Nitric Oxide Synthase Isotype Expression in Salt-Sensitive and Salt-Resistant Dahl Rats

Zhenmin Ni, Fariba Oveisi, Nosratola D. Vaziri

Abstract—Previous studies have suggested that salt-sensitive hypertension in humans and experimental animals may in part be due to dysregulation of the L-arginine/nitric oxide system. This study was conducted to determine the endothelial, inducible, and neuronal nitric oxide synthase expressions in the kidney, heart, aorta, and brain of salt-sensitive and salt-resistant Dahl rats. We studied salt-sensitive and salt-resistant Dahl rats maintained on high- (8%) and regular-(0.2%) salt diets for 3 weeks. Blood pressure was modestly elevated in both Dahl salt-sensitive and salt-resistant rats consuming regular diet and severely increased in sensitive but not resistant rats consuming the high-salt diet. The Dahl salt-sensitive animals showed a significant reduction in kidney, heart, and aorta inducible nitric oxide synthase protein abundance on the regular diet, with further reductions on the high-salt diet. In addition, the high-salt diet markedly downregulated endothelial nitric oxide synthase expression in the kidney and aorta but not in the heart of the Dahl salt-sensitive animals. The rise in blood pressure in the Dahl salt-sensitive rats on the high-salt diet was accompanied by a significant elevation of brain neuronal nitric oxide synthase protein. In contrast, salt-resistant animals showed no change in heart, kidney, and aorta endothelial or brain neuronal nitric oxide synthase and considerably less intense changes in inducible isotype than that seen in the salt-sensitive group in response to the high-salt diet. In conclusion, the study revealed a marked downregulation of inducible nitric oxide synthase in the Dahl salt-sensitive rats on the regular diet, with further reductions on the high-salt diet. Furthermore, Dahl salt-sensitive rats consuming the high-salt diet showed significant reductions of kidney and aorta endothelial nitric oxide synthase and an upregulation of brain neuronal nitric oxide synthase expression. (Hypertension. 1999;34:552-557.)

Key Words: nitric oxide • nitric oxide synthase • hypertension, sodium-dependent • sodium, dietary • blood pressure • heart • kidney

As in salt-sensitive hypertensive humans, blood pressure markedly rises in Dahl salt-sensitive (DS) rats maintained on a high sodium diet.1,2 The rise in blood pressure in response to a high-salt diet in the DS animals is associated with and largely due to the rise in peripheral and renal vascular resistance.3,4 This contrasts with the vasodilatory response to volume expansion in normal animals. The defective vascular response in DS rats to high-salt intake could be due to impaired production or action of vasodilatory factors. In this regard, alterations of atrial natriuretic peptide,3,5,6 arachidonic acid metabolites,7 the kallikrein-kinin system,8 and the L-arginine/nitric oxide (NO) pathway have been considered. Several studies have suggested that impaired metabolism of NO (otherwise known as endothelium-dependent relaxing factor) may play an important role in the pathogenesis of hypertension in DS rats. For instance, Chen and Sanders9,10 have shown that high salt–induced hypertension and nephrosclerosis can be prevented by the NO precursor L-arginine in DS rats. In another study, L-arginine supplementation was shown to normalize renal hemodynamic abnormality in hypertensive DS rats.11 In addition, the marked rise in total body NO production (discerned from urinary excretion of total nitrates and nitrates) seen in Sprague-Dawley rats on a high-salt diet does not occur in DS animals.9 Moreover, urinary excretions of NO metabolites and cGMP, the second messenger of NO, are reportedly reduced in hypertensive DS rats, and these abnormalities can be reversed by L-arginine administration.9 The role of impaired NO generation and/or action is further supported by marked reduction of endothelium-dependent relaxation response to acetylcholine, thrombin, and adenosine diphosphate in aortic rings taken from DS rats.12,13 Furthermore, pharmacological disruption of NO production by chronic nitric oxide synthase (NOS) inhibition in genetically normal animals leads to blunted pressure natriuresis,14 elevated renal vascular resistance,15,16 and hypertension.16–18 Together, these observations point to the role of NO in regulation of renal vascular resistance in normal animals and to defective L-arginine/NO response to high salt intake in DS animals. In fact, diminished renal tissue expression or activities of various NOS isotypes has been recently reported in DS rats on high salt intake.19–21

Received February 18, 1999; first decision March 11, 1999; revision accepted June 15, 1999.

From the Division of Nephrology, Department of Medicine, University of California, Irvine.

Reprint requests to N.D. Vaziri, MD, MACP, Division of Nephrology, Department of Medicine, UCI Medical Center, 101 The City Dr, Orange, CA 92868. E-mail tabotten@uci.edu

© 1999 American Heart Association, Inc.

Hypertension is available at http://www.hypertensionaha.org
Impaired NO production in response to salt loading in DS animals could be due to either decreased substrate availability, NOS deficiency, and/or accelerated NO inactivation. However, plasma l-arginine concentration is reportedly normal in DS animals, thereby excluding substrate deficiency as a likely possibility. 22 Although several recent studies have explored the effect of altered dietary salt intake on NOS isotype expression or activity in DS rats, comprehensive data on NOS isotype expression in various organs are lacking. This is particularly relevant since the contribution of different organs to the elevation of total vascular resistance is unequal and the pattern of regional hemodynamic changes varies greatly in this form of hypertension. 23 The present study was undertaken to determine the renal, vascular, cardiac, and brain tissue expressions of endothelial NOS, inducible NOS, and neuronal NOS (eNOS, iNOS, and nNOS, respectively) proteins in DS rats.

### Methods

#### Animal Models

Twelve-week-old male salt-sensitive Brookhaven strain of DS rats and age-matched male salt-resistant Dahl (DR) rats (Harlan Sprague-Dawley, Inc) were used. The animals were housed in a temperature-controlled, light-regulated space with 12-hour dark and light cycles. The DS and DR animals were randomly assigned to those fed a rat chow containing high-salt (8% NaCl) or a regular rat chow (0.2% NaCl). Six animals were included in each group. The animals were maintained on the designated diets for 3 weeks. Blood pressure was monitored with a tail cuff (Harvard Apparatus) as previously described, 24 and body weight, serum creatinine, and creatinine clearance were determined by standard methods. At the end of the 3-week period, the animals were anesthetized with an injection of pentobarbital sodium (50 mg/kg IP) and killed by exsanguination. Thoracic aorta, brain, heart (left ventricle), and kidney were promptly harvested and immediately frozen in liquid nitrogen and stored at −80°C until processed.

#### NOS Protein Assay

Homogenates were prepared from the frozen tissues for Western blot analysis and total protein measurement, as previously described. 24 Western blot analysis was used to determine the eNOS, nNOS, and iNOS proteins in the tissue preparations with the use of the respective monoclonal antibodies, as previously described. 24–27

#### Data Presentation and Analysis

ANOVA, Duncan’s multiple range test, and Student’s t test were used in statistical evaluation of the data, which are presented as mean ± SEM.

### Results

#### General Findings

Both the DS and DR rats showed moderate hypertension of similar magnitude on a regular diet. The DS rats consuming the high-salt diet showed a marked rise in blood pressure compared with the DS group receiving the regular diet (P<0.01). In contrast, the high-salt diet did not significantly affect blood pressure in the DR animals. No significant difference was found in either hematocrit or creatinine clearance between the groups (Table).

#### NOS Data

##### Aorta

No significant difference was found in the aorta eNOS protein expression between the DS rats and DR rats consuming a regular diet. Consumption of the high-salt diet resulted in a marked downregulation of the aorta eNOS protein in the DS animals but had no significant effect on eNOS expression in the DR group. Aorta iNOS protein abundance in the DS group consuming the regular diet was significantly lower than that of the DR group on the regular diet. Consumption of the high-salt diet for 3 weeks resulted in a dramatic fall in aorta iNOS abundance in the DS group and a significant but less intense fall in the DR group (Figure 1). Aorta total NOS activity in the DR animals consuming the regular diet was higher than that in the corresponding DS group. Consumption of the high-salt diet led to a severe fall in aorta NOS activity in the DS group and a less intense drop in the DR group (Table).

##### Heart

There was no significant difference in the cardiac tissue eNOS protein abundance between the DS and DR animals consuming the regular diet. The high-salt diet resulted in a mild but significant rise in cardiac eNOS protein expression in the DS group but had no significant effect in the DR group. Cardiac iNOS expression in DS and DR animals consuming the regular diet was similar. Consumption of the high-salt diet for 3 weeks led to a marked fall in cardiac iNOS protein abundance in the DS group but a significant rise in the DR group (Figure 2).

##### Kidney

Kidney eNOS protein abundance in DS and DR rats consuming the regular diet was similar. The high-salt diet did not significantly alter renal tissue eNOS protein in either DS or DR animals. Compared with DR animals, the DS animals

| Systolic Blood Pressure, Hematocrit, Creatinine Clearance, and Aorta Total NOS Activity in DS and DR Rats Consuming High-Salt and Regular Diets |
|-------------------------------|-----------------|-----------------|-----------------|
| **Systolic Blood Pressure, mm Hg** | **Hematocrit, %** | **Creatinine Clearance, mL (min)** | **Aorta NOS Activity, pmol (min - mg protein)** |
| DS-R | 155±10.0 | 43±2.6 | 2.1±0.2 | 0.49±0.03 |
| DS-H | 216±12.0* | 43±2.1 | 2.0±0.6 | 0.22±0.04* |
| DR-R | 160±3.1 | 41.5±0.65 | 2.2±0.7 | 0.74±0.11* |
| DR-H | 162±3.7 | 40.0±1.22 | 2.3±0.7 | 0.43±0.07 |

- *P<0.01 vs other groups.

R indicates regular diet; H, high-salt diet.
showed a severe reduction in kidney tissue iNOS protein abundance on the regular diet and virtually undetectable levels on the high-salt diet. The DR animals showed substantial iNOS expression on the regular diet and exhibited a significant reduction in kidney iNOS expression on the high-salt diet (Figure 3).

Brain
Brain nNOS in the DS rats consuming the regular diet was significantly higher than that of the DR rats consuming the regular diet. Consumption of the high-salt diet for 3 weeks resulted in a significant rise in brain nNOS protein abundance in the DS group but no significant change in the DR group, mirroring the changes in blood pressure (Figure 4).

Discussion
Previous studies undertaken to discern the possible involvement of NOS isotypes in salt-sensitive hypertension in rats have yielded contradictory results. For instance, Chen and Sanders\(^{22}\) have reported that administration of the iNOS inhibitor dexamethasone completely reversed salt resistance in DR rats, suggesting the regulatory role of iNOS in response to a high salt intake. In support of the latter conclusion, Ishimitsu et al\(^{28}\) have shown that administration of interleukin-2, an iNOS inducer, lowers blood pressure in hypertensive DS animals. In contrast, Westberg et al\(^{29}\) found no significant reduction in blood pressure in hypertensive DS rats with immune activation (using pertussis toxin in Freund’s
adjuvant) despite a significant increase in NO production (urinary total nitrates and nitrites). However, they showed that L-arginine administration prevented hypertension without a dramatic rise in NO production in these animals. They therefore concluded that whole body NO production may not be the determining factor in the pathogenesis of hypertension in DS animals. Persistent hypertension in immunized animals despite increased total NO production is most likely due to inactivation of NO by oxygen free radicals produced by activated leukocytes and macrophages.30 Interestingly, DS rats exhibit an increased renal vascular resistance in response to high salt intake before the onset of hypertension and the rise in total vascular resistance.3,4,31 The abnormalities in renal vascular response to high salt intake in DS rats are associated with diminished NO production,31 decreased renal NOS activity,21 and increased \( \text{N}^\text{G} \),\( \text{N}^\text{G} - \text{dimethyl-L-arginine} \), an endogenous NOS inhibitor.32 Furthermore, vasodilatory response to acetylcholine is impaired in hypertensive DS rats.12,13 Hypertensive DS rats also show a profound reduction of NO production by isolated perfused kidneys compared with normotensive controls, both at baseline and after acetylcholine stimulation.33 In addition, L-arginine supplementation normalizes renal hemodynamic abnormalities in DS rats with salt-induced hypertension.11 Together those observations point to the role of NO deficiency in the pathogenesis of salt sensitivity and provide indirect but compelling evidence for depressed NOS activity in DS animals on a high-salt diet.

Data on the effects of high salt intake on NOS isotype expression of extrarenal organs in DS rats are limited. In the present study we investigated the protein expressions of NOS isoforms in multiple organs, including heart, aorta, brain, and kidney. The data showed that iNOS protein expression is profoundly diminished in kidney, aorta, and heart of the DS rats compared with the DR group. Moreover, iNOS deficiency in these organs was aggravated by high salt intake. These findings strongly support the possible role of iNOS deficiency in the genesis of hypertension in DS rats. In this regard, Deng and Rapp34 recently demonstrated a strong cosegregation of iNOS alleles with blood pressure in the F2 population derived from a cross of inbred DS rats with Milan normotensive rats. On the basis of these observations, they suggested that iNOS may be a candidate for being the quantitative trait locus involved in the pathogenesis of hypertension in DS rats. However, a subsequent study by Deng35 using congenic strains in which regions of chromosome 10 in DS rats were substituted with the homologous regions of Milan normotensive rats containing iNOS gene failed to identify the iNOS gene as a candidate for being the quanti-

**Figure 3.** Representative Western blot and corresponding group data illustrating kidney iNOS (top) and eNOS (bottom) protein abundance in DS and DR rats on high-salt diet (DS-H and DR-H, respectively) and regular diet (DS-R and DR-R, respectively); n=6 in each group. *P<0.01, high-salt (H) vs regular diet within each group. #P<0.01, vs corresponding value in the other group.

**Figure 4.** Representative Western blot and corresponding group data illustrating brain nNOS protein abundance in DS and DR rats on high-salt diet (DS-H and DR-H, respectively) and regular diet (DS-R and DR-R, respectively); n=6 in each group. *P<0.01, high-salt (H) vs regular (R) diet within each group. #P<0.01, vs corresponding value in the other group.
tative trait locus capable of causing hypertension. He did, however, acknowledge the role of the NO system in the pathogenesis of hypertension in DS rats. Possible involvement of iNOS deficiency in the pathogenesis of salt-sensitive hypertension is further suggested by the observation that l-arginine–induced fall in blood pressure in DS rats maintained on high-salt diets was prevented by pretreatment with dexamethasone, which inhibits iNOS production. The results of the present study provide direct evidence for downregulation of iNOS expression in DS rats. The mechanism responsible for downregulation of iNOS expression in this model is unclear and awaits investigation.

Available data on the involvement of eNOS in the genesis of hypertension and functional dysregulation of kidney and other organs in DS rats are limited. In the present study we demonstrated marked alterations of eNOS protein expression in some but not all organs of DS animals. For instance, renal eNOS expression was similar in DS and DR rats on both diets. Thus, eNOS does not appear to explain the difference in their salt sensitivity. In contrast, high salt intake resulted in a marked reduction of aorta eNOS only in DS rats whose blood pressure rose but not in DR rats whose blood pressure did not rise. Thus, downregulation of aorta eNOS in DS rats may have been a consequence of severe extended hypertension. These observations support a recent view of the prevailing uncertainty, it is not clear whether the DS animals on high salt intake, shown here, are not clear. In particular, renal eNOS expression was similar in DS and DR rats on both diets. Thus, eNOS does not appear to explain the difference in their salt sensitivity. In contrast, high salt intake resulted in a marked reduction of aorta eNOS only in DS rats whose blood pressure rose but not in DR rats whose blood pressure did not rise. Thus, downregulation of aorta eNOS in DS rats may have been a consequence of severe extended hypertension with the high-salt diet. These observations support a recent study by Hayakawa and Raij that showed depressed eNOS activity in renal medulla and thoracic aorta of DS rats on high salt intake. Interestingly, heart eNOS protein increased in our DS but not in the DR rats maintained on the high-salt diet. The disparity between the heart and aorta eNOS protein expressions in DS rats consuming the high-salt diet, shown here, is consistent with the results of the recent study by Hayakawa and Raij, which demonstrated a marked reduction in eNOS enzymatic activity in the aorta but not the left ventricle of hypertensive DS rats.

Our DS, but not DR, animals maintained on the high-salt diet showed a marked elevation of brain nNOS protein. nNOS is normally expressed in several areas of the brain and is considered by some investigators to be involved in the neurogenic regulation of blood pressure. In particular, nNOS appears to be an integral component of the neuronal pathways that inhibit brain stem sympathetic outflow. Accordingly, nNOS-derived NO in the brain is thought to lower vascular resistance and blood pressure by diminishing central sympathetic outflow. However, acute and chronic pharmacological inhibition of NOS in eNOS knockout mice has been reported to paradoxically lower blood pressure. The precise mechanism responsible for and the functional significance of increased brain nNOS protein expression in DS animals on high salt intake, shown here, are not clear. In view of the prevailing uncertainty, it is not clear whether the observed upregulation of brain nNOS represents a compensatory response to high salt intake and/or the associated hypertension in these animals. However, the lack of a rise in brain nNOS, despite the high-salt diet in our DR rats who did not exhibit a further rise in blood pressure, tends to favor the role of blood pressure as opposed to a high-salt diet per se.

In summary, the DS rats consuming a regular diet exhibited a profound reduction of renal and cardiac iNOS protein and depressed aorta eNOS protein abundance. The high-salt diet resulted in marked downregulations of aorta iNOS and eNOS proteins and a significant upregulation of brain nNOS protein, coupled with further reductions of renal and cardiac iNOS abundance. Thus, DS animals exhibit profound alterations of various NOS isoform expressions in different tissues.

References
Nitric Oxide Synthase Isotype Expression in Salt-Sensitive and Salt-Resistant Dahl Rats
Zhenmin Ni, Fariba Oveisi and Nosratola D. Vaziri

Hypertension. 1999;34:552-557
doi: 10.1161/01.HYP.34.4.552

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1999 American Heart Association, Inc. All rights reserved.
Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://hyper.ahajournals.org/content/34/4/552

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Hypertension can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Hypertension is online at:
http://hyper.ahajournals.org//subscriptions/