Alterations of Nitric Oxide Synthase Expression With Aging and Hypertension in Rats

Tz-Chong Chou, Mao-Hsiung Yen, Chi-Yuan Li, Yu-An Ding

Abstract—The activity and protein expression of endothelial nitric oxide synthase (eNOS) and inducible NOS (iNOS) were investigated during the development of hypertension in spontaneously hypertensive rats (SHR). SHR and Wistar-Kyoto rats (WKY) were studied at three different ages: 4, 14 to 17, and 63 weeks of age. After treatment with saline or lipopolysaccharide (LPS, 10 mg/kg IV) for 3 hours, the aortas were removed for measurement of NOS activity and protein expression assay by [3H]-l-citrulline formation method and Western blot analysis, respectively. Plasma levels of nitrite/nitrate (NO$_2^-$/NO$_3^-$) and tumor necrosis factor-α (TNF-α) were also determined. At 14 to 17 weeks and 63 weeks, the basal activity and protein expression of eNOS in the aortas were significantly lower in SHR than in WKY. In addition, the aged WKY exhibited lower eNOS activity than that of adult WKY, but this change was not seen in SHR. By comparison, the basal activity and protein expression of iNOS were only observed in SHR of the 14-to-17-week group and in the 63-week group; SHR still exhibited higher activities, and these differences were further exaggerated by treatment with LPS. The basal and LPS-induced NO$_2^-$/NO$_3^-$ and TNF-α levels in the plasma were also higher in the SHR except the 4-week group. After treatment with quinapril, the basal and LPS-induced expressions of iNOS in SHR were significantly attenuated. Our results demonstrated that alterations of activity and protein expression of eNOS and iNOS occurred in SHR. In addition, aging may reduce the activity of eNOS in WKY but not in SHR. The decline of eNOS activity and/or expression may contribute to the development of hypertension, whereas the increase of iNOS expression may be a consequence of the pathological state of vessels associated with hypertension in SHR. However, the augmented expression of iNOS in SHR was attenuated by antihypertensive therapy, suggesting that the abnormal expression of iNOS is associated with hypertension. (Hypertension. 1998;31:643-648.)

Key Words: nitric oxide synthase • hypertension, experimental • rats, inbred SHR

The generation of NO from l-arginine by NOS is an important autocrine and paracrine signaling pathway in the regulation of various cell functions and in cell-cell communication.1,2 NO, also known as endothelium-derived relaxation factor, is produced endogenously in various types of cells.2 Molecular and enzymatic studies have indicated that there are at least two major isoforms of NOS: eNOS and iNOS, found in macrophages, VSMC, and other tissues,3 and the expression can be induced by bacterial endotoxin (LPS) and cytokines such as interleukin-1β, TNF-α,4 or by mechanical, viral, or bacterial injuries.5 NO produced from the iNOS acts as a cytotoxic agent against tumor cells, bacteria, fungi, and protozoa and plays an important role in pathological vasodilation and host tissue damage in endotoxic shock or altered immunologic status.5

Considerable evidence demonstrates that NO plays a critical role in the maintenance of blood pressure homeostasis.7,8 In addition, NO also prevents the activation and adhesion of platelets and neutrophils to the endothelium. NO induces vascular smooth muscle relaxation by activation of soluble guanylate cyclase, which leads to an increase of cGMP, causing a decrease in intracellular Ca$^{2+}$ with subsequent vasodilation.9 Therefore, alterations in NO synthesis may be related to the pathogenesis of hypertension. This hypothesis was supported by the finding that the vasodilatory response to ACh was impaired in SHR when compared with WKY.10–12 Presumably due to a decrease in synthesis and/or release of endothelium-derived relaxation factor. However, the effects of aging and hypertension on the activity and protein expression of NOS have not been investigated. Here we report direct measurement of the changes of NOS activity and protein expression of aortas at different age groups of the SHR and its control group, WKY. To further clarify the relationship between hypertension and the changes of NOS expression in SHR, we treated SHR with an antihypertensive agent, the ACEI quinapril, evaluating its effect on NOS expression.

Methods

In this study 81 male SHR and 60 male WKY were divided into three age groups: 4, 14 to 17, and 63 weeks old. Systolic blood pressure was measured in warmed, restrained, unanesthetized animals using the caudal artery tail-cuff MOD 59 plethysmograph (blood pressure meter/amplifier, Itic Inc). Animals were anesthetized with urethane.
Changes of NO Synthase Expression in SHR

(0.6 g/kg IP), and the carotid artery was cannulated for blood collection. A jugular vein was also cannulated for intravenous bolus administration of vehicle (saline) or LPS (10 mg/kg). After treatment for 3 hours, the aorta was removed and stored at −70°C for the determination of NOS activity and protein expression.

NOS Activity Assay
Aortas were homogenized on ice with a poltron (model PT MR 3000, Littau) according to the method of Klemm et al.13 NO activity was determined by measuring the conversion of [3H]-L-arginine to [3H]-L-citrulline. Tissue homogenates (approximately 60 μg protein) were incubated in 20 mmol/L HEPES buffer (pH 7.5) containing 10 μmol/L L-arginine and [3H]-L-arginine (3 μCi/mL), i-v-aline (60 mmol/L), NAPDH (1 mmol/L), calmodulin (30 nmol/L), tetrahydrobiopterin (5 mmol/L), and calcium (2 mmol/L) for 20 minutes at 37°C. Reaction was stopped by adding 1 mL of ice-cold HEPES buffer (pH 5.5) containing EDTA (2 mol/L) and EDTA (2 mol/L) and then applying to Dowex 50W (Na+ form) columns, and the amount of [3H]-L-citrulline eluted was quantified by liquid scintillation counter (Beckman, model LS3801). The activity of the Ca2+/calmodulin-dependent NO activity was determined from the difference between the [3H]-L-citrulline produced from samples containing 2 mmol/L calcium and samples without calcium and with EGTA (2 mmol/L); the activity of iNOS was determined from the difference between samples containing 2 mmol/L EGTA and samples without NADPH.13 Protein determination was made using dye-binding assay (Bio-Rad) with bovine serum albumin as a standard.

Western Blotting
Aorta lysate containing 10 μg protein was denatured and an equal amount of protein loaded on 7.5% SDS-polyacrylamide gel and then was transferred to nitrocellulose membranes using PharmSystem (Pharmacia Biotech). The membrane was blocked with 1% bovine serum albumin in Tris-buffer solution (TBS; pH 8.0) containing 0.1% Tween-20 for 2 hours at room temperature. Next, the membrane was incubated overnight at 4°C with mouse monoclonal anti-iNOS or anti-eNOS antibody (1:2000 dilution, Transduction Laboratories) in TBS containing 0.1% Tween-20. The membrane was washed and finally incubated with a 1:1000 dilution of anti-mouse IgG conjugated to horseradish peroxidase for 1 hour at room temperature. After successive washes with TBS, the immunocomplexes were detected using an enhanced horseradish peroxidase/luminol chemiluminescence reaction (Amersham International plc) and exposed to x-ray film for 3 to 5 minutes. The density of respective bands was quantified by densitometric scanning of the Western blots using Image-Pro Plus software. We took the density of the band representing 1 μg eNOS or iNOS positive control purified from human endothelial cells and mouse macrophages lysate (Transduction Laboratories), respectively, as 100% to calculate the relative density of other bands on the same gel.

Measurement of Plasma Nitrite/Nitrate Concentration
Plasma (100 μL) was mixed with 160 μL of 75 mmol/L ZnSO4 and 1.4 μL of 80 mmol/L NaOH for 10 minutes and centrifuged at 6000g for 10 minutes to deproteinize the plasma. The supernatant (100 μL)

| TABLE 1. Characteristics of Study Rats at 4, 14 to 17 and 63 Weeks of Age |
|-----------------|-----|-----|-----|
| Rat Strain      | n   | SBP, mm Hg | Body Weight, g |
| WKY, wk         |     |       |       |
| 4              | 25  | 117±5 | 105±4 |
| 14-17          | 24  | 122±4 | 256±6 |
| 63             | 11  | 135±5 | 302±5 |
| SHR, wk        |     |       |       |
| 4              | 28  | 124±3 | 97±4  |
| 14-17          | 37  | 198±4*| 246±3 |
| 63             | 16  | 196±5*| 298±5 |

SBP indicates systolic blood pressure. Values are mean±SEM.

Results
was removed and incubated with 25 μL substrate buffer (NADPH, 0.6 mmol/L; flavine adenine dinucleotide, 5 μmol/L; nitrate reductase, and 2 U/mL pH 7.6 at 37°C for 1 hour to convert NO2− to NO3−. The total nitrite (NO2−/NO3−) assay was determined by the method of Misko et al.14 The intensity of fluorescence was measured using a luminescence spectrometer (model LS-5, Perkin-Elmer) with excitation at 365 nm and emission at 450 nm. Nitrite levels in samples were calculated using a standard curve of nitrite to which NADPH had been added.

Measurement of Plasma Levels of TNF-α
Rats were anesthetized and instrumented as described above. Blood samples (0.5 mL) were taken at 0 and 90 minutes after the injection of LPS. The amount of TNF-α in the plasma was measured using a rat TNF-α ELISA kit (Genzyme Corporation).

In Vivo Study of Quinapril
SHR (14 to 17 weeks) were divided into two groups: one was treated with quinapril (10 mg/kg/day, PO) for 2 weeks, the other was treated with saline to serve as control. Each group was further divided into two groups, treated with or without LPS challenge. After the administration of LPS (10 mg/kg, IV) for 3 hours, the activity and protein expression of iNOS in aorta homogenates and plasma concentrations of NO2−/NO3− and TNF-α (at 90 minutes) were measured according to above methods.

Statistical Analysis
All data were analyzed by one-way ANOVA, and statistical significance was assessed by Student’s unpaired two-tailed t test. Differences were considered statistically significant when the value was P<.05.

Blood Pressure and Body Weight
The systolic blood pressure (mm Hg) of SHR was significantly higher than that of age-matched WKY except at 4 weeks of age (prehypertensive state). The body weight was similar between SHR and WKY in each age-matched group (Table 1).

Activity of eNOS and iNOS in the Aorta
In the prehypertensive state, the basal activity of eNOS (Ca2+/calmodulin-dependent) in the aorta was similar in SHR and WKY. However, in the 14-to-17- and 63-week groups, the activity of eNOS in SHR was greatly attenuated when compared with age-matched WKY. In addition, in WKY, the eNOS activity of the 63-week group was significantly lower than that in the 14-to-17-week group. In comparison, at young age (4 weeks), iNOS activity (Ca2+/calmodulin-independent) of aorta was undetectable in both SHR and WKY. It is important to note that in the

Selected Abbreviations and Acronyms
ACEI = angiotensin-converting enzyme inhibitor
ACh = acetylcholine
eNOS = Ca2+/calmodulin-dependent NOS expressed in endothelial cells
iNOS = inducible Ca2+/calmodulin-independent NOS
LPS = lipopolysaccharide
NOS = nitric oxide synthase
SBP = systolic blood pressure
SHR = spontaneously hypertensive rats
TNF-α = tumor necrosis factor-α
VSMC = vascular smooth muscle cells
WKY = Wistar-Kyoto rats
14- to 17-week group, the basal activity of iNOS was only observed in SHR. However, at elder age (63 weeks), the activity of iNOS also appeared in WKY, but it was much lower than that in SHR. On stimulation with LPS, the activity of iNOS of both strains was further enhanced, and the activity of iNOS in SHR remained higher than that in WKY except for the 4-week group (Table 2).

**Western Analysis of eNOS and iNOS in the Aorta**

The basal protein expression of eNOS in the aorta was similar in the 4-week group of both strains. However, it was significantly lower in SHR than in WKY at 14 to 17 and 63 weeks of age (Fig 1). Although in WKY the eNOS protein expression in the 63-week group was slightly lower than that in the 14-to-17-week group, the difference did not reach statistical significance. Interestingly, the basal protein expression of iNOS was only observed in SHR of the 14-to-17-week group; it was not detectable in the prehypertensive state in SHR and WKY. At 63 weeks, the protein expression of iNOS in WKY increased slightly but remained significantly lower than that in SHR. After LPS stimulation, the protein expression of iNOS was further elevated and remained significantly higher in SHR than in WKY in the 14-to-17- and 63-week groups (Fig 2). In addition, these observations were paralleled with the findings of NOS activity studies.

**Plasma NO2⁻/NO3⁻ and TNF-α Levels**

The basal plasma NO2⁻/NO3⁻ level was significantly greater in SHR than in WKY in the 14-to-17- and 63-week groups. After administration with LPS for 3 hours, the plasma NO2⁻/NO3⁻ level was markedly increased in both strains; however, the elevation of NO2⁻/NO3⁻ was higher in SHR than in WKY in the 14-to-17- and 63-week groups (Table 3). Similarly, the basal plasma TNF-α level was higher in SHR than in WKY in the 14-to-17- and 63-week groups (Fig 3A). The injection of LPS caused a bell-shape change in plasma TNF-α level that reached a peak value at 90 minutes; thereafter, it returned to the pretreatment level at 240 minutes. Therefore, the 90-minute time point was chosen for our study. The increase of TNF-α induced by LPS was also much greater in SHR than in WKY at 14-to-17- and 63-week groups (Fig 3B).

**Effects of Quinapril on Activity and Expression of iNOS**

After treatment with quinapril for 2 weeks, the systolic blood pressure of SHR was significantly reduced from 195±2 to 161±3 mm Hg (P<.01, n=7). Concurrently, the basal and LPS-induced activity and protein expression of iNOS were also significantly attenuated by quinapril treatment (Fig 4) and accompanied by a significant decrease in basal plasma NO2⁻/NO3⁻ (4.83±0.18 to 3.75±0.12 μmol/L, P<.05, n=7) and TNF-α (253±21 to 190±17 pg/mL, P<.05, n=7) levels when compared with the values of untreated group.

**Discussion**

In the development of hypertension, the SHR exhibits a decrease in the activity and protein expression of eNOS in the aorta with an elevation of activity and protein expression of iNOS.
iNOS, accompanied by greater plasma NO$_2^-$/NO$_3^-$ and TNF-α levels when compared with those from age-matched WKY. In addition, aged WKY exhibited a significantly lower eNOS activity than that in adult WKY. The augmented expression of iNOS in SHR was attenuated when blood pressure was reduced with quinapril.

In the 14-to-17- and 63-week groups, the basal eNOS activity of SHR was significantly lower than that of WKY, which was paralleled with a concurrent fall in eNOS protein expression. The changes of eNOS were associated with an elevation of blood pressure. There is evidence that there are no differences in eNOS activity and protein expression between SHR and WKY in the prehypertensive state. Many studies have reported that hypertension is associated with decreased endothelium-dependent relaxations.12,15 This has been demonstrated not only in large conduit arteries but also in resistant arteries obtained from hypertensive rats.16,17 The cause of the reduction of eNOS activity and expression in SHR is still unclear. It has been proposed that the damage of endothelium structure and/or functions may be an important factor, and this defect is closely related to hypertension. This hypothesis is supported by these findings, indicating that endothelial function is still normal in SHR at 4 weeks old but abnormal at 14 weeks of age when hypertension is established.18,19 Further more, because antihypertensive therapy with calcium channel antagonists or ACEI can improve the endothelium-dependent relaxation to ACh,20 it is likely that the endothelial dysfunction is a consequence of high blood pressure. However, the possibility that the endothelial dysfunction may also be a cause of hypertension cannot be ruled out.

Apart from hypertension, aging may be another important factor causing abnormal expression of eNOS. Evidence was

### Table 3. Plasma NO$_2^-$/NO$_3^-$ Levels in WKY and SHR Under Basal or LPS-Treated Condition

<table>
<thead>
<tr>
<th>Age Group</th>
<th>LPS Treatment</th>
<th>NO$_2^-$/NO$_3^-$, μmol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>WKY</td>
</tr>
<tr>
<td>4</td>
<td>–</td>
<td>3.56±0.23</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>6.63±0.28</td>
</tr>
<tr>
<td>14-17</td>
<td>–</td>
<td>3.62±0.37</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>7.85±0.75</td>
</tr>
<tr>
<td>63</td>
<td>–</td>
<td>3.85±0.29</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>8.56±0.90</td>
</tr>
</tbody>
</table>

Plasma NO$_2^-$/NO$_3^-$ level was assayed at 3 hours after normal saline or LPS (10 mg/kg IV). Results are expressed as mean±SEM for 7 to 8 rats.

*P<.05, †P<.01 compared with the age-matched WKY.
that our results demonstrated that aged WKY possessed lower eNOS activity than that of adult WKY, but no significant difference was found in the same age-matched SHR. These findings were consistent with the results of König and Lüscher, showing that senescent WKY (72-week-old) exhibited a reduced endothelium-dependent relaxation to ACh in aortas compared with that of 12-week-old WKY, but this change was not observed in aged SHR. The reasons are unclear. However, it is possible that the structure and/or function of the endothelium in the adult SHR has been impaired and that it is not being further damaged at a more aged state.

Our previous study showed that in endothelium-denuded preparations, L-arginine has no effect on rings from 16-week-old WKY rats but causes a slight relaxation in those from SHR. After treatment of rats with LPS, the L-arginine–induced relaxation was observed in both strains, with SHR showing a greater response. In addition, the L-arginine–induced relaxation was inhibited by aminoguanidine, a relatively selective inhibitor of iNOS, in both strains after LPS treatment or in SHR only before LPS treatment. It is well known that if iNOS is induced, the NO production from VSMC is mainly dependent on the entry of extracellular L-arginine. Therefore, it is reasonable to expect that L-arginine would evoke relaxations in both strains treated with LPS. These findings suggest that iNOS is present in the SHR, which is further enhanced by the stimulation with LPS, as evidenced by the results of activity and Western blot analysis of iNOS in SHR under basal conditions and after LPS challenge. In addition, Lee and Webb demonstrated that the addition of L-arginine caused a greater relative increase of L-NMMA–induced inhibition of ACh-induced relaxation in stroke-prone SHR, suggesting that this greater relaxation is mediated by iNOS, consistent with our findings. Furthermore, the VSMC of aorta and macrophages from SHR can produce greater amounts of NO stimulated by LPS than that from WKY, but VSMC of mesenteric artery induced by interleukin-1β and endothelial cells of aorta released less NO compared with those from WKY, showing the heterogeneity of vascular beds. Another finding was that the basal activity and expression of iNOS appeared in aged WKY, which may result from aging or vascular injury. The causes for the increased activity and expression of iNOS in SHR are still unknown.

Although the pathophysiological significance of iNOS in regulation of blood pressure is unclear, we propose that the appearance of iNOS in SHR may reflect the pathological state of vessels, presumably a consequence of injury due to hypertension. This is strongly supported by the finding that the exaggerated iNOS expression can be attenuated after reduction of blood pressure with chronic treatment of quinapril (Fig 4). In addition, antihypertensive therapy with ACEI or a calcium channel antagonist can also improve the endothelial-dependent relaxation to ACh. These results suggest that hypertension is an important factor in the modulation of eNOS and/or iNOS expression in vascular beds. The mechanisms by which quinapril reduces iNOS expression are still unclear. Recently, some studies have demonstrated that angiotensin II stimulates superoxide formation by activating NADPH and NADH oxidase both in cultured VSMC and in vivo studies. Reactive oxygen species have been implicated in the pathogenesis of hypertension and tissue damage. Enhanced oxygen species has been reported in the microvascular wall and endothelial cells of SHR. Furthermore, oxidative stress can induce transcription factor nuclear factor-κB–responsive genes, such as iNOS. Therefore, it is possible that the elevated iNOS observed in SHR may have been induced by the increased oxidative stress. These observations suggest that the mechanisms by which ACEI attenuates the iNOS expression and corrects the polyploidy of VSMC in SHR may also be associated with the reduction of angiotensin II–induced superoxide production. As we have mentioned above, in SHR with lower eNOS and higher iNOS, the sum of NO production may be unchanged or even enhanced. Therefore, the higher basal and LPS-induced NO2/NO3 levels in SHR were most likely mediated by iNOS rather than by eNOS. Despite the report by Sawada et al that release of NO in response to ACh is unaltered in SHR, the possibility that NOS in blood vessels is changed in SHR cannot be ruled out.

It is well known that cytokines such as TNF-α and interleukin-1β secreted by macrophages or VSMC are potent stimulators for iNOS. Our results showed that the plasma TNF-α level was higher in SHR both under basal conditions or LPS treatment, suggesting that an altered immune response may exist in SHR. In addition, according to our previous hemodynamic results, the overproduction of TNF-α may
play an important role in the more severe hypertensive effect induced by LPS and in activation of iNOS in SHR.

In conclusion, an early decline (from 4 to 14 weeks) in eNOS activity and protein expression was found in SHR but not in WKY. In addition, in the aging process (from 14 to 63 weeks), the decreased eNOS activity was observed in WKY, but no further decrease was observed in SHR. In contrast, the changes of iNOS activity and protein expression were opposite to those of eNOS in WKY and SHR. At 14 and 63 weeks, suggesting that the alteration of eNOS and iNOS is associated with aging and the development of hypertension. From the development of the hypertensive state (14 weeks), the iNOS in the aorta is present in SHR, and is further enhanced by stimulation with LPS. The higher basal levels of NO$_2^-$/NO$_3^-$ may result from higher expression of iNOS in SHR. We proposed that the pathological condition of vessels in hypertension may be responsible for an increase of iNOS expression. In addition, the abnormal expression of iNOS was attenuated by reduction of blood pressure with quinapril, which further indicates that hypertension may be an important factor in the regulation of iNOS expression. However, the “cause and effect” relationship between hypertension and iNOS expression needs to be further clarified.

Acknowledgments

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