Malignant Alterations following Early Blockade of Nitric Oxide Synthase in Hypertensive Rats

Yung Hsiang Hsu[1], Bang Gee Hsu[2], and Hsing I Chen[3]

[1]*Department of Pathology*
[2]*Department of Nephrology*
and
[3]*Institute of Integrative Physiology and Clinical Sciences*
Tzu Chi University
Hualien 97004, Taiwan, ROC

Abstract

Nitric oxide (NO) is important for the homeostasis of organ functions. We studied the structural and functional changes in the cardiovascular (CV) and renal systems following early NO deprivation by various nonspecific and specific NO synthase (NOS) inhibitors: N-nitro-L-arginine methyl ester (L-NAME), N-nitro-L-arginine (L-NA), S-methyl-isothiourea (SMT), and L-N^6-(1-iminoethyl)-lysine (L-Nil). The aim is to elucidate the involvement of NO through endothelial or inducible NOS (eNOS and iNOS). Drugs were given to spontaneously hypertensive rats (SHR) and age-matched normotensive Wistar Kyoto rats (WKY) from a young age (5-wk-old). Physiological, biochemical, and pathological examinations were performed. L-NAME and L-NA treatment caused a rapid increase in tail cuff pressure (TCP). The TCP of SHR reached a malignant level within 30 days with signs of stroke, proteinurea, severe glomerular sclerosis, and moderate ventricular hypertrophy (VH). The plasma nitrite/nitrate was reduced, while creatinine, urea nitrogen and uric acid were elevated. The renal tissue cyclic guanosine monophosphate (cGMP) was decreased with an elevated collagen content. The numbers of sclerotic glomeruli, arteriolar and glomerular injury scores were markedly increased, accompanied by reduction in renal blood flow, filtration rate, and fraction. Plasma endothelin-1 was increased following L-NAME or L-NA treatment for 10 days. The expression of eNOS and iNOS mRNA was depressed by L-NAME and L-NA. The relevant iNOS inhibitors, SMT and L-Nil depressed the iNOS expression, but did not produce significant changes in CV and renal systems. The continuous release of NO *via* the eNOS system provides a compensatory mechanism to prevent the genetically hypertensive rats from rapid progression to malignant phase. Removal of this compensation results in VH, stroke, glomerular damage, renal function impairment, and sudden death.

Key Words: arteriolar injury, nitric oxide synthase, stroke, glomerular injury, renal functions

Introduction

In human and animals, long-term hypertension can result in severe cardiovascular sequelae such as cerebral, coronary, and renal vascular disorders (15, 20, 26, 28, 33). It has long been established that endothelial function and nitric oxide (NO) play an important role in various physiological and pathological functions (5, 12, 16, 32). Acute or chronic administration of nitric oxide synthase (NOS) inhibitors caused systemic hypertension and increased total peripheral resistance as well as arterial impedance (1, 2, 4, 10, 20, 23, 26). Long-term NO deprivation in normotensive rats caused not only hypertension, but also vascular changes, glomerular damage, or even spinal infarct and sudden death (4, 6, 14, 20, 21, 42). The continuous formation of NO in either normotensive or hypertensive animal is important for the homeostasis

Corresponding author: Hsing I Chen, M.D., Ph.D., Institute of Integrative Physiology and Clinical Sciences, Tzu Chi University, No. 701, Section 3, Chung Yang Road, Hualien 97004, Taiwan. Tel: +886-3-8560824, Fax: +886-3-8573053, E-mail: chenhi@mail.tcu.edu.tw
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in vascular and organ functions (10, 16, 36).

Overt glomerular dysfunction and injury occurred in aged (73-wk-old) spontaneously hypertensive rats, suggesting that the development of renal damage is a lengthy process (26, 28, 33). However, treatment with L-NAME facilitated the structural and functional changes of renal glomeruli and cerebral arteries (17, 18, 20, 21, 26, 28, 36). These findings suggest that NO is involved in the alterations of glomerular dynamics and structures. However, the NO function and the involvement of various NO synthases in vascular or renal change following chronic hypertension of either genetic origin or NO deprivation remains undetermined (5, 10, 16). In addition, the role of endothelin in the hypertension induced by NO deprivation has not been well defined.

In the present study, we used nonspecific NOS inhibitors such as N^{ω}-nitro-L-arginine methyl ester (L-NAME) and N^{ω}-nitro-L-arginine (L-NA), as well as relatively specific inducible NOS (iNOS) blockers, S-methyl-isothiourea (SMT) and L-N^6-(1-iminoethyl)-lysine (L-Nil). These agents were given to spontaneously hypertensive rats (SHR) and normotensive Wistar Kyoto stain (WKY) from a young age (5-wk-old) at a prehypertensive stage. Tail cuff pressure (TCP), which reflects the systolic arterial pressure (AP), was measured. Plasma nitrite/nitrate, creatinine, uric acid and endothelin-1 (ET_1) were determined. We also examined the renal tissue cyclic guanosine monophosphate (cGMP), collagen volume fraction, pathological changes, and the expression of endothelial NOS (eNOS) as well as iNOS. Glomerular functions and injury were assessed. The purposes are to find the pathological changes of severe hypertension in the kidney, in particular the glomerular structure and functions, and to elucidate the involvement of eNOS, iNOS, and ET1 in these changes. We thus aim to understand the mechanisms and possible preventive and therapeutic regimens for malignant hypertension induced by NOS blockade in rats with genetic hypertension.

Materials and Methods

Animals

Inbred strains of SHR and WKY originated from Charles River (USA) were obtained from the National Animal Center. All study procedures for animal experiments were reviewed and approved by the University Committee of Laboratory Animal Research Center and were designed in accordance with the National Animal Center Guidelines. Rats were housed at 22 ± 1°C under a 12/12 h light/dark regimen. Food and water were supplied *ad libitum*. Standard rodent diet in pellet (NaCl content <1%) was purchased from PMI Feeds, Inc. (Richmond, IN, USA). The TCP was measured at conscious state with a photoelectric sphygmomanometer (UR-5000, Ueda, Tokyo, Japan). To minimize variation due to heating and restrain, TCP was determined four times daily. The average was taken for an individual data. The rats were placed in a metabolic cage to collect urine. Urine protein concentration was determined by the Bradford method (7).

Drugs

NOS inhibitors, L-NAME, L-NA, SMT and L-Nil were purchased from Sigma Chemical Co (St. Louis, MO, USA). The drugs were dissolved in pure water immediately before administration. L-NA is relatively indissoluble in water. The solution (50 mg in 1 ml water) required an addition of 0.2 ml NaOH (pH 8.6) for complete dissolution. Therefore, vehicle in the L-NA series had the same volume of water with NaOH instead of pure water. L-NAME might exert a muscarinic receptor antagonistic effect (8). To ensure that the effect would not be produced *via* muscarinic antagonism, an additional group receiving L-NA treatment was also included.

Histopathological Examination

Some rats died spontaneously, while a certain number of rats were euthanized by an overdose of intravenous pentobarbital sodium, perfused, and fixed with 4% paraformaldehyde in phosphate buffer solution. The heart, brain, spinal cord, and kidney were removed. The left ventricle (LV) was excised, freed from surrounding tissues, and weighed to obtain the LV weight (LVW). The LVW/body weight (BW) ratio was used as a measure of left ventricular hypertrophy (22). The kidney, brain, and spinal cord were inspected grossly for hemorrhagic or sclerotic lesions. For light microscopic examination, tissue specimens were embedded in paraformaldehyde, sectioned at 5 (m thickness, and stained with hematoxylin and eosin (H & E). Glomerular injury score (GIS) was determined by a method described by Raij (36). Arteriolar injury score (AIS) of the kidney was assessed by the method provided by Mai *et al*. (31) and modified by Ono *et al*. (33). The calculations were done in a blind fashion by several students to reduce subjectivity. The numbers of sclerotic glomeruli were examined from 100 sections in each kidney.

Determination of Plasma Nitrite/Nitrate, Creatinine (Cr), Blood Urea Nitrogen (BUN), Uric Acid (UA), and Endothelin-1 (ET-1)

Blood sample (0.5 ml) was collected. Plasma

nitrite/nitrate (the metabolites of NO) was measured with a high-performance liquid chromatography (HPLC) (29). The concentration of Cr, BUN, and UA was determined with an autoanalyzer (Vitros 750, Johnson & Johnson, New York, NY, USA). ET-1 concentration was measured with an ELISA kit (American Research Products, Belmont, MA, USA) every 5 to 10 days.

Tissue Cyclic Guanosine Monophosphate (cGMP), Collagen Volume Fraction, and Expression of iNOS and eNOS

Measurements of cGMP and collagen volume fraction in the kidney followed the procedures described by Arnal *et al*. (2) and Kolpakov *et al*. (27). The expression of eNOS and iNOS was determined with methods described by Pritchad *et al*. (35). In brief, reverse-transcriptase polymerase reaction (RT-PCR) was used to determine the eNOS and iNOS expression. The expression of GAPDH was used as an internal control for comparison.

Glomerular Dynamics

The effective renal plasma flow (ERPF), and glomerular filtration rate (GFR) were determined following standard clearance formulae (inulin and *p*-aminohippuric acid). The filtration fraction (FF) were calculated by the equation FF = GFR/ERPF (13, 26).

Experimental Protocols

SHR and WKY were used in four series of experiment. L-NAME has been commonly used for acute and long-term NOS inhibition (1, 2, 4, 10, 20, 21, 23, 28). Another nonspecific NOS inhibitor, L-NA, was also used. The L-NAME, L-NA, SMT, and L-Nil study series were carried out by different laboratory technicians to minimize the possible bias in data collection. SHR and WKY (4-wk-old, male and weighing 120-130 g) underwent a one-wk observation period before they were randomly divided into four groups to receive drugs and vehicle at the age of 5 wk. In each group, the number of animals was initially more than ten. Some rats were sacrificed for histopathological and RT-PCR examinations at day 25 after drug administration. The data for TCP measurements were obtained from 10 rats in each group. Thus, we had WKY and SHR receiving L-NAME and vehicle in the first series, L-NA and vehicle in the second series, SMT and vehicle in the third series, and L-Nil and vehicle in the fourth series. During the experiment, TCP was measured every 1 to 2 days. L-NAME, L-NA, SMT or L-Nil (a total daily dose of 50 mg/kg) was given twice a day (25 mg/kg at 8 am and 8 pm) by oral route with a gastric tube (1, 2, 4). Again, blind fashion was used to minimize subjectivity in data collection.

Statistical Evaluation

The data were expressed as mean ± SEM. Statistical evaluations of the differences among and between groups were done with analysis of variance and Scheffe's test. A modified paired *t*-test was used for comparison of the TCP and other measurements between treated and vehicle groups. Differences were considered significant at $P < 0.05$.

Results

Changes in TCP

Fig. 1 displays the TCP changes in SHR and WKY treated with L-NAME and vehicle (A), L-NA and vehicle (B), SMT and vehicle (C), and L-Nil and vehicle (D). L-NAME and L-NA treatment rapidly elevated TCP in SHR and WKY. The TCP reached a malignant level above 260 mmHg within 30 days following L-NAME and L-NA. Some of the rats showed signs of stroke and died within 42 days of the observation period. In contrast, SMT and L-Nil did not significantly alter the changes of TCP in both SHR and WKY.

Neurological Signs and Lesions

The SHR-L-NAME and SHR-L-NA rats at this stage of malignant hypertension soon manifested weakness, motor dysfunction and sudden death. The signs of motor dysfunction or stroke include monoplegia, paraplegia, and in some severe cases, tetraplegia (Fig. 2A). SMT and L-Nil did not cause signs of stroke in the observation period over 42 days.

In rats with signs of stroke and sudden death, pathological examination of the brain revealed ischemia, hemorrhage and infarction in the cerebellum, midbrain and spinal cord (Fig. 2B). Microphotographic picture showed hemorrhagic lesions, spongy edematous changes and loss of neurons with reactive cell infiltration (Fig. 2C).

Body Weight (BW), Heart Rate (HR), Left Ventricular Weight (LVW), and LVW/BW Ratio

The BW, HR, LVW, and LVW/BW were obtained at day 25 after administration of vehicle or drugs (Table 1). The BW of SHR was slightly, but not significantly lower, than that of WKY. The HR, LVW, and LVW/BW in SHR were higher than those in WKY. Administration of L-NAME or L-NA significantly reduced the BW and HR, but increased

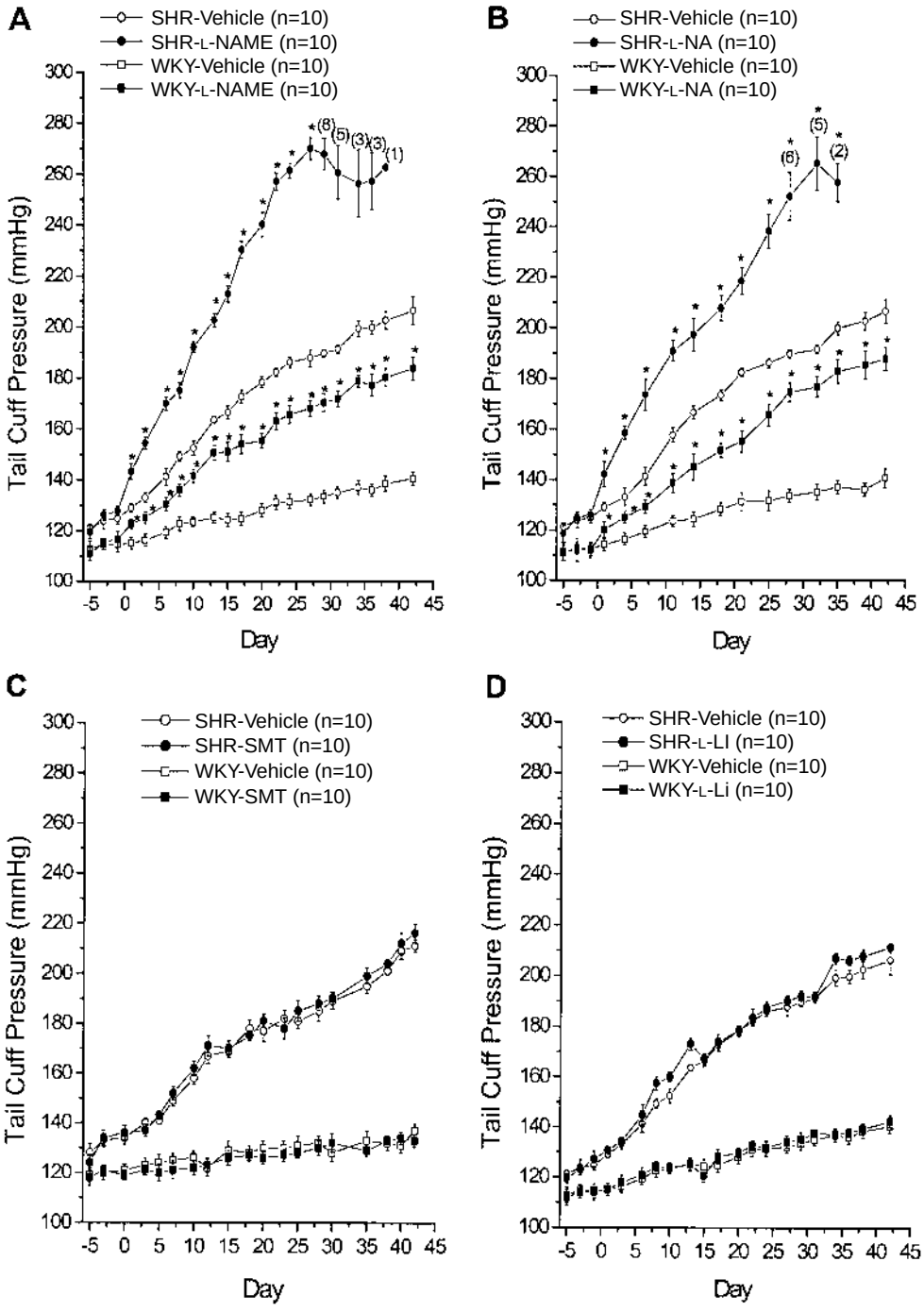

Fig. 1. Changes in tail cuff pressure (TCP, representing systolic arterial pressure) in spontaneously hypertensive rats (SHR) and normotensive Wistar Kyoto strain (WKY) in the L-NAME, L-NA, SMT, and L-Nil with vehicle groups. Daily administration of L-NAME or L-NA (starting from day 0) significantly increased TCP over the vehicle groups in both SHR and WKY (A and B, $*P < 0.05$). In SHR-treated with L-NAME or L-NA, the TCP soon reached a very high level at day 26. Thereafter, the animals showed signs of stroke and died during days 27 to 40. TCP was measured only in rats (number in parenthesis) without overt symptoms. Some rats were subjected to histopathological examinations before death. SMT (C) and L-Nil (D) essentially did not alter the TCP changes in SHR and WKY. In L-NAME and L-NA treated rats, the magnitude of TCP increase was greater in SHR than in WKY ($*P < 0.05$).

the LVW and LVW/BW in WKY and SHR. These effects were not observed in WKY and SHR treated with SMT or L-Nil. The data indicated that left ventricular hypertrophy (VH) occurred in SHR, and L-NAME or L-NA exacerbated the VH. Inhibition of iNOS with SMT or L-Nil did not exert such effects.

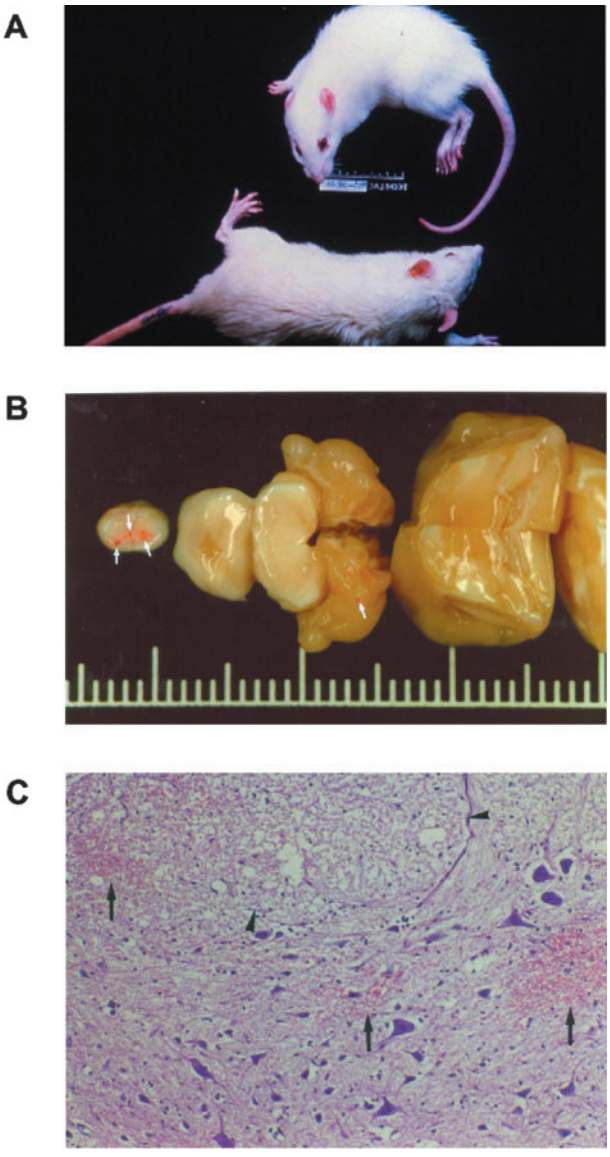

Fig. 2. Signs of stroke (paraplegia and tetraplegia) (A), spot hemorrhagic lesions in the spinal cord and a petechial hemorrhage in the cerebellum (B, arrows) in rats with spontaneous hypertension receiving L-NAME (original magnification × 5, small scale = 1 mm). Microphotography showing multiple foci of hemorrhagic lesions (C, arrows), spongy edematous changes (arrowheads), loss of neurons and reactive cells infiltration (hematoxylin and eosin stain, original magnification × 100).

Plasma Nitrite/Nitrate, Creatinine (Cr), Blood Urea Nitrogen (BUN), Uric Acid (UA), Renal Tissue Cyclic Guanosine Monophosphate (cGMP), and Collagen Volume Fraction (CVF)

The plasma nitrite/nitrate, Cr, BUN, UA, the renal tissue cGMP, and CVF in SHR were higher than those in WKY (Table 2). Treatment of L-NAME or L-NA in WKY and SHR for 25 days caused significant decreases in plasma nitrite/nitrate and tissue cGMP, but increases in Cr, BUN, UA, and tissue CVF. In WKY and SHR receiving SMT or L-Nil, these chemical factors were essentially unaltered. L-NAME and L-NA reduced the NO production and tissue cGMP. The nonspecific NOS inhibitors also impaired the renal functions as reflected in the increases in Cr, BUN, and UA. In addition, the collagen content was increased. Two relatively specific iNOS inhibitors did not produce these changes.

Plasma Endothelin-1 (ET-1) Concentration

The plasma ET-1 was increased at day 5 following treatment of L-NAME or L-NA. It reached a maximum at day 10. Thereafter, the ET-1 was slightly lower than the values at day 10. There was a tendency of progressive decrease after day 15. SMT or L-Nil did not affect the ET-1. In addition, it appeared that ET-1 was higher in SHR than that in WKY with or without treatment (Table 3).

Renal Pathology, Proteinurea, Glomerular Sclerosis, AIS, and GIS

Microscopic examination of the renal tissue sections revealed glomerular sclerosis. The lesions ranged from segmental to global sclerosis. Fig. 3 illustrates the severe renal damage in rats receiving L-NAME for 25 days. The pictures show numerous sclerotic glomeruli, adhesion of the capillary tuft to Bowman's capsule, fibrin deposition in the capsular epithelium, arteriolar hyalinosis, medial hypertrophy, and luminal obliteration. The vascular wall was obviously altered with eosinophilic and fibrin-rich deposits in the adventitial layer. In addition, severe luminal narrowing, encroachment, and tubulointerstitial changes with inflammatory cell infiltration were observed.

The 24-h urine protein, number of sclerotic glomeruli (NSG), AIS, and GIS were higher in SHR than in WKY (Table 4). L-NAME or L-NA increased the proteinurea by 92% and 167% in WKY, and by 315% and 300% in SHR. The NSG was increased from 0.6 % to 12.4 - 13.6% in WKY, and from 8.6% to 23.6 - 24.7% in SHR after treatment with L-NAME and L-NA. The increases in AIS were +306% and +318% with L-NAME and L-NA, respectively. L-NAME exacerbated the GIS by 599% in WKY, and by 405% in SHR. L-NA produced similar enhancement of GIS. Treatment with SMT and L-Nil in WKY and SHR virtually did not affect the proteinurea, NSG, AIS, and GIS.

Glomerular Dynamics: Effective Renal Plasma Flow (ERPF), Glomerular Filtration Rate (GFR), and Filtration Fraction (FF)

The ERPF and GFR in SHR were lower than the

Table 1. Body weight (BW), heart rate (HR), left ventricular weight (LVW), and LVW/BW ratio in various groups

	BW (g)	HR (bpm)	LVW (mg)	LVW/BW (mg/kg)
WKY–Vehicle	266 ±8	362 ±10	744 ±11	2.8 ±0.6
WKY–L-NAME	230 ±9*	302 ±6*	996 ±12*	4.3 ±1.0*
WKY–L-NA	229 ±6*	312 ±8*	982 ±14*	4.3 ±1.2*
WKY–SMT	258 ±8	358 ±9	758 ±8	2.9 ±0.8
WKY–L-Nil	261 ±9	368 ±10	752 ±10	2.9 ±1.1
SHR–Vehicle	254 ±6	424 ±12 †	1026 ± 10†	4.0 ± 0.8†
SHR–L-NAME	226 ± 9*	382 ±8*	1318 ±12*	5.8 ±1.2*
SHR–L-NA	221 ±10*	378 ± 10*	1296 ±14*	5.9 ±1.4*
SHR–SMT	249 ± 8	416 ±9 †	1012 ± 8†	4.1 ± 1.1†
SHR–L-Nil	256 ±9	428 ±14 †	998 ±10 †	3.9 ± 0.9†

Values are mean ± SEM (n = 10 for each group). BW, body weight; HR, heart rate; bpm, beat per min; LVW, left ventricular weight; LVW/BW, LVW to BW ratio. *$P < 0.05$ *vs*. Vehicle group; †$P < 0.05$ *vs*. the corresponding values in WKY.

Table 2. Plasma nitrite/nitrate, creatinine, blood urea nitrogen, uric acid, and renal tissue cyclic guanosine monophosphate and collagen volume fraction in various groups

	Nitrite/Nitrate (*p* mol)	Cr (mg/dl)	BUN (mg/dl)	UA (mg/dl)	cGMP (f mg/dl)	CVF (%)
WKY–Vehicle	582 ±12	0.72 ±0.04	0.68 ±0.04	0.62 ±0.02	3644 ±96	0.60 ±0.08
WKY–L-NAME	23 ±6*	2.38 ±0.09*	2.48 ±0.09*	1.98 ±0.06*	2126 ±86*	1.86 ±0.09*
WKY–L-NA	28 ±8*	2.42 ±0.06*	2.36 ±0.08*	2.02 ±0.09*	2120 ±78*	1.92 ±0.08*
WKY–SMT	544 ±10	0.80 ±0.06	0.74 ± 0.06	0.70 ± 0.05	3588 ±101	0.58 ±0.06
WKY–L-Nil	566 ±13	0.78 ±0.05	0.70 ±0.05	0.64 ±0.03	3642 ±98	0.61 ±0.05
SHR–Vehicle	988 ±14 †	1.24 ±0.06 †	1.82 ±0.12 †	1.20 ±0.03 †	4908 ±102 †	1.65 ±0.09 †
SHR–L-NAME	56 ±8*	3.49 ±0.12*	3.42 ±0.21*	3.02 ±0.06*	2008 ±88*	3.42 ±0.10*
SHR–L-NA	64 ±9*	3.52 ±0.18*	3.54 ±0.19*	3.08 ±0.08*	2198 ±96*	3.52 ±0.12*
SHR–SMT	972 ±22 †	1.32 ±0.08 †	1.84 ±0.14 †	1.18 ±0.04 †	4768 ±101 †	1.58 ±0.08 †
SHR–L-Nil	984 ±18 †	1.28 ±0.06 †	1.78 ± 0.12†	1.24 ±0.06 †	4822 ±98 †	1.62 ±0.06 †

Values are mean ± SEM (n = 10 for each group). Cr, creatinine; BUN, blood urea nitrogen; UA, uric acid; cGMP, cyclic guanosine monophosphate; CFV, collagen volume fraction. *$P < 0.05$ *vs*. Vehicle group; †$P < 0.05$ *vs*. the corresponding values in WKY.

Table 3. Change in plasma endothelin-1 concentration (pg/dl)

	Day						
	0	5	10	15	20	30	40
WKY–Vehicle	1.14 ±0.04	1.21 ±0.05	1.38 ±0.08	1.26 ±0.07	1.32 ±0.09	1.36 ±0.08	1.28 ±0.06
WKY–L-NAME	1.22 ±0.06	3.82 ±0.18 *	5.94 ±0.16 *	4.83 ±0.21 *	4.44 ±0.18 *	4.02 ±0.16 *	4.11 ±0.17 *
WKY–L-NA	1.29 ±0.07	3.76 ±0.16 *	6.02 ±0.22 *	4.96 ±0.19 *	4.56 ±0.21 *	4.32 ±0.18 *	4.28 ±0.20 *
WKY–SMT	1.28 ±0.09	1.31 ±0.08	1.40 ±0.09	1.51 ±0.12	1.54 ±0.14	1.48 ±0.09	1.51 ±0.11
WKY–L-Nil	1.16 ±0.08	1.42 ±0.09	1.44 ±0.11	1.48 ±0.16	1.50 ±0.15	1.52 ±0.14	1.48 ±0.13
SHR–Vehicle	2.42 ±0.09 §	2.38 ±0.08 §	3.28 ±0.19 †§	3.33 ±0.18 †§	3.42 ±0.19 †§	3.38 ±0.19 †§	3.42 ±0.18 †§
SHR–L-NAME	2.44 ±0.10 §	4.46 ±0.18 †*§	7.26 ±0.24 †*§	6.04 ±0.24 †*§	5.43 ±0.20 †*§	4.93 ±0.21 †*§	4.84 ±0.22 †*§
SHR–L-NA	2.39 ±0.12 §	4.52 ±0.21 †*§	7.38 ±0.21 †*§	6.19 ±0.22 †*§	5.66 ±0.23 †*§	5.02 ±0.20 †*§	4.96 ±0.19 †*§
SHR–SMT	2.26 ± 0.11§	2.31 ±0.14 §	2.42 ±0.15 §	2.51 ±0.18 §	2.48 ±0.17 §	2.53 ±0.18 §	2.48 ±0.12 §
SHR–L-Nil	2.32 ±0.08 §	2.42 ±0.16 §	2.46 ±0.16 §	2.38 ±0.14 §	2.41 ±0.16 §	2.46 ±0.16 §	2.51 ±0.15 §

Values are mean ± SEM (n = 10 for each group). *$P < 0.05$ *vs*. Vehicle group; †$P < 0.05$ *vs*. the corresponding values at day 0. §$P < 0.05$ *vs*. the corresponding values in WKY.

Table 4. Urine protein concentration, number of sclerotic glomeruli, AIS and GIS in various groups.

	UprotV (mg/24 h)	NSG (%)	AIS	GIS
WKY–Vehicle	24 ±6	0.6 ±0.2	34 ±6	27 ±4
WKY–L-NAME	46 ±5 *	12.4 ±0.8 *	138 ±19 *	189 ±12 *
WKY–L-NA	52 ±9 *	13.6 ±1.0 *	142 ±18 *	192 ±14 *
WKY–SMT	20 ±8	0.8 ±0.4	41 ±8	30 ±6
WKY–L-Nil	27 ±9	1.1 ±0.5	39 ±10	28 ±8
SHR–Vehicle	52 ±6 †	8.6 ±1.2 †	52 ±4 †	42 ±6 †
SHR–L-NAME	216 ±14 *†	24.7 ±2.2 *†	178 ±9 *†	212 ±12 *†
SHR–L-NA	208 ±12 *†	23.6 ±1.8 *†	180 ±10 *†	221 ±10 *†
SHR–SMT	62 ± 8†	9.1 ±1.1 †	54 ±6 †	44 ± 8†
SHR–L-Nil	68 ±9 †	7.8 ±0.9 †	58 ±9 †	50 ±9 †

Values are mean ± SEM (n = 10 for each group). UprotV, twenty-four hr urine protein concentration; NSG, number of sclerotic glomeruli per 100 sections; AIS, arteriolar injury score; GIS, glomerular injury score. *$P < 0.05$ *vs*. Vehicle group; †$P < 0.05$ *vs*. the corresponding values in WKY.

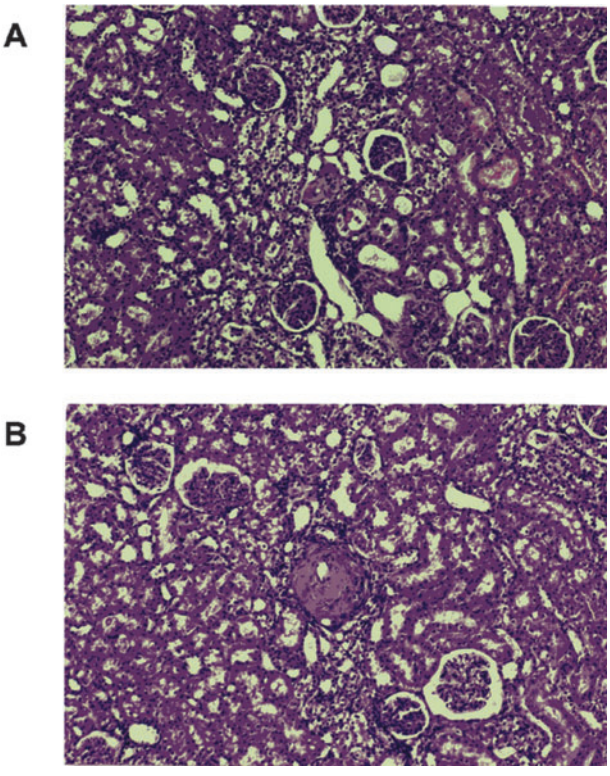

Fig. 3. Histopathological examination of the kidney in one L-NAME-treated SHR. The microphotography shows numerous sclerotic glomeruli (A), severe glomerular sclerosis, adhesion of the capillary tuft to Bowman's capsule, and fibrin deposition in the capsular epithelium. There are arteriolar hyalinosis, medial hypertrophy, and evidence of complete luminal obliteration (B). The structure of the vessel wall is strikingly altered with eosinophilic, and fibrin-rich deposits in the vascular circumference with severe luminal narrowing and encroachment. The picture also illustrates tubulointerstitial changes with inflammatory cell aggregation (hematoxylin and eosin stain, original magnification × 100).

values in WKY (Table 5). Treatment of L-NAME or L-NA greatly decreased the ERPF, GFR, and FF in WKY and SHR. In SHR receiving L-NAME or L-NA, the ERPF and GFR were seriously depressed to below 30% and 40% of the values in vehicle groups. The data indicated that nonspecific NOS inhibitors caused severe impairment of glomerular dynamics. Treatment of SMT or L-Nil did not produce significant alteration in glomerular dynamics.

Semiquantitation of the iNOS and eNOS Expression in Renal Tissue

Fig. 4 illustrates the expression of iNOS and eNOS in the kidney. For clarity's sake, we did not show the expression of GAPDH. Table 6 presents the ratio of iNOS/GAPDH in various groups. Both iNOS and eNOS were higher in SHR than in WKY. L-NAME and L-NA significantly depressed the iNOS and eNOS in WKY and SHR. SMT and L-Nil only decreased the iNOS, but not the eNOS.

Discussion

Hypertension of genetic origin and that caused by chronic NO deprivation are two different types of hypertension (20, 21, 42). The continuous release of NO is important for vascular homeostasis (5, 12, 16, 20, 21, 32). In the present study, we demonstrated that chronic NO deprivation in hypertension from the prehypertensive stage (5-wk-old) caused rapid and dramatic elevation of tail cuff pressure. In SHR receiving L-NAME or L-NA, the TCP soon reached a malignant level (above 260 mmHg) in 30 days. Cardiovascular and renal sequelae occurred with signs of stroke, ventricular hypertrophy, proteinurea and severe structural and functional changes of the glomeruli.

Table 5. Glomerular dynamics in various groups

	ERPF (ml/min/g)	GFR (ml/min/g)	FF %
WKY–Vehicle	3.17 ±0.22	0.64 ±0.06	20.2 ±2.0
WKY–L-NAME	1.42 ±0.18 *	0.22 ±0.08 *	15.5 ±1.6 *
WKY–L-NA	1.37 ±0.21 *	0.19 ±0.06 *	13.9 ±1.5 *
WKY–SMT	3.21 ±0.19	0.68 ±0.08	21.2 ±1.8
WKY–L-Nil	3.32 ±0.23	0.59 ±0.09	17.8 ±1.9
SHR–Vehicle	1.68 ±0.19 †	0.48 ±0.04 †	28.6 ±2.4 †
SHR–L-NAME	0.64 ±0.09 *	0.11 ±0.02 *	17.2 ±1.8 *
SHR–L-NA	0.58 ±0.10 *	0.12 ±0.03 *	20.7 ±1.6 *
SHR–SMT	1.71 ±0.22 †	0.50 ±0.06 †	29.2 ±2.1 †
SHR–L-Nil	1.56 ±0.18 †	0.46 ±0.03 †	29.5 ±2.0 †

Values are mean ± SEM (n = 10 for each group). ERPF, effective renal plasma flow; GFR, glomerular filtration rate; FF, filtration fraction. *$P < 0.05$ *vs*. Vehicle group; †$P < 0.05$ *vs*. the corresponding values in WKY.

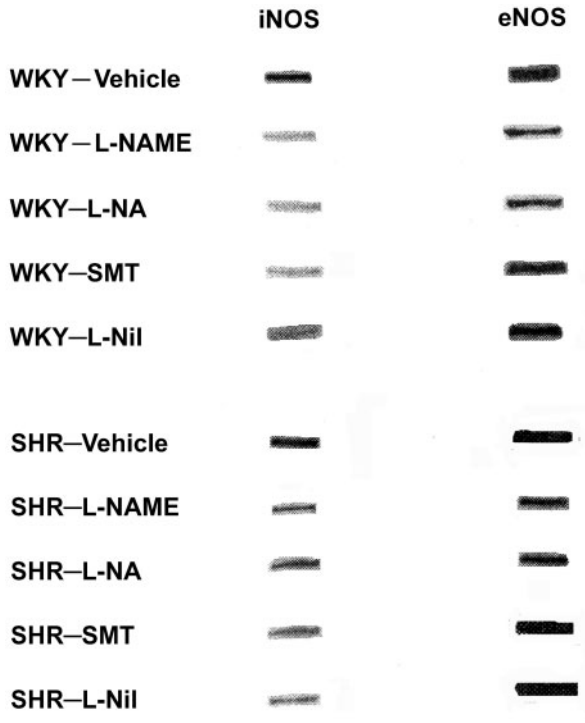

Fig. 4. Expression of inducible NOS (iNOS) and endothelial NOS (eNOS) in various groups. Note the iNOS reduction in WKY and SHR treated with L-NAME, L-NA, SMT and L-Nil. The iNOS expression is essentially not different between untreated WKY and SHR. The expression of eNOS is more obvious in untreated SHR than WKY. The eNOS expression is depressed by L-NAME and L-NA, but not by SMT and L-Nil in WKY and SHR. For clarity, the expression of GAPDH for internal control is not shown.

Table 6. The iNOS/GAPDH and eNOS/GAPDH ratios in various groups

	iNOS/GAPDH	eNOS/GAPDH
WKY–Vehicle	0.61 ±0.06	0.94 ±0.11
WKY–L-NAME	0.37 ±0.05 *	0.42 ±0.08 *
WKY–L-NA	0.32 ±0.06 *	0.50 ±0.07 *
WKY–SMT	0.28 ±0.03 *	0.48 ±0.06 *
WKY–L-Nil	0.30 ±0.05 *	0.53 ±0.09 *
SHR–Vehicle	0.71 ±0.05 †	1.32 ±0.13 †
SHR–L-NAME	0.29 ±0.08 *†	0.48 ±0.07 *
SHR–L-NA	0.25 ±0.05 *†	0.52 ±0.06 *
SHR–SMT	0.27 ±0.04 *	1.29 ±0.11 †
SHR–L-Nil	0.28 ±0.08 *	1.30 ±0.09 †

Values are mean ± SEM (n = 10 for each group). iNOS, inducible nitric oxide synthase; eNOS, endothelial nitric oxide synthase; GAPDH, glyeraldehyde-3-phosphate dehydrogenase. *$P < 0.05$ *vs*. Vehicle group; †$P < 0.05$ *vs*. the corresponding values in WKY.

Earlier work using isolated segments of blood vessels reveals that endothelium-dependent vasorelaxation was depressed in hypertensive animals and patients [for reviews, see refs. 11 and 32]. Li and Bukoski (30) found that the acetylcholine (ACh)-induced vasorelaxation in isolated mesenteric artery segments was suppressed in SHR. However, they provided evidence to indicate that the impairment in vascular response to ACh was not the result of hypertension. Some vasoconstrictor agents used for "precontraction" of the vessel segment might blunt the endothelium-dependent relaxation. They concluded that the endothelium-dependent vasorelaxation was not impaired in hypertensive rats. Huang *et al*. (24) reported that eNOS-knockout mice lost endothelium-dependent vasorelaxation to ACh, but developed only mild hypertension (BP 117 mmHg in mutant type *vs*. BP 97 mmHg in wild type). However, they did not

describe the age, and neither followed the time course of changes in blood pressure in these transgenic mice. It would be interesting to see whether the mutant mice lacking eNOS will display time-dependent severe or malignant hypertension with cardiovascular changes like we have reported in SHR with early NO deprivation.

Previous studies from our laboratory revealed that the acute and chronic NO blockade caused increases in AP and total peripheral resistance. These changes were greater in SHR than in WKY (10, 20, 21). The findings suggest that NO function or bioactivity was enhanced in SHR. In a study using perfused mesenteric vessel bed, we also found that endothelium-dependent or -independent vasoconstriction and vasodilatation were greater in SHR compared with those in WKY (9). Evaluation of the NOS bioactivity with conversion of L-[14C]arginine to L-[14C]citrulline revealed that the increase in calcium-dependent NOS activity was greater in SHR than in WKY (17, 18, 36). These experimental results suggest that NOS activity and NO formation are enhanced in genetic hypertensive rats. The compensatory increase in NO release or function is important to keep the arterial pressure at a lower level in SHR. Removal of the compensatory mechanism caused a rapid progression to the malignant phase of hypertension (Fig. 1). Our results also suggest that eNOS, not iNOS, was involved. It remains to be determined whether the change in NO function or bioavailability is genetically inherent or related to shear stress and cyclic strain (3).

Another question is whether NO function or bioactivity is enhanced or suppressed in other hypertensive models or in humans with hypertension. Raij reported that NO activity was up-regulated in SHR, but down-regulated in Dahl salt hypertensive rats (36). In humans with hypertension, the endothelium-dependent or -independent forearm vasodilatation was impaired (34, 39). The involvement of NO and divergent endothelial functions in various hypertensive animal models, human, and organ systems requires further investigation.

Arnal *et al.* (1) report that treatment of L-NAME in WKY for four wks causes hypertension with mild VH. Prolonged treatment for 8 wks caused more VH in some animals. They speculate that the development of VH was related to the duration of NO blockade and renin-angiotensin system. Several studies report that overt VH developed following chronic NO deprivation (36, 40). In the present study, we also found that the presence of VH was obvious in WKY and SHR following early deprivation of NO with nonspecific NOS blockers. However, iNOS inhibition did not produce significant effects on the heart (Table 1).

To the best of our knowledge, our animal experiment presented the most rapid and drastic progression of SHR from prehypertensive to malignant phase in a short period (25-42 days) of NO deprivation. Severe impairment of renal functions was reflected by marked increases in plasma Cr, BUN, and UA (Table 2) with proteinurea, glomerular sclerosis, AIS and GIS (Table 4). The ERPF, GFR, and FF were significantly reduced (Table 5). Ono *et al.* (33) and Komatsu *et al.* (28) reported that in SHR of 21-wk-old, the glomerular functions and morphology were not significantly different from the age-matched WKY. Severe functional and structural changes in the kidney were observed in old-age SHR (73-wk-old). Treatment with L-NAME in 20-wk-old SHR for 3 wks developed glomerular damage comparable with that occurred in old-age SHR. Angiotensin converting enzyme inhibition reduced the impairment of glomerular functions and pathological changes. These findings suggest that the balance in L-arginine-NO and renin-angiotensin systems plays an important role in the pathogenesis of glomerular damage. In fact, Hayakawa and Raij (17) speculated that NO generated within the kidney *via* eNOS system might participate in the regulation of glomerular microcirculation by modifying the tone of arterioles and mesangial cells. In the present study, we provided strong evidence that eNOS, but not iNOS, was the major factor for glomerular homeostasis. Early and short deprivation of eNOS produced glomerular disorders. The extent of impairment in glomerular damage and renal functions was even greater than those observed in old-age SHR (73-wk-old) or SHR (20-wk-old) receiving L-NAME for 3 wks as compared with the data by Ono *et al.* (33) and Komatsu *et al.* (28).

Raij (36) and Hayakawa and Raij (18) found that the NO activity evaluated by conversion of L-[14C] arginine to L-[14C]citrulline was increased in SHR and supported their early speculation that NO is important for the regulation of glomerular function. Hill *et al.* (19) revealed that the reduction of NO production in aging rats (17-22 months) caused an increase in renal vascular resistance and renal functions. Recent studies also support the importance of NO formation in the renal functions. In rats with streptozotocin-induced diabetes, an increase in superoxide anion production provoks oxidative stress that leads to NO degradation and impairment of renal functions in rat with diabetes (25). In rats with hyperhomocystemia, oxidative stress and NO inactivation promot renal hemodynamic dysfunctions (13). Scholze *et al.* (38) find that acetylcysteine reduces plasma homocysteine and improves the renal function in patients with end-stage renal failure. The changes are associated with improved endothelial function as evaluated by the reduction in arterial pulse wave reflectance. Sabbatini *et al.* (37) report that atovastatin exerted beneficial effects on the acute renal failure in young and old rats subjected

to renal ischemia. The improvement of renal functions after acute renal failure following kidney ischemia is accompanied by an increase in eNOS-mRNA expression and eNOS protein in the kidney.

Morphological alterations and increased collagen fraction in the kidney suggest an increase in extracellular matrix. The plasma ET-1 was elevated at day 10 after NO blockade. ET-1 is a profribrotic factor that may cause extracellular matrix and collagen accumulation (41). It is noteworthy to observe that the ET-1 concentration was not further elevated after 10 days, treatment with L-NAME or L-NA. Whether the structural and functional changes occurred at this short period of time is a subject for future study. Furthermore, the involvement of gelatinases or matrix metalloproteinases in the pathological changes remains to be determined.

In conclusion, we reported that early deprivation of NO *via* the eNOS system, but not the iNOS caused severe hypertension in WKY and SHR. Our findings suggest that upregulation of eNOS is essential for the maintenance of blood pressure at a lower level. This compensatory mechanism mitigates the progression from genetic hypertension to malignant phase. Deprivation of NO in SHR is one of the culprits in the consequent severe structural and functional changes in the cardiovascular, neurological, and renal systems. These abnormalities following early NO deprivation may result in stroke, proteinurea, and sudden death.

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