Vascular Smooth Muscle Nitric Oxide Synthase Anomalies in Dahl/Rapp Salt-Sensitive Rats

Pei Yan Chen, Reginald D. Gladish, Paul W. Sanders

Abstract—Salt-sensitive hypertension in the Dahl/Rapp rat (S strain) is prevented by L-arginine. Based on the observations that dexamethasone prevented the antihypertensive effect of L-arginine in these animals and the suggestion that a locus in or near an inducible nitric oxide synthase (NOS) gene on chromosome 10 cosegregated with hypertension in some F2 crosses that utilized the S rat, the present study explored the hypothesis that the vascular smooth muscle isoform of inducible NOS (NOS2) was abnormal in S rats. Primary cultures of aortic smooth muscle cells from S rats demonstrated impaired inducible NO production, which improved with increased L-arginine in the medium. Sequence analysis identified a single T→C transversion that produced an amino acid substitution (S714P) between the FAD and FMN binding sites and a restriction fragment length polymorphism. This restriction fragment length polymorphism was present only in S rats. The mutation of NOS2 and the role of this enzyme in the pathogenesis of salt-sensitive hypertension in the Dahl/Rapp rat require further investigation. (Hypertension. 1998;31:918-924.)

Key Words: endothelium-derived relaxing factor ■ genetics ■ genes ■ arginine ■ vasodilation

An increase in blood pressure that occurs with augmented dietary salt intake is considered an abnormal response that produces end-organ damage, including cerebrovascular accidents and renal failure. Perhaps as many as 25 million Americans manifest salt-dependent hypertension. Despite intensive efforts, the pathogenesis of salt-sensitive hypertension remains unknown. A compelling model of genetic salt-sensitive hypertension is the Dahl/Rapp rat. The Dahl/Rapp salt-sensitive (SS/Jr, termed S here) rapidly develops hypertension on high-salt chow, while the Dahl/Rapp salt-resistant (SR/Jr, termed R) remains completely resistant to the blood pressure effects of dietary salt.1,2

Blood pressure may be considered to represent the net effect of vasoconstrictor and vasodilator influences. Several studies3-8 have shown that the endogenous nitrovasodilator, or L-arginine:NO, pathway plays an important role in regulation of blood pressure in response to changes in dietary salt. In S rats, administration of L-arginine increased production of nitrite/nitrate and cyclic GMP and prevented hypertension that occurred in response to an increase in dietary salt.3-5 Simultaneous administration of dexamethasone, which prevents induction of the vascular smooth muscle isoform of the inducible NOS (NOS2),9-11 abolished the L-arginine–induced increase in NO production and permitted hypertension to develop.5 On basis of these data, the present studies were performed specifically to examine NOS2 in the S rat.

Methods

Blood Pressure Determination

All experimental protocols using animals were approved by the Animal Resources Program of the University of Alabama at Birmingham and followed institutional guidelines. To demonstrate that the regenerated S rats were hypertensive and had the same hypertensive response to L-arginine as they had previously,3,5 we repeated some of our original experiments.3 S, R, and SD rats (all from Harlan Sprague-Dawley, Indianapolis, Ind), 3 weeks of age, were placed on 0.3% NaCl chow (Dyets, Inc) for 2 weeks; the diet was then changed to 8.0% NaCl chow (Dyets, Inc) for an additional 2 weeks. The rats were then anesthetized using ethyl-1-methylpropylthiobarbiturate (Inactin, BYK-Gulden), 100 mg/kg IP, and prepared surgically in standard fashion.3,5 The left femoral artery was cannulated to record MAP with a pressure transducer (Cobe Laboratories, Inc) attached to an analog/digital converter (MacLab 8, ADInstruments, Inc) and a computer software program (MacLab II, ADInstruments, Inc). As we have done previously,3,5 300 mg/kg L-arginine (Sigma Chemical Co) was given as an intravenous bolus, followed by infusion of L-arginine (3 mg/kg per minute) in Ringer-bicarbonate. MAP was determined 30 minutes after the bolus was administered.

Analysis of Polymorphic Microsatellite-Based Genetic Markers

Previous studies by Lewis and associates12 had shown that three markers—R354, R721, and R1041—identified polymorphisms between genetically contaminated (S*) and S rats. To show that the rats used in these studies were completely inbred, three sets of PCR primers (MapPair, Research Genetics, Huntsville, Ala) were used to amplify the informative segments of genomic DNA that contained dinucleotide repeats and were potentially polymorphic. The primers

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Selected Abbreviations and Acronyms

ASMC = aortic vascular smooth muscle cell
IFN-γ = interferon gamma
LPS = E. coli lipopolysaccharide
MAP = mean arterial pressure
NO = nitric oxide
NOS2 = inducible vascular smooth muscle NOS enzyme
Nos2 = inducible vascular smooth muscle NOS gene
PCR = polymerase chain reaction
R = Dahl/Rapp salt-resistant rat strain
RFLP = restriction fragment length polymorphism
S = Dahl/Rapp salt-sensitive rat strain
SD = Sprague-Dawley rat

and methods to accomplish this analysis has been published. Briefly, genomic DNA was obtained from R, genetically contami-
nated S (S’), and regenerated S rats in standard fashion using phenol extraction and ethanol precipitation. Genomic DNA from S’ was a generous gift of Dr James L. Lewis, University of Alabama at Birmingham. After an initial melting at 95°C for 5 minutes, the DNA was amplified through 35 cycles of 94°C for 1 minute, 55°C for 1 minute, and 72°C for 1 minute, using an automated thermal cycler (Perkin Elmer Cetus). The PCR products were observed after electrophoresis on 2% agarose gels and ethidium bromide staining.

Preparation of ASMCs and Measurement of Nitrite Production

Primary cultures of ASMCs were established by pooling thoracic aortas from four rats in each group (SD, R, and S) using enzymatic digestion techniques and culture conditions as described. The cells were grown in Dulbecco’s modified Eagle’s medium (DMEM, Gibco) supplemented with 10% fetal bovine serum in a humidified 5% CO2-95% air atmosphere. ASMCs in the 5th passage were stimulated with 30 μg/mL LPS (Sigma Chemical Co) and 150 ng/mL IFN-γ (Gibco) for 20 hours. They were then incubated for 2 hours in DMEM without l-arginine and then an additional 8 hours with varying concentrations of L-arginine (0 to 500 μmol/L). Medium was collected for subsequent measurement of nitrite production, which was determined as described.

Immunoblot Analysis of Anti-NOS2 Expression

Cytosolic extracts from unstimulated and stimulated ASMCs were dissolved in SDS sample buffer and electrophoresed on 7.5% polyacrylamide gels. Each sample loaded into the wells contained 100 μg total protein. Proteins were then transferred to polyvinylidi-

Nonisotopic RNase Cleavage Assay

Nonisotopic RNase cleavage assay was performed using a kit (Mismatch Detect, Ambion, Inc) and methods as described in detail in the instruction manual provided by the manufacturer. Briefly, total RNA isolated from stimulated ASMCs was reverse transcribed to cDNA using oligo dT and SuperScript II RNase H Reverse Transcriptase (Life Technologies). A series of primers (Table 1) varying from 20 to 24 nucleotides (Operon) were designed using a computer program (MacVector, Oxford Molecular Group PLC) to PCR amplify cDNA fragments that overlapped the coding region of Nos2. Each fragment overlapped the adjacent fragments by at least 200 nucleotides to enhance sensitivity of this assay. The PCR reactions used a DNA polymerase (Ultima, Perkin Elmer Cetus) that possessed proofreading function. A second round of PCR amplifi-
cation was then performed using primers (44 to 47 nucleotides) that contained T1 (upstream primer) and SP1 (downstream primer) phage promoters (Table 2). These promoters allowed subsequent efficient in vitro transcription of sense and antisense mRNA to perform the RNase cleavage assay (Ambion). Using reagents provided in the kit, approximately 2 μg of each PCR product was used for in vitro transcription reactions to generate sense and antisense mRNA transcripts. Sense mRNA transcripts from one of the Nos2 segments of one strain of rat were hybridized to antisense transcripts of the same segment from the other two strains. After the annealing, these RNA/RNA duplexes were incubated with RNase A. This method was sufficiently sensitive to detect even single nucleotide sequence differences. The cleavage products were electrophoresed on 2% agarose gels and identified by ethidium bromide staining. These experiments were performed in triplicate using total RNA collected on two different occasions from a total of six rats in each group.

Cloning and Sequencing of Nos2 Fragment

PCR-amplified fragments between 1274 and 2364 bp of Nos2 from S and R rats were separately ligated into pCR-Script SK(+) phagemid vector using Sfi I (Stratagene) and cloned in standard fashion. Two clones containing each product were purified using Wizard Maxipreps (Promega) and sequenced by the dideoxy chain termination method (Sequenase, USB). Sequencing of the two isolated clones for the Nos2 fragments from S and R rats was performed, and the results were compared with those nos2 sequences published in Genbank (accession numbers X76881 and D14051).

RFLP of Nos2 in Genomic DNA

The nucleotide T→C transversion at position 2140 in S Nos2 eliminated a PstI restriction site. To determine whether this RFLP was present in genomic DNA from S rats and eight other strains that included R, SD, Brookhaven salt-resistant (DR), Brookhaven salt-sensitive (DS), Lewis (LE), spontaneously hypertensive (SH), salt-sensitive spontaneously hypertensive (S2), and Wistar-Kyoto (WKY), genomic DNA from four male animals of each strain was isolated as described previously. All strains were obtained from Harlan Sprague-Dawley, except for the salt-sensitive spontaneously hypertensive rat. Genomic DNA from these animals was obtained from kidney samples, which were generous gifts of Yui-Fai Chen, PhD, University of Alabama at Birmingham. Briefly, after sedating the animals with an injection of 50 mg/kg IP pentobarbital, genomic DNA was prepared from ~1.0 g of liver (or kidney) tissue, which was initially frozen in liquid nitrogen and fragmented with a mortar and pestle. The samples were incubated overnight in 50 mL of lysis buffer (0.4 mmol/L NaCl, 2 mmol/L EDTA, 10 mmol/L Tris-HCl, pH 8.0) and 0.5 mL of proteinase K (2 mg/mL of 20% SDS) at 54°C.
TABLE 2. Forward and Backward Primers Used in Second-Round Amplification and In Vitro Transcription of DNA for Use in Nonisotopic RNase Cleavage Assay

<table>
<thead>
<tr>
<th>Primers Location</th>
<th>Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>5’- CCT AAT ACG ACT CAC TAT AGG GCC AAG GTG ACC TTA AAG AGG AAA AG-3’</td>
<td>−42–634</td>
</tr>
<tr>
<td>5’- CCA TTT AGG TCA CAC TAT AGG ACC GAC CTG ATG TTG CGA CAT GAC GTC GGC-3’</td>
<td>613–1444</td>
</tr>
<tr>
<td>5’- CCT AAT ACG ACT CAC TAT AGG GAA CAG TGG CAA CAT CAG GTC GGC-3’</td>
<td>1190–1993</td>
</tr>
<tr>
<td>5’- CCA TTT AGG TCA CAC TAT AGG AGG AGT TTC ATG GGG ATC TGG CAA TGT TTG-3’</td>
<td>1649–2280</td>
</tr>
<tr>
<td>5’- CCT AAT ACG ACT CAC TAT AGG GAG CAG AAT GTG ACC ATC ATG GAC-3’</td>
<td>2165–2770</td>
</tr>
<tr>
<td>5’- CCA TTT AGG TCA CAC TAT AGG GAG ATC TGG CAA TGT TTG CAC-3’</td>
<td>2563–3194</td>
</tr>
</tbody>
</table>

The location of the amplified DNA segment is shown. The underlined regions represent the T7 (upstream primer) and SP6 (downstream primer) binding sites for DNA-dependent RNA polymerase.

Statistical Analysis
Data were expressed as mean±SE. Comparisons were analyzed for significant differences using paired or unpaired t tests or ANOVA, where appropriate. Statistical significance was set at 5%.

Results

Phenotyping and Genotyping Dahl/Rapp Rats
Our previous studies have shown that S but not R rats rapidly developed sustained hypertension while on a high-salt (8.0% NaCl) diet. L-Arginine corrected salt-sensitive hypertension in S rats. Genetic contamination of this commercial S rat strain (Harlan Sprague-Dawley) was discovered in late 1993 but was subsequently corrected. In the present study, S rats regenerated by Harlan Sprague-Dawley were tested to confirm the phenotype of salt-sensitive hypertension corrected by l-arginine. After 2 weeks on the 8.0% NaCl diet, initial mean MAP was higher (P<.05) in S rats than in R rats (144±5 versus 108±5 mm Hg). Intravenous infusion of l-arginine dramatically decreased (P<.05) MAP from 144±5 to 112±6 mm Hg in S rats (Fig 1). l-Arginine had no effect on MAP of SD and R rats (from 109±5 to 108±5 mm Hg and 108±6 to 110±5 mm Hg, respectively). In addition, S rats used in this study were also genotyped at three different loci.

![Figure 1. Effect of l-arginine infusion on MAP in SD, R, and S rats. After 2 weeks on an 8% NaCl chow, S rats developed hypertension (144±5 mm Hg); SD and R rats remained normotensive (108±5 and 108±5 mm Hg, respectively). Intravenous infusion of l-arginine decreased (P<.05) MAP to 112±6 mm Hg in S rats, whereas l-arginine had no effect on MAP in both SD and R rats (109±5 and 110±5 mm Hg, respectively).](http://hyper.ahajournals.org/doi/10.1161/HYPERTENSIONAHA.114.069172)
(R354, R721, and R1041), which had been shown to be polymorphic in the genetically contaminated S (termed S*) rats. These microsatellite-based genetic markers confirmed that unlike the S* rat (kindly provided by Dr James L. Lewis, University of Alabama at Birmingham), the reestablished S strain did not show polymorphisms at any of the three different alleles (Fig 2). The combined data show that this regenerated S strain of rat was the same as the original strain.

**NOS2 Production Was Reduced in ASMCs of S Rats**

Primary cultures of ASMCs, which have been reported to express only NOS2 but not the constitutively expressed isoforms of NOS, from R, SD, and S rats were stimulated in the 5th passage with 30 μg/mL LPS and 150 ng/mL IFN-γ for 20 hours. Unstimulated NO production was undetectable in the medium of ASMCs from all three strains of rat. After 20 hours of stimulation, nitrite production increased as a function of the concentration of L-arginine in R rats (Fig 3). The EC50 was 11.5±1.7 μmol/L for ASMCs from R rats. In contrast, ASMCs from S rats demonstrated a fivefold greater EC50 (55.5±4.6 μmol/L). Maximum nitrite production by ASMCs from R and S rats were comparable (30.0±0.2 versus 29.8±0.6 nmol/h per 10^6 cells). Inducible nitrite production by cultured ASMCs from SD rats was similar to that seen from ASMCs of R rats (data not shown). L-arginine-dependent inducible NO production in these cultured ASMCs was completely inhibited by addition of 100 μmol/L N^ω^-nitro-L-arginine, an inhibitor of NOS, to the medium.

Cytosol from unstimulated ASMCs of SD, R, and S rats did not react with rabbit polyclonal anti-NOS2 antibody. Western blot analysis of three different samples revealed similar amounts of an ∼130-kD band in stimulated ASMCs from SD, R, and S rats (Fig 4).

**Mutation in Nos2 Transcript of S Rats**

To determine whether a mutation was present in the coding region of Nos2 from S rats, total RNA was isolated from ASMCs of S and R rats after stimulation with LPS and IFN-γ to induce NOS2. Amplification of seven overlapping cDNA fragments covering the coding region (∼3.5 kb) of Nos2 was accomplished using gene-specific primers and a thermostable DNA polymerase that possessed 3’-5’ exonuclease (proof-reading) activity. The PCR primers contained SP6 or T7 promoter sequences that allowed efficient in vitro transcription of these cDNA segments. After in vitro transcription of Nos2 fragments, both sense and antisense mRNA transcripts from the seven Nos2 fragments obtained from experimental (S) rats were hybridized to complementary antisense and sense mRNA transcripts obtained from control (SD or R) rats. These RNA/RNA duplexes were then exposed to RNase A digestion. Because RNase A cleaves even single unpaired bases (mismatches) in the RNA/RNA duplexes, subtle differences between the transcripts were identified. Only two of the seven Nos2 fragments, which mapped the region between 1274 and 2364 bp, contained a mismatch in the sequence between S and both R and SD strains (Fig 5). S and R Nos2.

![Figure 2. Three different loci previously shown to be polymorphic in genetically contaminated S (termed S*) rats, including R354, R721, and R1041, were examined in the S rats used in this study. In contrast to the S* rat, polymorphisms in these microsatellite-based genetic markers were not present in the reestablished S strain.](Image)

![Figure 3. Production of nitrite in cultured ASMCs stimulated by LPS+IFN-γ from R and S rats. Nitrite production was measured in the presence of varying concentrations of L-arginine (n=4 in each group). ASMCs from S rats demonstrated a fivefold greater EC50 (P<.05) than cells from R rats.](Image)

![Figure 4. Western blot analysis of NOS2 obtained from cytosolic extracts from stimulated ASMCs of SD, R, and S rats demonstrated an ∼130-kD band of similar densities in all three samples. Equivalent amounts of total protein were loaded into each well.](Image)
NOS2 Abnormalities in Dahl/Rapp Rats

Remarkable advances in understanding NO production have occurred since the initial report of endothelium-derived relaxing factor in 1980. Three known classes of NOS have been identified. The first enzyme cloned was the so-called neuronal NOS or NOS1. The second NOS was derived from macrophages and has been termed NOS2. The third was obtained from endothelial cells and is known as eNOS or NOS3. NOS1 and NOS3 have been determined to be constitutively expressed enzymes, while NOS2 was considered an inducible protein. However, recent evidence suggests that NOS2 is present in normal unstimulated kidney. Thus, nomenclature that avoids the term “inducible” is perhaps more appropriate. Because of our previous reports and the observation that expression of NOS2, documented by Western blotting, was increased in kidneys of rats on high-salt chow, we hypothesized that NOS2 was abnormal in the Dahl/Rapp salt-sensitive (S) rat. In our present studies, primary cultures of ASMCs from S rats demonstrated impaired NO production after stimulation with LPS and IFN-γ, compared with cells obtained from SD and R rats; this defect was overcome by increasing the concentration of l-arginine in the medium. Sequence analysis identified a single point mutation that produced an important amino acid substitution (S714P) lying between the FAD and FMN binding sites. This point mutation resulted in an RFLP that characterized the S NOS2 allele and was absent in Nos2 of the other strains of rats that were examined.

The study of salt-sensitive hypertension was pioneered by Lewis K. Dahl, who produced from the Sprague-Dawley line two strains of rats that were either susceptible or resistant to the hypertensive effects of a high-salt (8.0% NaCl) diet. These Dahl salt-susceptible and salt-resistant strains were completely inbred by John P. Rapp to yield three microsatellite-based genetic markers that were polymorphic between the normal and contaminant S rats. Animals derived from that colony were used in this study. We confirmed initially that these S rats demonstrated the important phenotype of salt-sensitive hypertension and died within weeks. In contrast, Dahl/Rapp salt-resistant (R) rats remained normotensive despite a high salt intake. Although the S strain commercially available from Harlan Sprague-Dawley became genetically contaminated, the foundation colony was not affected. All three markers demonstrated that the S rats used in these studies were homozygous at these alleles.

The l-arginine:NO pathway is uniquely poised to regulate blood pressure in response to dietary salt. This important vasodilator and natriuretic system is located in the arterial wall and in several tubule segments of the kidney. Shultz and Tolins demonstrated that in normotensive SD rats, an increase in salt intake increases plasma concentration and urinary excretion of nitrite and nitrate, the metabolic end products of NO, and cGMP, which is produced in response to NO. In addition, accentuated NO production is physiologically important in blood pressure regulation because that component of blood pressure that was...
sensitive to NO inhibition was enhanced by the high salt intake. In contrast to normotensive rats, S rats did not increase NO production while on 8% NaCl chow, allowing development of salt-sensitive hypertension in this strain. Even though plasma concentrations of L-arginine of S and R rats did not differ and dietary salt did not affect plasma L-arginine levels of S rats, this defect in NO synthesis was overcome by providing L-arginine. S rats given oral L-arginine did not develop hypertension despite treatment for over 8 weeks with 8% NaCl chow. Administration of L-arginine also improved salt excretion and returned the pressure-natriuresis curve to normal in S rats. The antihypertensive effect of L-arginine appeared to be specific for this genetic model of hypertension and was dramatic.

In summary, our present study demonstrated a solitary sequence abnormality in the coding region of Nos2 of S rats. Administration of dexamethasone, which has been shown to prevent induction of Nos2 but not alter the function of the constitutive isoforms of Nos, prevented the increase in nitrate/nitrite and cGMP production and abolished the antihypertensive effect of L-arginine in these animals. Along with demonstration of regulation of Nos2 in the rat kidney by dietary salt, these findings suggest a role of Nos2 in the blood pressure response to dietary salt. Deng and Rapp recently used polymorphic markers in or near the macrophage inducible Nos (Nos2) to show that the S allele, but not the constitutive neuronal isoform (Nos1), cosegregated with hypertension in F2 crosses obtained from S rats bred with either Wistar-Kyoto or Milan normotensive rats. Interestingly, this allele did not cosegregate with blood pressure in an F2 cross from S and R rats. The explanation for this apparent discrepancy is uncertain. However, the unique presence of the Nos2 RFLP in the S strain, but not in other strains of rat including Wistar-Kyoto (Fig 6), suggests that the normotensive strain that was used to produce the F2 cross with S rats was not the factor that produced different results with respect to cosegregation of S Nos2 with blood pressure. More recently, Nos3 was shown not to participate in hypertension in these animals. The role of Nos2 in the genesis of salt-sensitive hypertension merits further investigation. One or more genes in the L-arginine:NO pathway may possess mutations in the S strain and contribute to salt-sensitive hypertension in these animals.

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