**Oxidative Inactivation of Nitric Oxide and Endothelial Dysfunction in Stroke-Prone Spontaneous Hypertensive Rats**

XIN-LIANG MA, FENG GAO, ALLEN H. NELSON, BERNARD L. LOPEZ, THEODORE A. CHRISTOPHER, TIAN-LI YUE, and FRANK C. BARONE

Department of Surgery, Division of Emergency Medicine, Thomas Jefferson University, Philadelphia, Pennsylvania (X.-L.M., B.L.L., T.A.C.); Department of Physiology, Fourth Military Medical University, Xian, People’s Republic of China (F.G.); and Department of Cardiovascular Pharmacology, SmithKline Beecham Pharmaceuticals, King of Prussia, Pennsylvania (A.H.N., T.-L.Y., F.C.B.)

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**ABSTRACT**

This study tested the hypothesis that increased nitric oxide (NO) inactivation and concurrent peroxynitrite formation is responsible for endothelial dysfunction in the spontaneously hypertensive stroke-prone rat (SHRSP). In SHRSP, the aortic vasorelaxation to acetylcholine (ACh) was decreased (p < 0.05), but NO production was unchanged. Nitrotyrosine staining, a footprint of peroxynitrite (ONOO−) formation, was detected. Exposure of SHRSP to a high-salt, high-fat diet (SFD) further exacerbated hypertension and accelerated end-organ disease. A severe endothelial dysfunction [maximal ACh relaxation: 49.8 ± 2.1 versus 94.5 ± 1.8% in Wistar-Kyoto rats (WKY), p < 0.01], increased basal NO production (482 ± 17 versus 356 ± 21 nM, p < 0.01), decreased ACh-stimulated NO production (57 ± 6 versus 112 ± 6 nM, p < 0.01), extensive inducible NO synthase and nitrotyrosine staining, elevated nitrotyrosine content (21-fold increase over WKY), and a high percentage of cells with DNA damage were observed in the aortic tissues from these animals. Treatment of SHRSP on SFD with carvedilol restored ACh-induced vasorelaxation and NO production, inhibited nitrotyrosine formation, reduced vascular cell DNA damage, and reduced end-organ injury. These data demonstrate that endothelial dysfunction was caused by increased NO inactivation alone (SHRSP) or in combination with decreased NO production from endothelial NO synthase (SHRSP on SFD). Antioxidant treatment with carvedilol exerted significant vascular protective effects, attenuated end-organ damage, and decreased mortality under these conditions.

The vascular endothelium plays a key role in cardiovascular homeostasis through its diverse influences on blood vessel structure and function. In addition to its importance as a physical permeability barrier and as a site for metabolism of certain vasoactive substances, the endothelium elaborates proteins and other factors that confer antithrombogenic properties to the vessel wall (Mombouli and Vanhoutte, 1999). The endothelium also releases a host of paracrine and autocrine factors that not only influence vascular tone and permeability, but also play an important role in long-term vascular growth and remodeling. Among the various endothelium-derived molecules, nitric oxide (NO), a molecule produced in the endothelium by NO synthase (NOS) from l-arginine, has been demonstrated to play a pivotal role in the maintenance of normal cardiovascular function under physiologic conditions and the adaptation of the cardiovascular system under pathologic conditions (Arnal et al., 1999).

Numerous studies have demonstrated that endothelium-dependent vasorelaxation is markedly decreased in hypertensive patients and in experimental hypertensive animal models (Dominiczak and Bohr, 1995) (Grunfeld et al., 1995). However, the underlying mechanisms of this endothelial dysfunction are unknown, and the existing explanations are often controversial and seem to vary depending on the model studied. In Dahl salt-sensitive rats, the decrease in endothelium-dependent relaxation is associated with impaired endothelial NOS (eNOS) activity and decreased NO production (Boulanger, 1999). However, in other animal models of hypertension [such as the spontaneously hypertensive rat of the stroke-prone strain (SHRSP)] and in patients subjected to essential hypertension, endothelium-dependent vasorelaxation is markedly decreased even though eNOS expression and NO production have been found to be significantly increased (McIntyre et al., 1999). These results suggest that endothelial-generated NO may have been inactivated before it could reach its desired target (e.g., guanylate cyclase in...
vascular smooth muscle cells), thus resulting in a decreased bioactive NO concentration. In this regard, several studies have recently demonstrated that production of superoxide anion (O$_2^-$), a free radical that reacts with NO in a diffusion-limited rate, is markedly increased in vascular endothelial cells from SHRSP and essential hypertensive patients (Ts-chudi et al., 1996; Swei et al., 1997; Kerr et al., 1999). However, direct evidence demonstrating the inactivation of NO by superoxide anion and its concurrent production of peroxynitrite (ONOO$^-$), an extremely cytotoxic radical, in vascular tissue subjected to hypertension is not yet available.

Carvedilol, 1-[carbazol-4-(4-oxyl)-3-(2-methoxy-phenoxyethyl) amino]-propanol-(2), was originally introduced as a vasodilating $\beta$-adrenoceptor antagonist and has been used for the treatment of mild to moderate hypertension. Several recent clinical trials have consistently demonstrated that carvedilol exerts a superior cardiovascular protection over other $\beta$-adrenoceptor antagonist, such as bisoprolol and metoprolol (Packer et al., 1996; Metra et al., 2000). However, the mechanisms underlying carvedilol’s superior protective effects remain undefined. Moreover, we have recently demonstrated that in SHRSP with high-salt, high-fat diet (SF), treatment with carvedilol markedly decreased cardiomyopathy (Barone et al., 1998), renal damage, and mortality (Barone et al., 1996). Once again, the mechanism underlying its protection remains unclear.

Therefore, the aims of the present study were 1) to determine the mechanism of endothelial dysfunction (i.e., decreased NO production or increased NO destruction) in SHRSP; 2) to provide direct evidence of NO inactivation by O$_2^-$ and its concurrent formation of ONOO$^-$; and 3) to determine the effects of treatment with carvedilol on the severity of endothelial dysfunction and ONOO$^-$ formation in SHRSP, thus providing further insight into the mechanisms of cardiovascular injury and the end-organ protection exerted by carvedilol. Our general hypothesis was that oxidative inactivation, but not reduced production of NO, is responsible for endothelial dysfunction in SHRSP, and carvedilol, an antioxidant $\beta$-adrenoceptor antagonist, may reduce NO inactivation and improve endothelial function in these animals.

Materials and Methods

SHRSP. SHRSP progeny from the strain developed by Okamoto et al. (1974) were obtained from the National Institutes of Health and were bred in the Department of Laboratory Animal Science at Smith-Kline Beecham Pharmaceuticals. Animals were housed and cared for in accordance with the Guide for Care and Use of Laboratory Animals (Department of Health, Education, and Welfare, 1985). Procedures using laboratory animals were approved by the Institutional Animal Care and Use Committee of Smith-Kline Beecham Pharmaceuticals and Thomas Jefferson University. Male SHRSP at 10 weeks of age were adapted to individual cages and fed powdered National Institutes of Health-07 diet for 2 weeks.

When the rats reached 12 weeks of age, SHRSP were assigned to one of three groups on the basis of body weight and age (i.e., both parameters were balanced equally between groups) as described in our previous studies (Barone et al., 1996, 1998). One group of rats received normal drinking water (i.e., without added salt) ad libitum and regular National Institutes of Health-07 diet (SHRSP on normal diet, $n = 10$). The second group of rats received 1% NaCl as drinking water and were fed with National Institutes of Health-07 diet supplemented with 24.5% fat as described previously (SHRSP on SFD, $n = 12$) (Ogiku et al., 1993; Cosentino and Katusic, 1995; Dominiczak and Bohr, 1995). The third group of rats received the same SFD, but supplemented with 2400 ppm carvedilol (carvedilol SHRSP on SFD, $n = 12$). All diets were milled and formulated by Zeigler Brothers, Inc. (Gardiners, PA). An additional group of Wistar-Kyoto rats (WKY), at an age and weight that were comparable with that of SHRSP, served as normotensive controls on normal diet ($n = 10$). The general health of rats was monitored daily. Over the first 11 weeks of observation, SHRSP on SFD developed signs of morbidity known to result in mortality as described in our previous study (Barone et al., 1996). During the next 2 weeks, in vitro studies were performed in tissues from surviving rats that were sacrificed in groups of four (i.e., with tissue prepared from one rat from each group for comparison) as described below.

Plasma NOx Assay. Nitric oxide has a very short half-life (<10 s) and is oxidized to form NO$_2$ and NO$_3$ in vivo. Measurement of NOx (NO + NO$_2$ + NO$_3$) concentration in plasma has been demonstrated to reflect NO formation in vivo. When rats were prepared for sacrifice, they were anesthetized with pentobarbital sodium, 0.5 ml of blood was withdrawn from the left carotid artery, and plasma was obtained after centrifugation. To each 0.2 ml of plasma, 0.4 ml of ice-cold 100% alcohol was added and placed in ice for 30 min. The plasma-alcohol mixture was recentrifuged, and the supernatant was used to measure the concentration of NOx by using the vanadium reduction method (Ma et al., 1997). Briefly, 50 $\mu$l of sample was injected into a water-jacketed, oxygen-free purge vessel containing 5 ml of 0.1 M vanadium (III) chloride (Aldrich, Milwaukee, WI) in 2 N HCl (Sigma, St. Louis, MO). Acidic vanadium (III) at >80°C quantitatively reduces both nitrite and nitrate to NO, which is quantified by a chemiluminescence detector (SIEVERS 270B nitric oxide analyzer, SIEVERS, Boulder, CO) after reaction with ozone. Signals from the detector were collected and analyzed using a computer-based data acquisition and analyzing system (MacLab, ADInstruments, Inc., Millford, MA). Standard curves were obtained using the area under the curve after each injection of 50 $\mu$l of 0, 12.5, 25, 50, 75, and 100 $\mu$M sodium nitrate. The calculations to determine the NOx content of the plasma were done by the slope of the regression analysis using the linear formula $y = a + bx$.

Determination of Endothelium-Dependent, Nitric Oxide-Mediated Vasorelaxation. After blood was withdrawn, rats were then overdosed with pentobarbital. The chest was then opened and the thoracic aortas were carefully removed and placed into ice-cold Krebs-Henseleit (K-H) buffer consisting of (mmol/l) NaCl 118, KCl 4.75, CaCl$_2$-2H$_2$O 2.54, KH$_2$PO$_4$ 1.19, MgSO$_4$·7H$_2$O 1.19, NaHCO$_3$ 25, and glucose 10.0. Isolated vessels were cleaned of adhering fat and connective tissue and cut into rings 3 to 4 mm in length. The rings were then mounted onto stainless steel hooks, suspended in 7.5-ml tissue baths, and connected to FORT-10 force transducers (WPI, Sarasota, FL) to record changes via a MacLab data acquisition system. The baths were filled with 7.5 ml of K-H buffer and aerated at 37°C with a gas mixture of 95% O$_2$ and 5% CO$_2$. Aorta rings were initially stretched to give an optimal preload of 1 g of force and equilibrated for 60 min. During this period, the K-H buffer in the tissue bath was replaced every 20 min.

After equilibration, 100 $\mu$mol/l U-46619 (9,11-epoxyeicosanoids-prostaglandin H$_2$, BIOMOL Research Laboratories, Plymouth Meeting, PA), a thromboxane A$_2$ mimetic, was added to generate a maximal vasoconstriction. After the response stabilized, the rings were washed several times, and force was allowed to return to baseline values. Rings were then contracted submaximally (about 90% of maximal) by addition of 50 $\mu$mol/l U-46619, and cumulative relaxation curves to Ach (10$^{-9}$–10$^{-6}$ mol/l) were obtained to assess endothelium-dependent vasorelaxation. After the response stabilized, the rings were washed and allowed to equilibrate to baseline once again. The procedure was repeated with acidified NaNO$_2$, an endothelium-independent vasodilator (25–100 $\mu$mol/l). Acidified NaNO$_2$ was prepared by dissolving the compound in 0.1 N HCl and titrating it to pH 2.0. Titrating distilled water to pH 2.0, and adding aliquots
to the tissue bath did not produce any vasorelaxation (unpublished observation).

Determination of Total NO Release in Response to ACh Stimulation in Aortic Segments. In a separate study, rat aorta were isolated as described above and cut into segments 5 to 6 mm in length. Aortic segments were weighed and then placed individually in 24-well plates containing serum-free modified Eagle’s medium (1 ml/100 mg of tissue) supplemented with 2 mM l-arginine (Ing et al., 1999). To each well, ACh (final concentration: 10−5 M) or its vehicle (PBS) was added. The plate was covered and incubated at 37°C for 24 h in a humidified cell culture incubator. At the end of incubation, NOx concentration in culture buffer was determined in a similar manner as described above, except that a larger volume of sample solution was injected into the reaction vessel (i.e., 200 μl instead of 50 μl when plasma NOx was determined), and a lower concentration of sodium nitrate (0.3, 1, 3, and 10 μM) was used for constructing the standard curves. NOx concentration in vehicle-treated segments was defined as basal NO production. Differences in NOx concentration between vehicle-treated and ACh-treated segments were calculated and defined as ACh-stimulated total NO production.

Immunohistochemical Detection of iNOS Expression and Nitrotyrosine Formation in Aortic Vascular Tissue. In a separate study, four pentobarbital-anesthetized rats from each group were whole-body perfused with 200 ml of PBS followed by 300 ml of 10% formalin in PBS. Thoracic aorta was removed and stored in 10% formalin for less than 48 h. Fixed aortic segments were dehydrated and embedded in paraffin, and sections were cut at 6 mm and mounted onto glass slides. Immunohistochemical procedures for detecting iNOS and nitrotyrosine formation were performed according to the procedure published by Liu et al. (1997) and Beckman et al. (1994), respectively. Rabbit polyclonal antibody against nitrotyrosine was provided by Dr. Joseph Beckman as a gift (University of Alabama at Birmingham) and rabbit polyclonal antibody against iNOS was purchased from Upstate Biotechnology Inc. (Lake Placid, NY). The DAKO avidin-peroxidase kit (DAKO Corporation, Carpinteria, CA) was used for both iNOS and nitrotyrosine immunostaining.

Detection of Vascular Cell Apoptosis by Terminal Deoxynucleotidyl Transferase-Mediated dUTP-Biotin in Situ Nick-End Labeling (TUNEL). The TUNEL assay was performed in formalin-fixed and paraffin-embedded thoracic aorta tissue slides by using an apoptosis detection kit (Roche Molecular Biochemicals, Ridgefield, CT) according to the manufacturer’s instructions. The digoxigenin-conjugated dUTP was incorporated to the ends of DNA fragments by terminal deoxynucleotidyl transferase. The signal of terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling was then detected by an anti-fluorescein antibody conjugated with alkaline phosphatase, a reporter enzyme that catalytically generates a red-colored product from Vector red substrate.

Quantification of Nitrotyrosine in Aortic Tissue. Quantification of aortic tissue nitrotyrosine levels was performed by using a modified ELISA procedure (Tanaka et al., 1998; Ronson et al., 1999). In brief, aortic tissue was homogenized in ice-cold PBS (1:10 w/v) using a PRO 200 homogenizer first (PRO Scientific Inc., Monroe, CT, 60 s at 7000 rpm) followed by sonication with a dismembrator (Fisherton, CA) and expressed as nanograms per milliliter. The amount of nitrotyrosine present in the peroxynitrite-treated BSA solution was measured at 430 nm (ε₉₀ = 4400 M⁻¹ cm⁻¹) using a spectrophotometer (Beckman DU 640, Fullerton, CA) and expressed as nanograms per milliliter. The stock solution of the peroxynitrite-treated BSA was diluted with PBS (final nitrotyrosine concentration, 0.75-75 ng/ml). These standard samples, along with tissue samples from hearts (protein concentration, 4 mg/ml) were applied to disposable sterile ELISA plates (Corning Glassworks, Corning, NY) and allowed to bind for 1 h at 37°C in a microincubator shaker (Teltec Co., San Jose, CA). After blocking nonspecific binding sites with 1% BSA in PBS, the wells were incubated for 60 min at 37°C with a rabbit polyclonal anti-nitrotyrosine primary antibody (from Dr. Joseph Beckman, 1:500 in 10% goat serum PBS) and subsequently for 60 min at 37°C with a peroxidase-conjugated goat anti-rabbit IgG secondary antibody (1:1000, Amersham Pharmacia Biotech, Inc. Piscataway, NJ). After washing the plates, the peroxidase reaction product was generated using O-phenylenediamine dihydrochloride (2.2 mM) (Abbott Diagnostics, Abbott Park, IL). The plate was incubated for 20 min in the dark at room temperature, and the reaction was stopped by addition of 20 ml of 2 M H₂SO₄. The optical density was measured at 460 nm with a microplate reader (Bio Tek Instruments, Inc., Winooski, VT). The amount of nitrotyrosine content in tissue samples was calculated using standard curves generated from nitrated BSA containing known amounts of nitrotyrosine.

Statistical Analysis. All values in the text, table, and figures are presented as means ± S.E. of n independent experiments. All data were subjected to analysis of variance followed by the Scheffe’s correction for post hoc t test comparison. For nonparametric (%) data, the x² test for independent samples was utilized. Probabilities of 0.05 or less were considered to be statistically significant.

Results

Systolic Blood Pressure and Mortality. Table 1 summarizes systolic blood pressure, heart rate, and morbidity/mortality for the four groups of rats at week 11. The SFD significantly increased blood pressure further in the already significantly hypertensive SHRSP. Carvedilol did not reduce the extremely elevated blood pressure induced by SFD. Carvedilol did decrease heart rate in SHRSP, probably due to its β-adrenoreceptor antagonist activity. It also significantly eliminated the morbidity/mortality produced in SHRSP by SFD. These results were similar to those reported previously (Barone et al., 1996). During the next 2 weeks (weeks 12 and 13), rats were sacrificed in groups of four (i.e., one rat from each group), and in vitro tissue studies were conducted. In this manner, tissue was removed from healthy rats after group differences in morbidity were determined and tissues between groups were compared appropriately under the different experimental conditions.

Endothelium-Dependent Vasorelaxation in Aortic Rings. As summarized in Fig. 1, aortic rings isolated from WKY exhibited full relaxation to the endothelium-dependent
Nitric Oxide Production. To determine whether decreased NO production was responsible for the observed endothelial dysfunction in the aortic rings from SHRSP and SHRS on SFD, we measured NOx concentration in plasma, a reliable index for in vivo NO production. Plasma NO concentration was not significantly changed in SHRS on normal diet (36.6 ± 2.2 versus 37.6 ± 3.1 μM in WKY, p > 0.5), and was markedly increased in SHRS on SFD (59.2 ± 3.7 μM, p < 0.01 versus WKY and SHRS). In SHRS on SFD treated with carvedilol, plasma NO concentration was significantly decreased (44 ± 2.1 μM, p < 0.01 versus SHRS on SFD without carvedilol treatment). These results demonstrated that whole-body total NO production in the hypertensive animals was not decreased, and even significantly increased in hypertensive rats with SFD exposure.

Tracer studies in humans have demonstrated that as much as 90% of circulating NOx is derived directly from the metabolite of L-arginine by NOS, indicating that plasma NOx is a reliable quantitative index of NO production in vivo (Rhodes et al., 1995; Kelm et al., 1999). However, plasma NOx does not provide information regarding the cellular or organ origination of NO generation. To directly determine the effects of systemic hypertension on NO production in vascular endothelial cells, we further examined basal and Ach-stimulated NO release in aortic vascular segments isolated from the four experimental groups. In vascular segments from WKY, addition of Ach markedly increased NOx concentration (+112 ± 6 nM over vehicle). Consistent with plasma NOx concentration, basal NOx (366 ± 18 versus 354 ± 21 nM in WKY) and Ach-stimulated NOx (+105 ± 7 nM over basal) in aortic vascular segments were not significantly changed in SHRS with normal diet, suggesting that production from endothelial NOx was not decreased in aortic segments from SHRS. However, in vascular segments isolated from SHRS on SFD, the basal NOx concentration was markedly increased (482 ± 26 nM, p < 0.01 versus WKY and SHRS), but Ach-stimulated NOx was significantly decreased (+57 ± 6 nM, p < 0.01 versus WKY and SHRS). In addition, treatment with carvedilol significantly blunted the increase in basal NOx concentration (415 ± 23 nM, p < 0.05 versus vehicle-treated SHRS on SFD) and enhanced Ach-stimulated increase in NOx (+89 ± 5 nM, p < 0.01 versus vehicle-treated SHRS on SFD) (Fig. 2).

Inducible NOS Expression and Nitrotyrosine Formation in Aortic Vessels. To provide further insight into the mechanisms of endothelial dysfunction in hypertensive rats, we studied the expression of iNOS (Fig. 3, top) and formation of nitrotyrosine (Fig. 3, middle), a footprint of NO oxidation by reactive oxygen species in vivo, in the aortic vessels. Immunohistological staining showed that iNOS and nitrotyrosine residues were undetectable in aortic samples from WKY, suggesting that NO generated in this tissue by eNOS was not significantly oxidized by O$_2^-$ to produce a detectable nitrotyrosine formation. However, in the aortic samples isolated from SHRS, clear immunostaining was observed. Both iNOS and nitrotyrosine staining were primarily located on the luminal surface of the aortic segments, as well as in the adventitial tissues. Most notably, when SHRS were exposed to SFD, there were significantly more vessels staining with greater intensity for iNOS and nitrotyrosine in aortic vascular samples. Moreover, iNOS and nitrotyrosine staining were not only observed on the endothelial side of the
vessel, but also observed in the vascular wall, suggesting that ONOO− was present in the vascular smooth muscle cells. Treatment with carvedilol, an antioxidant β-blocker, in SHRSP on SFD markedly decreased iNOS expression and nitrotyrosine staining.

To determine further the alteration of nitrotyrosine formation in hypertensive animals and the effects of antioxidant treatment, tissue nitrotyrosine contents were measured quantitatively using an ELISA method. A very low nitrotyrosine concentration was detected in aortic tissues from WKY rats (36.8 ± 6.4 ng/g of protein). The nitrotyrosine content was significantly increased in SHRSP (104 ± 14.9 ng/g of protein, p < 0.05 versus WKY). Exposure of hypertensive rats to SFD further markedly increased nitrotyrosine content to a level that was 21 times higher than that of normal control (i.e., WKY) and 7.5 times higher than that of SHRSP on normal diet (775 ± 79 ng/g of protein, p < 0.01 versus WKY and SHRSP on normal diet). Moreover, treatment with carvedilol significantly attenuated nitrotyrosine increase in SHRSP on SFD (236.9 ± 38 ng/g of protein, p < 0.01 versus SHRSP on SFD receiving only vehicle). Taken together, these results demonstrate that although NO generation by the endothelium was unchanged in SHRSP on SFD, NO production were markedly decreased in SHRSP on SFD, which was significantly decreased in the same preparation. In addition, our immunohistochemical results demonstrate that clear nitrotyrosine staining can be observed in vessels from SHRSP, although carvedilol-induced vasorelaxation was significantly decreased in the same preparation. In addition, our immunohistochemical results demonstrate that clear nitrotyrosine staining can be observed in vessels from SHRSP, although carvedilol-induced vasorelaxation was significantly decreased in the same preparation. In addition, our immunohistochemical results demonstrate that clear nitrotyrosine staining can be observed in vessels from SHRSP, although carvedilol-induced vasorelaxation was significantly decreased in the same preparation. In addition, our immunohistochemical results demonstrate that clear nitrotyrosine staining can be observed in vessels from SHRSP, although carvedilol-induced vasorelaxation was significantly decreased in the same preparation.

Vascular Cell Apoptosis. Data from the NOX production study in isolated aortic segments showed that although basal NOX production was markedly increased in aortic segments from SHRSP on SFD, ACh-stimulated NOX production in these vessel segments was significantly decreased. This result suggested that endothelial cells might have been injured by the high concentration of ONOO−. As illustrated in Fig. 3 (bottom), no significant TUNEL positive cells were observed in the aortic section from either control WKY or SHRSP. However, a large number of cells were TUNEL positive in the aortic segments from SHRSP on SFD, which was significantly inhibited by carvedilol treatment.

Discussion

The major novel findings of the current study are that 1) in SHRSP on SFD, ACh-stimulated vasorelaxation and vascular NO production were markedly decreased, but whole-body NO production and vascular basal NO production were significantly enhanced; 2) high salt and high fat intake in SHRSP resulted in a marked induction of iNOS expression, massive formation of the highly toxic peroxynitrite, and significant endothelial as well as smooth muscle cell death, likely via apoptosis; and 3) carvedilol treatment in SHRSP on SFD significantly suppressed iNOS expression, decreased nitrotyrosine formation, improved endothelial function, and reduced vascular cell death.

Growing evidence suggests that an increased breakdown of NO by free radicals is a major mechanism for hypertension-induced impairment of NO-mediated vasorelaxation (Harri-son, 1997). Our present study confirms previously published results that in SHRSP on normal diet, endothelium-dependent vasorelaxation is significantly decreased but total plasma NOX concentration is unchanged (McIntyre et al., 1999). Because plasma NOX reflects whole-body total NO production and is not vascular specific, we further directly measured the basal and ACh-stimulated NO production in aortic segments. Our results showed, for the first time, that ACh-stimulated NO production was normal in vascular segments from SHRSP, although ACh-induced vasorelaxation was significantly decreased in the same preparation. In addition, our immunohistochemical results demonstrate that clear nitrotyrosine staining can be observed in vessels from SHRSP, but not from WKY. Quantitative assays indicate that nitrotyrosine content in vessels from SHRSP was 3 times higher than that in vessels from WKY. These results provide additional evidence that oxidative inactivation, not decreased production, of NO is responsible for endothelial dysfunction in SHRSP on normal diet.

Elevating dietary sodium intake for SHRSP over a prolonged period (>6 weeks) has been consistently shown to
Accelerate the onset of stroke and increase mortality (Cama-\text{r}o et al., 1993). In our previous study, we provided a high-fat and high-salt diet to SHRSP of a specific age range and established optimal conditions for accelerated morbidity and mortality (Barone et al., 1996, 1998). The mechanisms underlying these profound deleterious effects of adverse diet are not completely understood. Although SFD further increased blood pressure, it seems unlikely that the lethal effects of SFD could be completely attributed to the systolic blood pressure change since carvedilol does not affect this

Severe hypertension. It has been recently reported that in hypertensive rats, high-salt diet (4 weeks) further increased $O_2^-$ generation in the microvessels of the mesentery (Swei et al., 1997), suggesting that overproduction of reactive oxygen species may play an important role. However, effect of high-salt loading on NO production is not clear, and previous results obtained from short-term high-salt loading (<2 weeks) are controversial (Shultz and Tolins, 1993; Campese et al., 1996; Higashi et al., 1996; Fujiwara et al., 2000). More importantly, whether highly toxic ONOO$^-$ was formed in a large quantity under this condition, thus contributing to the end-organ damage and high mortality, has not been previously studied.

In the present study, we have demonstrated that sustained exposure of SHRSP to SFD markedly increased iNOS expression and total NOx concentrations in the plasma. Moreover, although basal NOx concentration was markedly increased, ACh-stimulated increase in NOx production was significantly decreased in the incubation buffer containing aortic segments isolated from these animals. ACh-induced vasorelaxation, a measure of the level of NO bioactivity, decreased to a level that was only about 50% of WKY and 70% of SHRSP without SFD exposure. These results suggest that NO production from the normal constitutive eNOS was significantly reduced, whereas NO production from inducible calcium/calmodulin-independent nitric-oxide synthase (i.e., iNOS) that was highly expressed in the vascular wall in these animals was dramatically increased. Therefore, the severe endothelial dysfunction observed in hypertensive animals exposed to SFD probably results from a combination of increased inactivation of NO by reactive oxygen species and decreased NO production from eNOS. Our present experiment has provided clear evidence supporting this conclusion. In vascular segments from SHRSP on SFD, nitrotyrosine content was dramatically increased (21-fold versus WKY control animals and 7.5-fold versus SHRSP on normal diet), indicating that increased oxidative NO inactivation occurred in these vessels. On the other hand, positive TUNEL staining, an index of DNA fragmentation and cell death, was significantly increased in vascular endothelial cells in the vascular segments isolated from SHRSP on SFD, indicating that not only functional, but also structural, injury has occurred in the vascular endothelial cells.

Superoxide and NO react at a diffusion-limited rate ($k_{\text{r}} = 6.7 \times 10^9 \text{ M}^{-1} \text{s}^{-1}$) (Beckman and Koppenol, 1996). This bi-radical reaction can be harmful in at least two ways, first by removing the beneficial effects of NO, and second by producing a highly toxic product, ONOO$^-$. In in vitro and cell culture studies, ONOO$^-$ has been shown to be highly reactive with a wide variety of molecules, including deoxyribose, cellular lipids, and protein sulfhydryl moieties, and it causes direct oxidative tissue damage apparently similar to that caused by $\cdot$OH in vitro (Beckman and Koppenol, 1996). In a recent study, Arstall et al. (1999) demonstrated that iNOS expression and subsequent ONOO$^-$ formation plays a pivotal role in myocyte apoptosis induced by cytokine stimulation. In the present study, we have provided evidence that iNOS expression and ONOO$^-$ formation is probably a major cause of vascular endothelial as well as smooth muscle cell death occurring in SHRSP on SFD and may thus play a significant role in the end-organ injury and morbidity/mortality demonstrated in this animal model.
In our previous studies using the same animal model, we demonstrated that administration of carvedilol in SHRSP on SFD prevented hypertensive cardiomyopathy, attenuated renal damage, and reduced mortality (Barone et al., 1996, 1998). The present study provides direct evidence that the organ-protective effects of carvedilol observed in our previous study may be related to its vascular protective effects. We have demonstrated that carvedilol treatment markedly reduced nitrotyrosine formation, decreased vascular cell death, and improved endothelial function. In addition, we have provided the first evidence that in addition to its well defined superoxide anion scavenging property, carvedilol markedly inhibits iNOS expression in vascular segments. Therefore, carvedilol can inhibit NOO− formation by reducing not only O2−, but also NO production from iNOS. However, treatment with carvedilol failed to reduce the systolic blood pressure in these animals. This was likely due to the extreme hypertention in SHRSP on SFD. Therefore, α1-adrenergic receptor blockade and endothelial functional improvement afforded by carvedilol treatment was not sufficient to effectively reduce the systolic blood pressure in these animals. Moreover, we recently demonstrated that endothelin plays a significant role in the premature morbidity/mortality exhibited in SHRSP on SFD; mixed inhibition of endothelin A and endothelin B receptors reduced end-organ damage (Barone et al., 2000). It is thus possible that the combined treatment of carvedilol with endothelin receptor inhibitor may exert the best protection in SHRSP on SFD.

In summary, our present experimental results suggest that oxidative inactivation, but not reduced production of NO, is responsible for endothelial dysfunction in SHRSP on normal diet. However, when SHRSP animals were exposed to SFD, a severe endothelial dysfunction occurred, probably because of a combination of increased NO inactivation and a decreased NO production from eNOS. A massive production of NOO− in the vascular wall under this pathologic condition is a major cause of vascular endothelial and smooth muscle cell injury. Pharmacological intervention to inhibit NOO− formation is important and may be useful in the prevention and treatment of a host of cardiovascular diseases common to the Western world.

References


Address correspondence to: Dr. Xin-Liang Ma, Department of Surgery, Division of Emergency Medicine, Jefferson Medical College, 1020 Walnut St., Philadelphia, PA 19107-5004. E-mail: xin.ma@mail.tju.edu