*Original Article*

# Effects of Angiotensin II Type 1 Receptor Blockade on the Systemic Blood Nitric Oxide Dynamics in $N^{\omega}$-Nitro-L-Arginine Methyl Ester–Treated Rats

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**We previously succeeded in measuring the nitrosylhemoglobin (HbNO) level as an index of blood nitric oxide (NO) by the electron paramagnetic resonance (EPR) HbNO signal subtraction method. In this study, we examined the effects of olmesartan, an angiotensin II type 1 receptor blocker (ARB), on NO dynamics in $N^{\omega}$-nitro-L-arginine methyl ester (L-NAME)–treated rats by the EPR-subtraction method. Oral administration of L-NAME for 2 weeks induced serious hypertension, and the HbNO concentration was reduced to 37.6% of the level in controls. Coadministration of olmesartan improved hypertension and increased the blood HbNO concentration of L-NAME–treated rats. In contrast, coadministration of hydralazine improved hypertension but did not affect the blood HbNO concentration. In conclusion, our findings suggested that chronic administration of olmesartan ameliorated the endothelial dysfunction in L-NAME–treated rats. (*Hypertens Res* 2006; 29: 369–374)**

***Key Words*: nitric oxide, nitrosylhemoglobin, electron paramagnetic resonance, olmesartan**

## Introduction

It has been reported that endothelium-derived vasodilatation is impaired in hypertensive animals and patients compared with normotensive subjects (*1*–*3*). This endothelial dysfunction may be associated with a decreased production of nitric oxide (NO). Angiotensin-converting enzyme inhibitors (ACEIs) and angiotensin II (AII) type 1 receptor blockers (ARBs) have been shown to significantly restore endothelial function and systemic NO production in experimental and clinical studies (*4*–*8*). In these studies, the systemic NO level was inferred from the Griess and cGMP methods for NOx and cGMP measurement. However, plasma levels of NOx and cGMP do not always reflect the systemic NO concentration because of their poor specificities for blood NO. In a previous study, we succeeded in measuring the nitrosylhemoglobin (HbNO) levels of rat blood as an index of blood NO concentration by the electron paramagnetic resonance (EPR) HbNO signal subtraction method (*1*). This method is more specific for NO compared with other methods, such as the Griess and cGMP methods, and does not require any pretreatment of the blood sample. It can thus be considered a simple and relevant method for measurement of the NO concentration in blood.

Using the EPR HbNO signal subtraction method, we previously reported that temocapril, an ACEI, ameliorated the decrease in blood NO levels in L-NAME–treated rats (*1*). In the present study, we examined whether chronic treatment

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with an ARB would ameliorate the endothelial dysfunction and augment the blood HbNO level in L-NAME–treated rats using the same method.

## Methods

### Materials

$N^{\omega}$-Nitro-L-arginine methyl ester (L-NAME) was purchased from Nacalai Tesque (Kyoto, Japan). Olmesartan was provided by Sankyo (Tokyo, Japan). Other reagents were of the highest grade available from Wako Pure Chemical Industries (Tokyo, Japan). Olmesartan (0.5 mg/ml) was suspended in carboxyl methyl cellulose (0.5%) and given to the animals by oral gavage.

### Animals

Male Sprague-Dawley rats (12 weeks old) were obtained from Japan SLC (Shizuoka, Japan) and kept in plastic cages at a controlled temperature (25°C) under controlled lighting conditions (12:12-h light-dark cycle). The animals were fed a commercial diet and had access to tap water ad libitum until the day of the experiments. All animal care and treatments were conducted in accordance with the guidelines of the animal use and care committee of the University of Tokushima.

### Animal Experiments

Rats were divided into four groups (group 1, control group; group 2, L-NAME group; group 3, L-NAME plus olmesartan group; and group 4, L-NAME plus hydralazine group) (Fig. 1). Groups 1 and 2 had seven rats. Groups 3 and 4 had eight rats. The animals received distilled water (group 1) or distilled water containing L-NAME (1 g/l, groups 2–4) from day 0 to day 13. Water consumption was 30–40 ml/rat/day in group 1, and 20–30 ml/rat/day in the L-NAME–treated groups; these values were constant throughout the experiment. On the basis of the drug solution intake, the effective daily dose of L-NAME was estimated to be about 65 mg/kg/day.

In group 3, olmesartan (2 mg/kg/day) was administered twice a day by oral gavage from day 7 to day 15. Group 4 received L-NAME and hydralazine (200 mg/l) in its drinking water. Groups 1, 2 and 4 were given the vehicle instead of olmesartan (Fig. 1). Body weight, systolic blood pressure (SBP), and heart rate (HR) were measured at days 0, 7, 14, and 16. SBP and HR were measured by the tail-cuff method(MK-1000; Muromachi Kikai, Tokyo, Japan). To evaluate the endothelial function in the release of NO, we stopped the L-NAME treatment 2 days before the blood sampling. Rats were anesthetized with pentobarbital sodium (40 mg/kg body weight i.p.) on day 16, and venous blood was obtained from the vena cava and stored in liquid nitrogen until the EPR measurement.

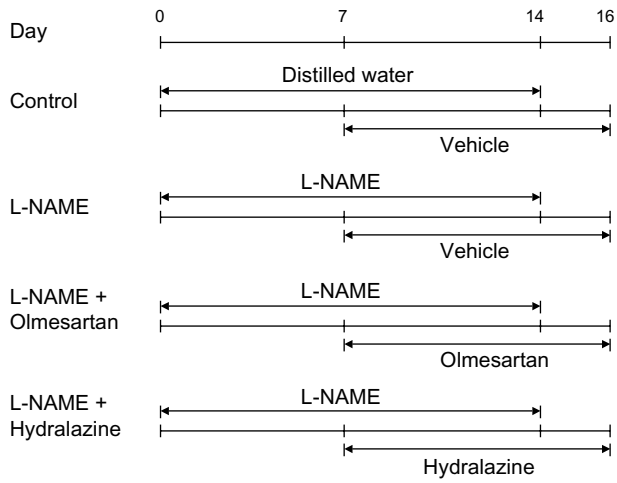


**Fig. 1**. *Experimental design. L-NAME,* $N^{\omega}$*-nitro-L-arginine methyl ester.*

### EPR Measurement and Data Processing

We used a method similar to that described previously to measure and evaluate EPR signals (*1*, *9*, *10*). All EPR measurements were carried out at liquid nitrogen temperature. The frozen sample was directly transferred to a liquid nitrogen–filled quartz finger dewar, which was placed in the cavity of the EPR measurement device. A JES TE 300 ESR spectrometer (JEOL, Tokyo, Japan) with an ES-UCX2 cavity (JEOL) was utilized to collect EPR spectra at the X band (9.5 GHz). Each sample was measured four times and normalized using ESPRIT 432 software (JEOL) to improve the signal-to-noise ratio. Typical EPR conditions were as follows: power, 20 mW; frequency, 9.045 GHz; field, 3,200±250 gauss; modulation width, 6.3 gauss; sweep time, 60 min; time constant, 1 s; and amplitude, 250. Spectra were stored on an IBM personal computer for analysis.

The HbNO signal was obtained by subtracting the EPR spectrum of HbNO-depleted venous blood from that of each sample. EPRMAIN computer software obtained from the National Institute of the Environmental Health Sciences (http://epr.niehs.nih.gov/pest.html) was used to accomplish this calculation.

### Statistical Analysis

All data are expressed as the means±SEM. Data were analyzed by a two-way ANOVA, followed by the Bonferroni test for comparisons between groups. Values of $p<0.05$ were accepted as statistically significant.

**Table 1. Heart Rate and Body Weight in Control and Treated Groups**

| | Day 0 | Day 7 | Day 14 | Day 16 |
|---|---|---|---|---|
| Control group ($n=7$) | | | | |
| Body weight (g) | 373±8 | 405±4 | 423±6 | 423±6 |
| Heart rate (beats/min) | 345±6 | 344±6 | 341±8 | 337±5 |
| L-NAME group ($n=7$) | | | | |
| Body weight (g) | 389±4 | 404±5 | 428±6 | 422±6 |
| Heart rate (beats/min) | 356±7 | 294±3* | 306±6* | 315±7* |
| L-NAME + olmesartan group ($n=8$) | | | | |
| Body weight (g) | 381±5 | 408±5 | 404±7 | 404±7 |
| Heart rate (beats/min) | 333±3 | 316±4 | 328±7 | 338±1 |
| L-NAME + hydralazine group ($n=8$) | | | | |
| Body weight (g) | 377±5 | 399±6 | 400±8 | 406±9 |
| Heart rate (beats/min) | 336±5 | 315±4 | 326±3 | 321±3 |

Values are expressed as means±SEM; $n=7$ in the control group, $n=7$ in L-NAME group, $n=8$ in L-NAME + olmesartan and $n=8$ in L-NAME + hydralazine group. *$p<0.05$ *vs*. Day 0. L-NAME, $N^{\omega}$-nitro-L-arginine methyl ester.

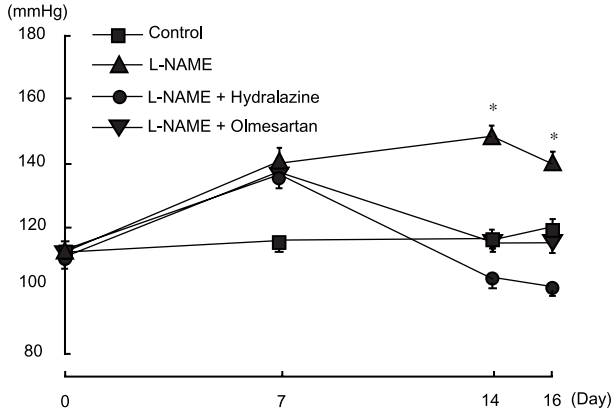


**Fig. 2.** *Changes in systolic arterial pressure (SBP). Rats received distilled water (control;* n=*7), distilled water containing L-NAME alone (L-NAME,* n=*7), L-NAME plus olmesartan (L-NAME + olmesartan,* n=*8), or L-NAME plus hydralazine (L-NAME + hydralazine;* n=*8). Olmesartan was administered twice a day by oral gavage.* *p<*0.05* vs. *the control group. Values are expressed as the means±SEM.*

## Results

### Body Weight and Hemodynamic Variables

Body weight did not differ significantly among the groups before treatment and increased continuously in all groups during the experimental period (Table 1). When the experiment was started, basal SBP was ~110 mmHg, and there were no significant differences among groups (Fig. 2). In the control group, SBP and HR did not change throughout the experiment. Administration of L-NAME induced hypertension and bradycardia; these phenomena were consistent with previous findings (*11*, *12*) (Table 1). Olmesartan and hydralazine treatment significantly lowered SBP in the L-NAME–treated rats ($p<0.05$) (Fig. 2).

### HbNO Concentration

Figure 3 shows the HbNO concentration for each group. In the control group, the HbNO concentration was 60.9±3.7 arbitrary units (A.U.). Treatment with L-NAME alone significantly reduced the blood HbNO concentration ($p<0.05$) (22.9±1.4 A.U.). Olmesartan significantly reversed the L-NAME–induced HbNO reduction ($p<0.05$) (40.1±1.7 A.U.), but hydralazine did not (17.6±2.7 A.U.).

## Discussion

It has been reported that endothelial dysfunction may result in the progression of vascular structural changes, cardiac hypertrophy, and renal failure, which may be associated with a decreased production of NO (*13*). ACEIs and ARBs have been shown to significantly ameliorate the endothelial dysfunction and reduced structural changes of some organs, and these phenomena are expected to be responsible for the improvement in circulating NO levels shown in experimental and clinical studies (*14–20*). However, there is no definite proof of these phenomena because NO is a quite labile molecule whose direct measurement is technically difficult. NO does exist as a relatively stable HbNO adduct in the circulation (*21*), which means that the amount of HbNO may reflect the blood NO concentration. HbNO is detectable both optically (*22*) and magnetically. Optical detection of HbNO in blood is quite difficult because other heme compounds, such as deoxyhemoglobin, oxyhemoglobin and methemoglobin, show absorption spectra similar to that of HbNO (*22*). On the other hand, magnetic EPR spectroscopy has many advantages for measurement of systemic HbNO: 1) the EPR spectrum of HbNO has a distinct three-line structure, and hence endoge-

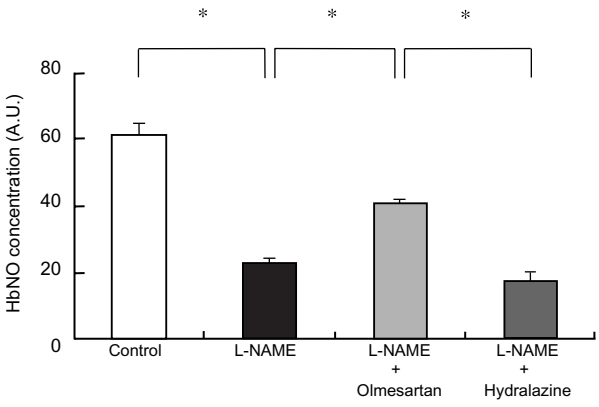


**Fig. 3.** *Effect of chronic L-NAME treatment in combination with olmesartan and hydralazine on HbNO concentration. HbNO concentrations are expressed as the means±SEM. Statistically significant changes in HbNO concentration, *p < 0.05 (Bonferroni test).*

nous Hb acts as a natural spin-trapping agent for NO (*23*); 2) neither oxyhemoglobin nor deoxyhemoglobin produces an EPR signal; and 3) the shapes of the EPR signals of methemoglobin and HbNO are completely different, which enables us to distinguish these spectra. Nonetheless, there were still some difficulties in obtaining a fine HbNO signal because of the existence of paramagnetic compounds that give a strong EPR signal overlapping the same magnetic region as HbNO (*24–29*). Therefore, we developed an EPR HbNO signal subtraction method based on subtraction of the EPR spectrum of NO-depleted whole blood from that of each sample. This method enabled us to estimate the physiological concentration of HbNO in a previous study (*1*). This method also enabled us to detect small changes of circulating NO concentration, because intravenous L-arginine infusion increased the EPR signal of HbNO in a dose-dependent manner without SBP changes (*1*).

In this study, we utilized this method to examine whether the ARB olmesartan would restore endothelial function and systemic NO production in L-NAME–treated rats. The oral administration of L-NAME (1 g/l) for 2 weeks induced a time-dependent hypertension (Fig. 2), and the HbNO concentration was reduced to 37.6% of that in the controls (Fig. 3). The administration of olmesartan significantly increased the HbNO concentration in L-NAME–treated rats and restored their SBP to that of control rats (Figs. 2 and 3). We already reported that chronic treatment with an ACEI also increased the HbNO concentration in L-NAME–treated rats (*1*). In both studies, it was found that the SBP-lowering effect of ACEI and ARB were associated with an increase in circulating NO levels. This improvement in circulating NO level may result from the endothelial protective effects of these agents (*14*). To elucidate the causal relationship between the reduction in SBP and improvement in circulating NO levels, we choose hydralazine as a reference because hydralazine is reported to decrease blood pressure without NO production (*30*). We found that olmesartan and hydralazine were equally effective in reducing systolic arterial pressure, but the administration of hydralazine did not increase the HbNO concentration (17.6±2.7 A.U.) to the extent that olmesartan did (40.1±1.7 A.U.) (Fig. 3). This phenomenon suggested that the SBP-lowering effect was not always associated with an improvement in systemic NO levels in the L-NAME–treated rats.

The mechanism by which L-NAME induces hypertension and cardiovascular diseases has not been fully elucidated. However, the renin-angiotensin system, the sympathetic nervous system, prostaglandins, and superoxide anions have all been reported to contribute to the organ damages induced by chronic L-NAME administration. The renin-angiotensin system is activated in the rat model of chronic administration of L-NAME (*31*). AII-stimulated vascular NAD(P)H oxidase may be responsible for the increases in superoxide production and chronic L-NAME–induced endothelial dysfunction. Thus, in the present study, ARB administration may have prevented the increased vascular superoxide production and attenuated the endothelial dysfunction induced by chronic L-NAME administration. Katoh *et al*. (*11*) and Takemoto *et al*. (*32*) showed that chronic NOS inhibition facilitates the upregulation of cardiac AII receptors and causes subsequent cardiovascular inflammatory changes, and that treatment with an ARB, but not hydralazine, prevented increases in the inflammatory changes. These data suggest that not only inhibition of arterial hypertension but also prevention of cardiovascular inflammatory changes is crucial for the promotion of NO synthesis by ARBs.

Several reports have indicated that ARBs, *via* stimulation of AII type 2 receptors, may activate the bradykinin-NO cascade (*33–37*). De Gennaro Colonna *et al*. reported that losartan was effective at reversing L-NAME–induced endothelial vasodilator dysfunction and enhancing endothelial NO production, as shown by the ability of losartan to increase the eNOS mRNA in the aortic tissue and the circulating level of nitrite/nitrate (*34*). In addition, they indicated that the enhanced endothelial generation of prostacycline induced by losartan in L-NAME–treated rats was also mediated by bradykinin $B_2$–receptor activation. The mechanisms by which the renin-angiotensin system affected the dynamics of NO after the administration of L-NAME were not explored in our study. Further studies will be needed to elucidate the pathophysiological factors responsible for the improvement in systemic NO levels in our experimental model.

In conclusion, this is the first study to demonstrate directly that olmesartan reversed the decrease of blood NO in L-NAME–treated rats by using the EPR HbNO signal subtraction method. Our present findings suggest that olmesartan may have a blood pressure–independent effect of improving endothelial function and systemic NO production in L-NAME–treated rats.

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