

# Upregulation of Renal and Vascular Nitric Oxide Synthase in Young Spontaneously Hypertensive Rats

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**Abstract**—The available data on the role of the L-arginine/nitric oxide (NO) pathway in the genesis of hypertension in spontaneously hypertensive rats (SHR) are limited and contradictory. In an attempt to address this issue, male SHR were studied during the early phase of evolution of hypertension (age 8 to 12 weeks) to distinguish the primary changes of NO metabolism from those caused by advanced hypertension, vasculopathy, and aging late in the course of the disease. A group of age-matched male Wistar-Kyoto rats (WKY) served as controls. The SHR exhibited a marked rise in arterial blood pressure and a significant increase in urinary excretion and plasma concentration of NO metabolites (nitrite/nitrate [NOx]). Likewise, the SHR showed a significant elevation of thoracic aorta NO synthase (NOS) activity coupled with significant increases of kidney, aorta, inducible NOS (iNOS), and endothelial NOS (eNOS) proteins. In an attempt to determine whether the enhanced L-arginine/NO pathway is a consequence of hypertension, studies were repeated using 3-week-old animals before the onset of hypertension. The study revealed significant increases in urinary NOx excretion as well as vascular eNOS and renal iNOS proteins. In conclusion, the L-arginine/NO pathway is upregulated in young SHR both before and after the onset of hypertension. Thus, development of hypertension is not due to a primary impairment of NO production in SHR. On the contrary, NO production is increased in young SHR both before and after the onset of hypertension. (*Hypertension*. 1998;31:1248-1254.)

**Key Words:** nitric oxide ■ nitric oxide synthase ■ endothelium-derived relaxing factor ■ kidney

Spontaneously hypertensive rats originated from the mating of a normotensive pair of WKY. These animals exhibit severe progressive hypertension that begins at ≈5 weeks of age and leads to severe vasculopathy. The course of genetic hypertension in SHR bears a resemblance to that of essential hypertension in humans. Thus, SHR have been widely used as a model to study the mechanism, pathophysiology, and management of idiopathic hypertension. These investigations have revealed several abnormalities of vaso-regulatory factors, including the renin-angiotensin system, catecholamines, vasopressin, and vasoactive intestinal peptide in SHR.<sup>1-5</sup>

Endothelium-derived NO plays a major role in regulation of vascular tone, hence vascular resistance and arterial blood pressure. NO is produced from the conversion of L-arginine to L-citrulline by a family of enzymes known as NOS. The available data on the L-arginine/NO pathway in SHR are limited and apparently contradictory. Both decreased<sup>6-10</sup> and increased<sup>11-15</sup> L-arginine/NO pathway activities have been reported by different investigators. The present study was designed to explore NO production as well as renal and vascular NOS expression in young SHR before and after the onset of hypertension. The study revealed strong evidence for upregulation of NO production together with increased iNOS and eNOS protein expressions in prehypertensive and hyper-

tensive SHR. These findings exclude a depressed L-arginine/NO pathway as the primary cause of hypertension in SHR. On the contrary, the study points to the activation of this pathway in these animals.

## Methods

### Animals

Eight-week-old male SHR and WKY were purchased from Harlan Sprague-Dawley, Inc (Indianapolis, Ind). The animals were fed a low-nitrate basic diet (Purina Mills) and water ad libitum. They were housed in a climate-controlled, light-regulated space with 12-hour light (>500 lux) and dark (<5 lux) cycles. Eight animals were included in each group. Tail arterial blood pressure was determined using a tail sphygmomanometer (Harvard Apparatus) at baseline (8 weeks of age) and at weeks 10 and 12. At the conclusion of the study, animals were placed in metabolic cages for 24-hour urine collection. The urine samples were collected in sterilized containers that were chilled over ice and stored at -70°C until assayed. The animals were then killed by exsanguination using cardiac puncture between the hours of 9 AM and 11 AM, and blood, kidney, and thoracic aortas were harvested immediately. The tissues were snap-frozen in liquid nitrogen immediately and stored at -70°C until processing.

### Prehypertensive Group

In an attempt to discern the effect of hypertension per se on NO metabolism, we studied a group of 3-week-old SHR during the prehypertensive phase and compared the results with those obtained in their WKY counterparts. These animals were purchased from

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**Selected Abbreviations and Acronyms**

EDRF = endothelium-derived relaxing factor  
 eNOS = endothelial nitric oxide synthase  
 iNOS = inducible nitric oxide synthase  
 NOS = nitric oxide synthase  
 NOx = nitrite/nitrate  
 SHR = spontaneously hypertensive rat(s)  
 WKY = Wistar-Kyoto rat(s)

Harlan Sprague-Dawley, Inc. They were placed in metabolic cages for timed urine collection after which they were killed by exsanguination, and blood and tissues were harvested and processed as described above.

**Tissue Preparation**

Thoracic aorta and kidney were used for determination of NOS. Rats were killed by cardiac puncture, and thoracic aorta and kidney were immediately excised, cleaned with PBS, frozen in liquid nitrogen, and stored at  $-70^{\circ}\text{C}$ . Homogenates (25% wt/vol) were prepared in 10 mmol/L HEPES buffer, pH 7.4, containing 320 mmol/L sucrose, 1 mmol/L EDTA, 1 mmol/L DTT, 10  $\mu\text{g/mL}$  leupeptin, and 2  $\mu\text{g/mL}$  aprotinin at  $0^{\circ}\text{C}$  to  $4^{\circ}\text{C}$  with the aid of a tissue grinder fitted with a motor-driven ground glass pestle. Homogenates were centrifuged at 12 000g for 5 minutes at  $4^{\circ}\text{C}$  to remove tissue debris without precipitation of plasma membrane fragments.<sup>10,11</sup> The supernatant was used for determination of NOS activity and protein mass. Protein concentration was determined with a Bio-Rad kit.

**NOS Activity Assay**

NOS activity was measured as previously described.<sup>16</sup> In brief, enzyme reactions were conducted at  $37^{\circ}\text{C}$  for 30 minutes in 40  $\mu\text{L}$  of the supernatant and 100  $\mu\text{L}$  of 40 mmol/L potassium phosphate buffer, pH 7, containing 4.8 mmol/L DL-valine, 1 mmol/L NADPH, 1 mmol/L  $\text{MgCl}_2$ , 2 mmol/L  $\text{CaCl}_2$ , 20  $\mu\text{mol/L}$  L-arginine, 1  $\mu\text{g/mL}$  calmodulin, and 1.25  $\mu\text{L/mL}$  L-[ $^3\text{H}$ ]arginine (59 Ci per mmol/L, Amersham Life Science Inc). On each occasion, parallel measurements were obtained in the presence and absence of 1 mmol/L  $N^G$ -methyl-L-arginine. The reactions were terminated by 0.86 mL ice-cold stop buffer containing 0.2 mmol/L EDTA. Dowex 50W-X8 resin (250 mg,  $\text{Na}^+$  form) was added to a 0.25-mL aliquot of the reaction mixture and shaken for at least 5 minutes to remove the remaining L-arginine. The  $\text{Na}^+$  form of Dowex 50W was prepared by washing the  $\text{H}^+$  form of the resin (100 to 200 mesh, Bio-Rad) with 1 mol/L NaOH four times and then washing with  $\text{H}_2\text{O}$  until the pH fell below 7.5. The above mixture was then centrifuged, and a 100- $\mu\text{L}$  aliquot of the supernatant containing L-citrulline was mixed with 10 mL of scintillation cocktail in a 20-mL scintillation vial and counted by a Beckman LS-9000 counter. Net radioactivity was

determined by substrating the counts per minute observed in the presence of  $N^G$ -methyl-L-arginine from that observed in the absence of  $N^G$ -methyl-L-arginine. NOS activity was determined from the production of [ $^3\text{H}$ ]citrulline per minute per milligram of protein.

**Western Blot Analysis**

These measurements were carried out to determine the eNOS and iNOS protein mass as previously described.<sup>17,18</sup> Anti-eNOS monoclonal antibody, peroxidase-conjugated goat anti-mouse IgG antibody, anti-Mac NOS-I, human endothelial-positive control, and mouse macrophage-positive control were supplied by Transduction Laboratories. Briefly, aorta and kidney tissue preparations (50  $\mu\text{g}$  of protein for the aorta and 100  $\mu\text{g}$  for the kidney) were size-fractionated on 4% to 12% Tris-Glycine gel (Novex) at 120 V for 3 hours. In preliminary experiments, we found that the given protein concentrations were within the linear range of detection for our Western blot technique. After electrophoresis, proteins were transferred onto Hybond-ECL membrane (Amersham Life Science Inc) at 400 mA for 120 minutes using the Novex transfer system. The membrane was prehybridized in 10 mL of buffer A (10 mmol/L Tris hydrochloride, pH 7.5, 100 mmol/L NaCl, 0.1% Tween 20, and 10% nonfat milk powder) for 1 hour and then hybridized for an additional 1-hour period in the same buffer containing 10  $\mu\text{L}$  of the given anti-NOS monoclonal antibody (1:1000). The membrane was then washed for 30 minutes in a shaking bath, with the wash buffer (buffer A without nonfat milk) changed every 5 minutes before 1 hour of incubation in buffer A plus goat anti-mouse IgG-horseradish peroxidase at the final titer of 1:1000. Experiments were carried out at room temperature. The washes were repeated before the membrane was developed with a light-emitting nonradioactive method using ECL reagent (Amersham Inc). The membrane was then subjected to autoluminography for 1 to 5 minutes. The autoluminographs were scanned with a laser densitometer (model PD1211, Molecular Dynamics) to determine the relative optical densities of the bands. In all instances, the membranes were stained with Ponceau stain before prehybridization. This step verified the uniformity of protein load and transfer efficiency across the test samples.

**Measurements of Total Nitrate and Nitrite**

The concentration of total nitrate and nitrite in the test samples was determined by a modification of the procedure described by Braman and Hendrix<sup>19</sup> using the purge system of a Sievers Instruments model 270B nitric oxide analyzer (NOA 228, Sievers Instruments Inc). Briefly, plasma samples were first diluted and deproteinized using chilled 100% ethanol (sample/ethanol, 1:2 [vol/vol]), and urine samples were diluted 10 times in distilled water before analysis.<sup>20</sup> A saturated solution of  $\text{VCl}_3$  in 1 mol/L HCl was prepared and filtered before use. This reagent (5 mL) was added to the purge vessel and purged with nitrogen gas for 5 to 10 minutes before use. The purge vessel was equipped with a cold-water condenser and a water jacket to permit heating of the reagent to  $95^{\circ}\text{C}$  with a circulating water bath.

**TABLE 1. Initial (Obtained at 8 Weeks of Age) and Final (Obtained at 12 Weeks of Age) Measurements of Body Weight, Hematocrit, Plasma Creatinine, and Creatinine Clearance in SHR and WKY**

Group	Time Period			
	WKY (n=6)		SHR (n=6)	
	Initial	Final	Initial	Final
Body weight, g	290.00 $\pm$ 7.11	366.40 $\pm$ 15.60*	231.70 $\pm$ 6.35†	267.00 $\pm$ 13.20*†
Hematocrit, %	0.40 $\pm$ 0.04	0.40 $\pm$ 0.01	0.44 $\pm$ 0.02	0.43 $\pm$ 0.02
Plasma creatinine, mmol/L	0.054 $\pm$ 0.008	0.040 $\pm$ 0.004	0.059 $\pm$ 0.008	0.039 $\pm$ 0.006
Creatinine clearance, mL $\cdot$ min $^{-1}$ $\cdot$ kg body wt $^{-1}$	3.2 $\pm$ 0.5	6.0 $\pm$ 0.91*	2.8 $\pm$ 0.2†	4.4 $\pm$ 0.4*†

\* $P < .05$ , difference within each group, final vs initial.

† $P < .01$ , difference between two groups, SHR vs WKY.

**TABLE 2. Body Weight, Hematocrit, Plasma Creatinine, and Creatinine Clearance in Prehypertensive 3-Week-Old SHR and Age-Matched WKY**

	WKY (n=6)	SHR (n=6)
Body weight, g	59.30±0.80	63.80±1.30
Hematocrit, %	0.30±0.01	0.30±0.06
Plasma creatinine, mmol/L	0.025±0.002	0.024±0.001
Creatinine clearance, mL · min <sup>-1</sup> · 100 g body wt <sup>-1</sup>	1.1±0.17	0.8±0.12

The hydrochloric acid vapors were removed by a gas bubbler containing ≈15 mL of 1 mol/L NaOH. The gas flow rate into the chemiluminescence detector was controlled using a needle valve adjusted to yield a cell pressure of ≈7 mm Hg. The flow rate of nitrogen into the purge vessel was adjusted to prevent vacuum distillation of the reagent.

Samples were injected into the purge vessel to react with the VCl<sub>3</sub>/HCl reagent, which converted nitrate, nitrite, and *S*-nitroso compounds to NO. The NO produced was stripped from the reaction chamber (by purging with nitrogen and vacuum) and detected by ozone-induced chemiluminescence in the chemiluminescence detector. The signal generated (NO peak and peak area) was recorded and processed by a Hewlett Packard model 3390 integrator. In a typical assay, 5 μL of the test sample was injected into the purge vessel, and all samples were run in triplicate.

Standard curves were constructed using various concentrations of NO<sub>3</sub><sup>-</sup> (5 to 100 μmol/L), relating the luminescence produced to the given NO<sub>3</sub><sup>-</sup> concentrations of the standard solutions. The amount of NO<sub>2</sub><sup>-</sup>/NO<sub>3</sub><sup>-</sup> in the test sample was determined by interpolation of the result into the standard curve.

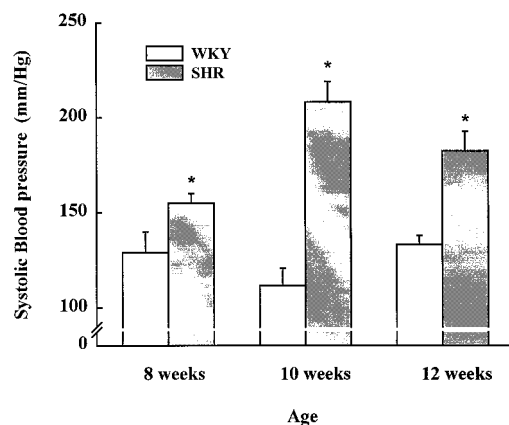
### Data Presentation and Analysis

Data are given as mean±SEM. Multiple-measure ANOVA, Student's *t* test, and regression analysis were used in the statistical evaluation of the data as appropriate. Values of *P*≤.05 were considered significant.

## Results

### General Data

Data are shown in Tables 1 and 2 and Figure 1. Initial body weight and creatinine clearance in the 8-week-old SHR were significantly lower than the corresponding values found in the WKY animals. Although body weight and creatinine clearance increased in both groups during the observation period,



**Figure 1.** Tail systolic blood pressure obtained at ages 8, 10, and 12 weeks in SHR and WKY. n=6 in each group. \**P*<0.01 vs WKY group.

they remained significantly lower in the SHR than in the WKY group. Initial arterial blood pressure obtained at 8 weeks of age in the SHR was significantly higher than that found in the WKY group. During the observation period, blood pressure rose significantly above the initial value in the SHR but remained practically unchanged in the WKY group. No significant difference was found in hematocrit level between the two groups.

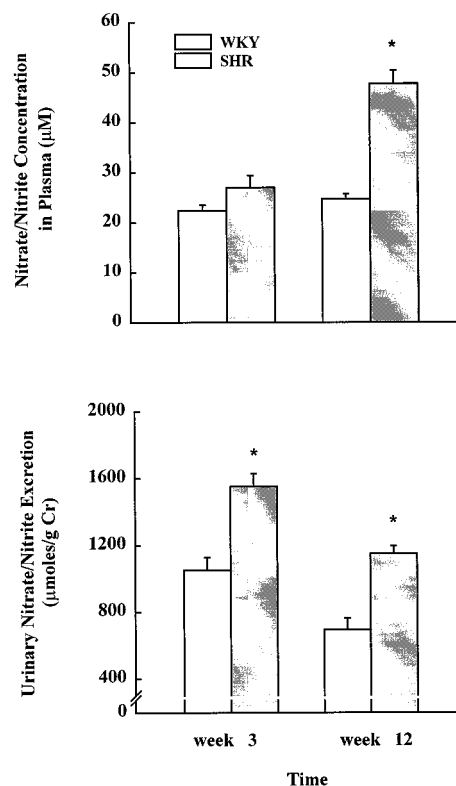
Body weight in the 3-week-old prehypertensive SHR group was slightly but significantly higher than the corresponding value observed in the WKY group. No significant difference was found in either hematocrit, plasma creatinine, or creatinine clearance between the two 3-week-old groups.

### Plasma and Urinary NOx

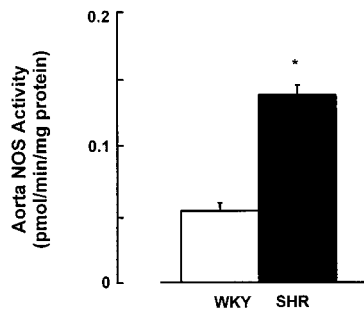
Data are shown in Figure 2. Urinary excretion of NOx in both prehypertensive (3-week-old) and hypertensive (12-week-old) SHR was significantly greater than in the corresponding WKY groups. Likewise, plasma NOx concentration was significantly higher in the SHR when compared with the corresponding value found in the WKY group.

### NOS Activity and eNOS and iNOS Proteins

Data are shown in Figures 3 through 11. At 12 weeks of age, thoracic aorta NOS activity was significantly greater in the SHR than that found in the WKY group. This was accompanied by a significant increase in the thoracic aorta iNOS and eNOS proteins in the 12-week-old SHR. In addition, renal tissue iNOS and eNOS protein abundance was markedly



**Figure 2.** Plasma concentration (top) and urinary excretion of stable nitric oxide metabolites (NOx; bottom) in SHR and WKY. n=6 in each group. \**P*<0.01.



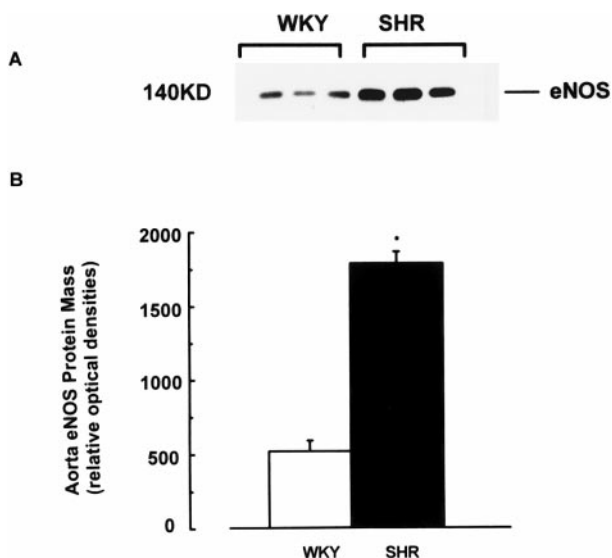
**Figure 3.** Aorta NOS activity in the SHR and WKY groups.  $n=6$  in each group. \* $P<0.01$ .

increased in these animals compared with that in the WKY group. Interestingly, a marked increase in kidney iNOS protein abundance as well as thoracic aorta eNOS protein abundance was observed in 3-week-old prehypertensive SHR compared with the corresponding values found in the age-matched WKY animals. However, kidney tissue eNOS and aorta iNOS, which were markedly elevated in the 12-week-old SHR, were not yet increased in the prehypertensive 3-week-old SHR.

### Discussion

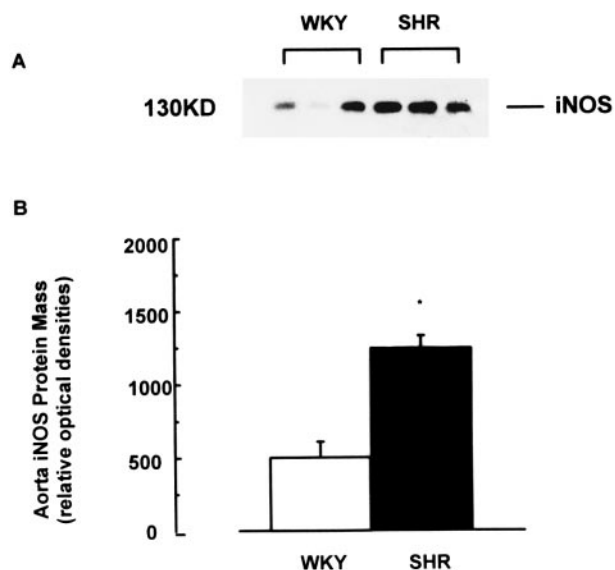
Data on the status of the L-arginine/NO pathway in SHR are contradictory. Although several studies have found evidence for depressed NO production, others have suggested the opposite. For instance, Cuevas et al<sup>6</sup> have recently demonstrated that the percentage of endothelial cells with immunostainable eNOS is greatly reduced in the thoracic aorta of aged SHR but not in normotensive WKY animals. In addition, Dubois<sup>7</sup> has recently shown that NO production and L-citrulline release in response to stimulation with 5% fetal calf serum, endotoxin, and interleukin-1 $\beta$  is reduced in cultured vascular smooth muscle cells obtained from SHR when compared with cells derived

from normotensive WKY animals. On the basis of these findings, the author concluded that vascular smooth muscle iNOS activity is depressed in SHR. Likewise, Malinsky et al<sup>8</sup> have demonstrated depressed NO production in response to bradykinin stimulation in cultured endothelial cells from SHR when compared with cells obtained from normotensive WKY animals. Furthermore, Crabos and coworkers<sup>9</sup> have recently shown that compared with WKY, 20-week-old SHR exhibit decreased vasodilatory response to bradykinin, reduced sensitivity to NOS inhibition, and diminished immunohistochemically detectable eNOS in the coronary arteries. In another study, Sunano and associates<sup>10</sup> showed a significant impairment of vasodilatory response to  $\alpha_2$ -adrenoreceptor agonist and, to a lesser extent, acetylcholine in precontracted aorta rings from SHR compared with those of WKY. They further demonstrated that these vasodilatory responses were completely inhibited by NOS inhibitor, pointing to the role of NO in this process. On the basis of these observations, the authors concluded that the impaired vasodilatory response to  $\alpha_2$ -adrenoreceptor agonist and acetylcholine is due to depressed NO production in SHR. They further speculated that impaired  $\alpha_2$  agonist-mediated NO production may in part contribute to hypertension in SHR.<sup>10</sup> In contrast, Akiba et al<sup>11</sup> have recently produced indirect evidence for increased NO production in young (6-week-old) and adult (20-week-old) SHR compared with WKY of the same age. This conclusion was based on the greater hypertensive response to NOS blockade in SHR than in WKY.<sup>11</sup> Similarly, Gil-Longo and coworkers<sup>12</sup> found a significantly greater hypertensive response to NOS blockade, suggesting increased resting NO production in SHR compared with WKY. However, the magnitude of contractile response to NOS inhibitor and methylene blue (an NO quencher) was significantly lower in submaximally contracted (25 mmol/L KCl) aortic rings from SHR than from WKY. On the basis of these observations, they suggested that hypertension enhances NO tone in vivo but impairs vascular NO production in vitro.<sup>12</sup> Further support for the hypertension-induced increased NO production in SHR comes from recent studies of Tomita et al,<sup>13</sup> who showed a direct correlation between blood pressure and acetylcholine-induced vasorelaxation in aortic rings of 9-week-old SHR, WKY, and F1-hybrid rats. They further showed that correction of hypertension with antihypertensive therapy for 5 weeks reverses the exaggerated acetylcholine-mediated vasorelaxation of the aortic rings in SHR, hence substantiating the role of hypertension per se.<sup>13</sup> These observations are further supported by recent studies of Hayakawa et al,<sup>14</sup> who have shown increased NOS activity in the aorta and renal medulla of 16-week-old SHR compared with normotensive WKY. In addition, Wu and associates<sup>15</sup> have found higher plasma nitrite and tumor necrosis factor- $\alpha$  concentrations and greater cGMP production and iNOS expression by aorta smooth muscle in SHR than in WKY at baseline and after lipopolysaccharide stimulation. This was associated with a greater hypotensive response to lipopolysaccharide in SHR as opposed to WKY. They concluded that NO production is increased in



**Figure 4.** A, Representative Western blot of aorta eNOS protein in three 12-week-old SHR and three WKY. B, Group data illustrating the optical densities of eNOS protein bands in the study groups.  $n=6$  in each group. \* $P<0.01$ .

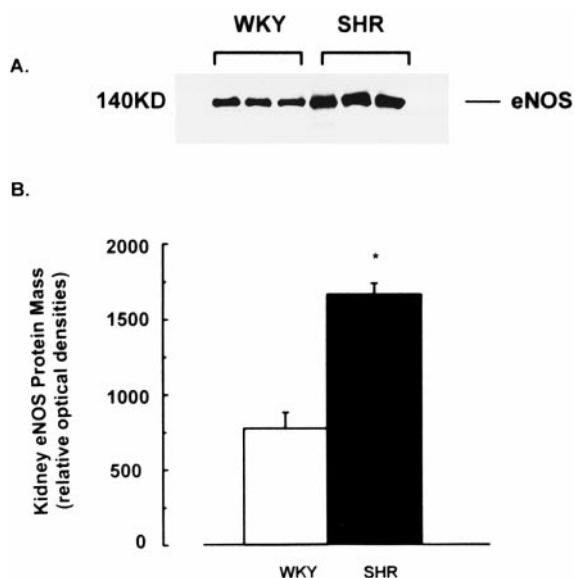




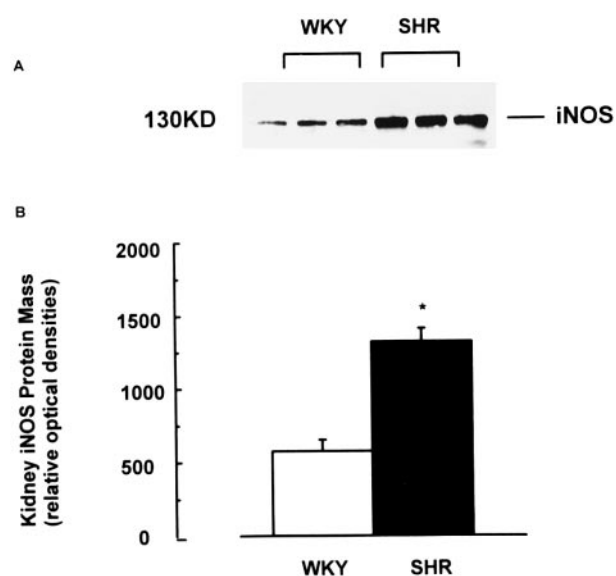
**Figure 5.** A, Representative Western blot of aorta iNOS in three 12-week-old SHR and three WKY. B, Group data demonstrating the optical densities of aorta iNOS protein bands in the study groups. n=6 in each group. \* $P<0.01$ .

SHR and attributed this phenomenon to basal expression of iNOS.<sup>15</sup>

The original group of SHR used here was studied between the ages of 8 and 12 weeks, representing the developmental phase of hypertension in this model. We elected to study the animals at the early phase of evolution of hypertension to distinguish the primary changes of NO metabolism from those caused by long-standing progressive hypertension and the resultant vasculopathy. The 8-week-old SHR showed a significant lower initial body weight and a significantly slower rate of growth during the 4-week observation period compared with their WKY



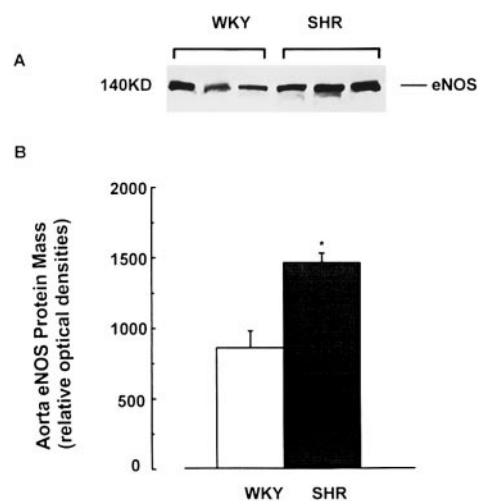
**Figure 6.** A, Representative Western blot of kidney eNOS in three 12-week-old SHR and three WKY. B, Group data illustrating relative optical densities of kidney eNOS bands in the study groups. n=6 in each group. \* $P<0.01$ .



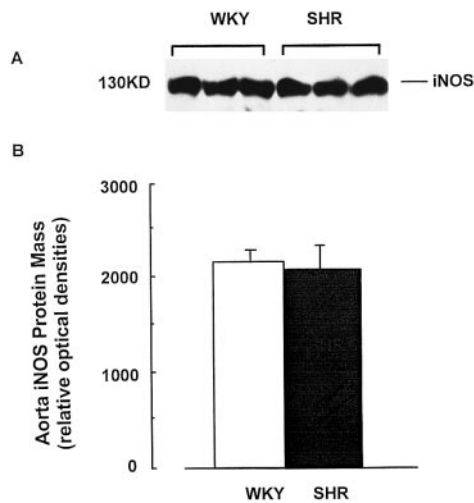
**Figure 7.** A, Representative Western blot of kidney iNOS in three 12-week-old SHR and three WKY. B, Group data depicting relative optical densities of the kidney iNOS protein bands in the study groups. n=6 in each group. \* $P<0.01$ .

counterparts. However, differences in body weight should not affect the validity of the biochemical measurements presented here. This is because efforts were made to normalize the given parameters when possible. For instance, urinary NO<sub>x</sub> was normalized against creatinine excretion, creatinine clearance was corrected for body weight, and NOS activity and protein mass data represent amounts present in fixed amounts of total tissue protein.

The SHR showed a marked increase in urinary NO<sub>x</sub> excretion, pointing to increased total body NO production during the early phase of hypertension. In addition, NOS activity as well as eNOS and iNOS proteins were significantly increased in the vascular tissue of SHR. Likewise, both iNOS and eNOS protein contents of the kidney tissue were markedly elevated in the 12-week-old SHR. These



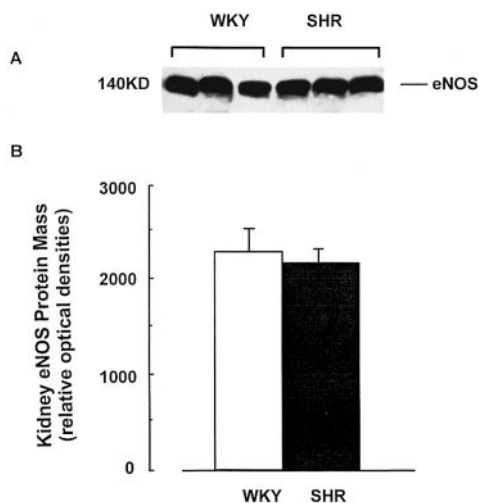
**Figure 8.** A, Representative Western blot of aorta eNOS protein in three prehypertensive 3-week-old SHR and three WKY. B, Group data illustrating the optical densities of eNOS protein bands in the study groups. n=6 in each group. \* $P<0.05$ .



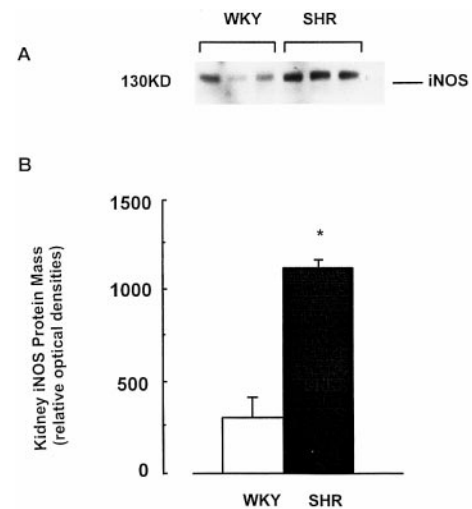
**Figure 9.** A, Representative Western blot of aorta iNOS in three prehypertensive 3-week-old SHR and three WKY. B, Group data demonstrating the optical densities of aorta iNOS protein bands in the study groups.  $n=6$  in each group.

findings point to the upregulation of NOS protein expression and increased NO production in the early phase of the evolution of hypertension in SHR. Accordingly, the onset of hypertension in SHR is not due to depressed NO production or NOS deficiency.

In an attempt to determine the possible role of hypertension per se in the upregulation of NOS expression, we studied a group of prehypertensive SHR and their normotensive WKY counterparts. We were surprised to find a marked increase in urinary NO<sub>x</sub> excretion together with a significant increase in the aorta eNOS and kidney iNOS protein abundance in prehypertensive 3-week-old SHR when compared with age-matched WKY. The mechanism and biological significance of upregulation of the L-arginine/NO system in prehypertensive SHR is not certain. However, it may be due to the hyperdynamic state preceding the onset of progressive hypertension in these animals.



**Figure 10.** A, Representative Western blot of kidney eNOS in three prehypertensive 3-week-old SHR and three WKY. B, Group data illustrating relative optical densities of kidney eNOS bands in the study groups.  $n=6$  in each group.



**Figure 11.** A, Representative Western blot of kidney iNOS in three prehypertensive 3-week-old SHR and three WKY. B, Group data depicting relative optical densities of the kidney iNOS protein bands in the study groups.  $n=6$  in each group.  $*P<0.05$ .

It is of interest that upregulation of the L-arginine/NO pathway in SHR involved both eNOS and iNOS. While the vasoregulatory role of eNOS as the source of EDRF is well understood, the role of low-level iNOS expression is less clear. It should be noted, however, that contrary to the conventional view, iNOS is constitutively expressed in several tissues, including kidney (thick ascending limb of loop of Henle, glomeruli, and interlobular and arcuate arterioles), heart, and arterial wall.<sup>21–23</sup> The constitutive expression of this NOS isotype in the kidney and other tissues points to its homeostatic role apart from that associated with its inducible immunologically mediated activation.

It should be noted that increased NOS protein abundance and NO production during the early phase of evolution of hypertension in SHR do not necessarily denote their persistent elevation during the advanced phase of the disease. On the contrary, with progressive vasculopathy and endothelial dysfunction, NOS expression and NO production may fall, leading to true NO deficiency in animals with advanced hypertension. The resulting NO deficiency can in turn contribute to the worsening of hypertension and the associated progressive vasculopathy. In fact, Cuevas et al<sup>6</sup> have shown a dramatic decline in the percentage of endothelial cells with detectable eNOS on histochemical examination of thoracic aorta in aged SHR. In contrast, age-matched normotensive WKY showed no discernible decline in immunostainable eNOS of thoracic aorta endothelial cells. Thus, the results of the present study and those of other investigators showing increased L-arginine/NO pathway activity in young spontaneously hypertensive animals<sup>10–15</sup> do not necessarily contradict those of Cuevas et al in aged SHR.<sup>6</sup> Instead, the data most likely represent different points in the natural course of progressive hypertension and vasculopathy in this model. Further studies are required to examine this possibility.

In conclusion, development of hypertension in young SHR is preceded by and associated with enhanced total body NO production and increased vascular and renal NOS protein expressions. These findings tend to exclude an impaired L-arginine/NO pathway as the primary cause of hypertension in SHR.

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