), noradrenaline bitartrate (NA, Sigma), acetylcholine hydrochloride (ACh, Wako Chem., Osaka, Japan), sodium nitroprusside (SNP, Wako Chem.), ethylenediaminetetraacetate (EDTA, Dojindo, Kumamoto, Japan), verapamil hydrochloride (Wako Chem.) and papaverine hydrochloride (Wako Chem.).

**Statistics**

The relaxation was expressed as a percentage of the amplitude of the precontraction induced by NA. These values were expressed as the mean ± SE with the number of experiments in parenthesis. The differences between values were analyzed using one-way or two-way analysis of variance (ANOVA) followed by Bonferroni/Dunn’s *post hoc* test. *P* values less than 0.05 were considered significant. EC₅₀ values (concentration of agonist required to induce half the maximum response) were obtained from individual concentration-response curves by fitting the data to the logistic equation. The curve fitting was carried out by use of Prizm 3 software (GraphPad Software Inc., San Diego, CA, USA). Logarithmic values of EC₅₀ \((\text{pD}_2)\) were used for the statistical analysis.

**Results**

**Body weight and systolic blood pressure**

The body weight and systolic blood pressure of untreated and L-NAME-treated Wistar rats, and WKY and SHRSP at 15 weeks of age are shown in Table 1. The body weight of the L-NAME-treated Wistar rats and SHRSP were significantly smaller than that of control rats (untreated Wistar rats and WKY, respectively).

The systolic blood pressure of L-NAME-treated Wistar rats was significantly higher than that of untreated Wistar rats but lower than that of SHRSP. When untreated Wistar and WKY rats were compared, neither body weight nor systolic blood pressures were different.

**Effect of treatment of Wister rats with L-NAME on blood pressure**

Fig. 1 shows the effect of treatment of Wistar rats with L-NAME on blood pressure through the 6 week duration of the experiment. The dose was described in the Methods. The treatment with L-NAME caused immediate elevation of blood pressure that continued to increase to a peak at 13 weeks. At 15 weeks of age, the blood pressure of L-NAME-treated Wistar rats was still significantly higher than that of untreated Wistar and WKY control rats (Table 1). Although the
Response in arteries of L-NAME-treated rats

 effects of chronic treatment of Wistar rats with L-NAME on systolic blood pressure at weekly intervals after the beginning of treatment. Treatment was started at the age of 9 weeks and continued until 15 weeks, just before sacrifice. L-NAME was given orally in doses described in the Methods. Asterisks indicate significant difference from the value of untreated rats (**, \( P < 0.001 \)).

Table 1  Body weight and systolic blood pressure of rats at 15 weeks of age.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Body weight (g)</th>
<th>Systolic blood pressure (mmHg)</th>
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</thead>
<tbody>
<tr>
<td>Wistar rats</td>
<td></td>
<td></td>
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<tr>
<td>untreated rats (n=20)</td>
<td>452.0 ± 21.0</td>
<td>137.4 ± 1.7</td>
</tr>
<tr>
<td>L-NAME-treated rats (n=12)</td>
<td>357.5 ± 15.8**.†</td>
<td>196.8 ± 2.4**.††</td>
</tr>
<tr>
<td>WKY (n=15)</td>
<td>411.7 ± 8.3</td>
<td>137.8 ± 1.0</td>
</tr>
<tr>
<td>SHRSP (n=15)</td>
<td>326.0 ± 8.6**.††</td>
<td>241.7 ± 3.1**.††</td>
</tr>
</tbody>
</table>

The values are the mean ± SEM. *: vs. untreated rats (**: \( P < 0.001 \)), †: vs. WKY (**: \( P < 0.05 \), ††: \( P < 0.001 \)), †: vs. SHRSP (**: \( P < 0.001 \)).

Effects of chronic treatment of Wistar rats with L-NAME on endothelium-dependent relaxation

The application of ACh to the preparations from the aorta and carotid artery of untreated Wistar rats induced concentration-dependent relaxation (Figs. 2a and 2b). In the iliac artery of untreated Wistar rats, the relaxation was peaked at \( 10^{-5} \) M ACh and was reduced by an increasing concentration of ACh to \( 10^{-4} \) M (Fig. 2c). Such a relaxation was not observed in the endothelium-removed preparations or in the presence of L-NAME (\( 10^{-4} \) M, data not shown). It was also observed that once L-NAME was applied, it was extremely difficult to wash out even with at least 5 changes of solution over a period of 2 to 4 h (data not shown).

In the arteries from L-NAME-treated Wistar rats, the ACh-induced relaxation was almost abolished, although a small relaxation was observed at \( 10^{-4} \) and \( 10^{-5} \) M (Fig. 2). This small relaxation was completely inhibited by application of L-NAME (\( 10^{-4} \) M). In these experiments,
Fig. 2. Effects of L-NAME chronic treatment on ACh-induced relaxation of arteries and of an application of L-NAME (10⁻⁴ M) on the relaxation in the preparations from L-NAME-treated rats. a, b and c were results for the aorta, carotid and iliac arteries, respectively. Precontraction of the preparations from the aorta, carotid and iliac arteries was initiated respectively by 5 × 10⁻⁷ M, 5 × 10⁻⁷ M and 2 × 10⁻⁶ M noradrenaline. The relaxation induced by ACh was expressed as a percentage of the height of precontraction (mean ± SE of the values obtained from indicated number of preparations in the parentheses). Untreated rats and L-NAME-treated rats indicate non-treated and L-NAME-treated Wistar rats, respectively. Asterisks and daggers indicate significant differences from the values of “untreated rats, control” and “L-NAME-treated rats, control”, respectively (**: P<0.001, ††: P<0.001).
both carotid and iliac arteries showed a contractile response at higher concentrations of ACh ($10^{-5}$ and $10^{-4}$ M), which was inhibited by removal of the endothelium or by an application of indomethacin ($10^{-5}$ M) (data not shown). The contractile response was more marked in the iliac artery.

**Endothelium-independent relaxation induced by sodium nitroprusside (SNP)**

Fig. 3 shows the relaxation induced by SNP in endothelium-removed aortae of L-NAME-treated and untreated Wistar rats. In the preparations from untreated Wistar rats, SNP-induced relaxation was not affected by application of L-NAME ($10^{-4}$ M) ($pD_2=8.51 \pm 0.08$ (n=10) without L-NAME c.f. $pD_2=8.50 \pm 0.05$ (n=7), $P>0.05$ with L-NAME). The relaxation was also not affected by chronic treatment with L-NAME ($pD_2=8.28 \pm 0.08$ (n=6), $P>0.05$).

**Endothelium-dependent relaxation of preparations from WKY and SHRSP**

Application of ACh to aortae, carotid and iliac arteries of WKY and SHRSP (precontracted with noradrenaline) induced concentration-dependent relaxation (Fig. 4). The relaxation was blocked by removal of the endothelium or by application of L-NAME ($10^{-4}$ M) (data not shown). The concentrations of ACh for the initiation of relaxation and for the maximum relaxation were identical in preparations from both WKY and SHRSP. However, the amplitude of endothelium-dependent relaxation was significantly smaller in preparations from SHRSP, when compared with that from WKY (Fig. 4), but this impairment of the relaxation was less prominent than that in arteries of L-NAME-treated Wistar rats shown in Fig. 2.
Discussion

It is known that the vascular endothelium controls the tone of vascular smooth muscle by releasing several factors (see Furchgott and Vanhoutte, 1989; Pearson and Vanhoutte, 1993). EDRF is one of these factors, which is known to be released spontaneously in response to shear stress and to some intrinsic substances. Therefore, it plays an important role (or roles) in the control of blood pressure.

Since Ignarro et al. (1987; see also Ignarro, 1989) reported that EDRF is NO, a number of reports regarding the action of NO have been published (see Moncada et al., 1991). NO is synthesized from L-arginine by the action of NO synthase (Palmer et al., 1988). It is also known that NO synthesis is inhibited by L-arginine analogues such as L-NMMA, L-NAME and L-NNA, and that these compounds elevate blood pressure, when applied in vivo, as will be described.

![Diagram](image-url)
below. We have also observed that the infusion of L-NNA elevated the blood pressure of WKY and various spontaneously hypertensive rats (SHR, SHRSP and malignant SHRSP (M-SHRSP)), the effect being dependent of their blood pressure (Yamamoto et al., 2001, in press).

In the present study, it has been shown that chronic treatment of normotensive Wistar rats with L-NAME elevated their blood pressure. Similar results have been reported in rats treated with L-NAME (Arnal et al., 1992; Baylis et al., 1992; Dowell et al., 1996; Manning Jr. et al., 1993) and with L-NNA (Ribeiro et al., 1992; Dananberg et al., 1993; Takahashi et al., 1995). Since these drugs are known to inhibit NO synthesis (Palmer and Moncada, 1989; Rees et al., 1990; Ishii et al., 1990), these authors attributed the elevation of blood pressure to the elevated vascular tone due to the inhibition of vascular smooth muscle relaxation which was induced by NO. In fact, endothelium-dependent relaxation of vascular smooth muscle has been reported to be depressed after the chronic treatment with these drugs (Vargas et al., 1996; Bryant et al., 1995; Takase et al., 1996).

The strong inhibition of the endothelium-dependent relaxation of the aorta, carotid and iliac arteries by chronic treatment with L-NAME agrees with results reported by Bryant et al. (1995) and by Takase et al. (1996). The inhibition may be explained by the inhibition of NO synthesis in the endothelium (Takase et al., 1996). The observation that relaxation of the Wistar rat arteries was strongly inhibited by chronic treatment with L-NAME and that the residual relaxation observed in arteries from L-NAME-treated Wistar rats was completely inhibited by the additional application of L-NAME, would indicate that NO is responsible for a major part of the relaxation in these arteries.

The relaxation in response to SNP of rat aorta was not affected by chronic treatment with L-NAME as Küng et al. (1995) and Zhao et al. (1999) have observed. Bryant et al. (1995) have also reported similar results in perfused kidney. This result suggests that the inhibition of the relaxation to ACh by chronic treatment with L-NAME is not due to the impairment of the pathway producing cyclic GMP or its action (Katsuki et al., 1977; Gruetter et al., 1981) but due to inhibition of endothelial NO production. In the mesenteric artery, on the other hand, it has been reported that the response to SNP was rather sensitized by chronic treatment with L-NNA (Dowell et al., 1996) or was impaired by treatment with L-NAME (Jolma et al., 2000). These discrepant results may be due to differences of dose or duration of treatment, or the preparations used in their studies.

The observation that the strong inhibition of the endothelium-dependent relaxation by chronic treatment with L-NAME persisted even after soaking the preparations in physiological salt solution for a long period is in accord with the report that endothelial NO synthase activity cannot recover even when L-NNA was washed out with normal buffer (Mayer et al., 1993). We have also observed in the present study that the effect of acute application of L-NAME did not disappear even after prolonged wash out. Thus, strong inhibition of endothelium-dependent relaxation by chronic application of L-NAME is thought to be due to the accumulation of the drug in the endothelium.

In the aorta, carotid and iliac arteries, relaxing factors other than NO, such as endothelium-derived hyperpolarizing factor (EDHF) and/or prostacyclin (see Furchgott and Vanhoutte, 1989; see also Pearson and Vanhoutte, 1993), are unlikely to be involved in endothelium-
dependent relaxation, since the relaxation could be completely inhibited in the presence of L-NAME. On the contrary, the elevation of tension at higher concentrations of ACh observed in both carotid and iliac arteries may be induced by endothelium-derived contracting factor (EDCF, see Furchgott and Vanhoutte, 1989; see also Pearson and Vanhoutte, 1993), since it was abolished by the removal of the endothelium or by application of indomethacin. The observation that the degree of the elevation of tension varied in the different arteries indicates that the release or action of EDCF may also vary in different kinds of arteries. Involvements of such factors, however, requires further investigation.

As mentioned above, the inhibition of NO synthesis causes the elevation of blood pressure as has been shown in experiments involving the acute administration of NO synthase inhibitors (Takahashi et al., 1995; Manning Jr. et al., 1993; Ribeiro et al., 1992; Baylis et al., 1992; Arnal et al., 1992; Vargas et al., 1996). However, it is uncertain whether the impairment of endothelium-dependent relaxation in preparations from rats chronically treated with L-NAME, is due to specific action of L-NAME or to the continued hypertension. The latter possibility has been reported in spontaneously hypertensive rats including SHRSP (Shimamura et al., 1991; Sunano et al., 1993). The impairment of endothelium-dependent relaxation in these rats was improved by antihypertensive treatment (Shimamura et al., 1991; Sunano et al., 1993), indicating the involvement of maintained hypertension in the impairment. In support, Takase et al. (1996) have reported that both hypertension and impairment of ACh-induced relaxation of mesenteric and renal arteries of L-NAME-treated rats could be prevented by antihypertensive treatment with a Ca-antagonist and an angiotensin-converting enzyme inhibitor. In the present experiment, however, it was shown that the blood pressure of L-NAME-treated Wistar rats (lower than 200 mm Hg) was much lower than that of SHRSP (higher than 240 mm Hg); nevertheless, the impairment of endothelium-dependent relaxation in the arteries from L-NAME-treated Wistar rats was much greater than that in those from SHRSP. These results suggest that the elevated blood pressure that occurs during chronic treatment with L-NAME does not contribute to the impairment of endothelium-dependent relaxation in the arteries from L-NAME-treated Wistar rats.

We have also observed that the release of NO from mesenteric resistance arteries was not altered in preparations from SHRSP when compared with those from WKY (Sunano et al., 2001). In addition, Dowell et al. (1996) have reported that the maximum amplitude of the relaxation to ACh was not altered in the mesenteric artery from rats chronically treated rats with L-NAME, although the sensitivity was slightly reduced. Since blood pressure is controlled by resistance changes in blood vessels of this size, the discrepancy between the elevation of blood pressure and inhibition of NO-induced relaxation may be explained by the difference in the involvement of factors such as EDCF and EDHF. Dissociation of endothelial cell dysfunction and blood pressure has also been reported in SHR (Tesfamariam and Ogeletree, 1995). Thus, the results obtained in the present experiment and the above mentioned reports suggested that the impaired NO synthesis of endothelium is not the main cause of the elevation of blood pressure in SHRSP.

It has been shown in the present experiment that endothelium-dependent relaxation in all preparations from SHRSP was impaired as has been reported (Shimamura et al., 1991; Sunano et
al., 1989 and 1993; see Vanhoutte and Boulanger, 1995). The blood pressure of SHRSP was, however, much higher than that of chronically treated Wistar rats with L-NAME. Nevertheless, the impairment of endothelium-dependent relaxation was less prominent than in preparations from L-NAME-treated Wistar rats. From these results, it can be concluded that the impairment of NO synthesis of endothelium does not play a major role in the initiation of hypertension in SHRSP.

References


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