Nitric Oxide Synthase (NOS2) Mutation in Dahl/Rapp Rats Decreases Enzyme Stability

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Abstract—The pathogenesis of salt-sensitive hypertension remains poorly defined, but a role for nitric oxide (NO) has been suggested. The Dahl/Rapp salt-sensitive rat possesses a defect in NO synthesis that is overcome by supplementation with l-arginine, which increases NO and cGMP production and prevents salt-sensitive hypertension. An S714P mutation of inducible NO synthase (NOS2) was subsequently identified. The current report examined the functional significance of an S714P mutation in NOS2. COS-7 cells were transiently transfected with cDNA of wild-type NOS2 and S714P and S714A mutants of NOS2, and enzyme function was determined. Whereas steady-state mRNA levels did not differ, immunoblot analysis demonstrated decreased levels of NOS2 protein. Metabolic labeling experiments confirmed a reduced half-life of the S714P mutation. Nitrite production, which was dependent on the concentration of l-arginine in the medium, was diminished in cells transfected with the S714P mutant, compared with the wild type and the S714A mutant. These data provide a biochemical explanation of the physiological abnormalities of NOS2 in the Dahl/Rapp salt-sensitive rat and suggest that a posttranslational mechanism involving the proteasome may be responsible for the diminished NO production observed in response to increased dietary salt intake in these animals. (Circ Res. 2001;89:317-322.)

Key Words: hypertension ■ nitric oxide ■ nitric oxide synthase ■ proteasome ■ lactacystin

Almost a quarter of the population of the United States has hypertension.1 Although African-Americans are much more likely to exhibit salt sensitivity, 50% to 75% of the total hypertensive population demonstrate salt sensitivity; that is, blood pressure is altered as dietary salt intake is changed.2 Breeding studies in rats have confirmed that there is a genetic susceptibility to develop salt-sensitive hypertension.3,4 Whereas multiple genes are involved in the blood pressure response to changes in dietary salt intake, increasing evidence supports an important role of altered synthesis of nitric oxide (NO) in salt sensitivity. NO synthesis occurs in the vessel wall5-7 and in the kidney8,9,10 and serves as a locally produced vasodilator8,9,11 and natriuretic factor.12-16 Kidney production of NO appears to be particularly important in blood pressure regulation, because infusion of L-citrulline and L-arginine methyl ester directly into the renal medulla causes sodium retention and increased blood pressure.17 Thus, NO is uniquely situated to control blood pressure responses to increased salt intake.

Dietary salt modulates NO production in healthy humans18 and in normotensive rats.19,20 An increase in salt intake increases NO production.19,20 Along with determining the blood pressure response to a high-salt diet,12,19,21,22 the renal vascular resistance and renal functional changes that occur with an increase in dietary salt are also modulated by NO.12,13,23 In contrast to normotensive rats, the increase in NO production is absent in Dahl/Rapp salt-sensitive (S) rats, an inbred strain of rat that manifests salt-sensitive hypertension. However, when provided with l-arginine, production of NO and cGMP increases and salt-sensitive hypertension is completely prevented.19,24-26 Decreased serum levels of L-citrulline and L-arginine and alteration of the rate-limiting enzymes of the urea cycle have not been identified in S rats.27 The combined studies leave little doubt about a central role of NO synthesis in development of salt-sensitive hypertension in S rats.

The three known isoforms of NO synthase (NOS) are present in the kidney8,9,28-31 and respond to changes in dietary salt,28-30,32 NOS1, the neuronal isoform, is localized primarily in the macula densa and actually decreases when dietary salt is increased; therefore, activity of NOS1 cannot explain the increase in NO production that occurs with an increase in salt intake. NOS3, which is also referred to as the endothelial isoform, increases in aorta and glomeruli as dietary salt is increased; NOS3 is expressed by cytokine induction, but interestingly is present in the normal kidney, suggesting a different mechanism of regulation in the kidney.9,29,31 Both NOS3 and NOS2 are involved in the renal response to changes in dietary salt.
NOs2 appears to be involved in salt-sensitive hypertension. An increase in dietary salt increased the renal expression of NOs2.23 Lässcher’s group demonstrated that, on a diet that contained a high salt content, renal cortical and medullary calcium-independent NO production increased in Dahl/Rapp salt-resistant (R) rats but was impaired in S rats.33 Administration of highly specific inhibitors of NOs2 to R rats produced salt-sensitive hypertension in these animals, which are known to be very salt-resistant.21 Careful studies by Tan et al34 also showed an important role of NOs2 in increasing NO production and preventing salt-sensitive hypertension in R rats and in decreasing salt sensitivity in S rats. In early studies, this laboratory hypothesized a role for NOs2 in salt-sensitive hypertension, because dexamethasone, which has been shown to inhibit induction of NOs2,35 also prevented the antihypertensive effects of L-arginine in S rats.19 NOs2 from vascular smooth muscle cells in culture produced less NO than NOs2 from cells of Sprague-Dawley and R rats. Subsequently, a single point mutation in NOs2 cDNA from S rats was identified. This T→C transversion resided between the flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) binding sites of the molecule and produced an amino acid substitution of proline for serine at position 714.26 The purpose of the present study was to determine the role of the S714P mutation in NOs2 function.

Materials and Methods

Site-Directed Mutagenesis

Rat NOs2 cDNA was a generous gift from Prof Göran K. Hansson (Karolinska Institute, Stockholm, Sweden). Site-directed mutagenesis was accomplished using a kit (QuikChange Site-Directed Mutagenesis Kit, Stratagene). Briefly, using a 523-bp cloned fragment of NOs2 as template, synthetic complementary oligonucleotide primers (Operon) containing the desired mutations were extended using Phi30 Turbo DNA polymerase during low-cycle temperature cycling. Primers used to generate the S714P mutation were the following: 5′-CCCCAGGCCGAGCTGCCTAGACTTCAA-CAAAGC-3′ and 5′-GCTTTGTTGAGGTTGCTAGAGCT-CTGGGCTCTGGG-3′. Primers used to generate the S714A mutation were the following: 5′-CCCCAGGCCGAGCTGCCTAGACTTCAA-CAAAGC-3′ and 5′-GCTTTGTTGAGGTTGCTAGAGCT-CTGGGCTCTGGG-3′. Primers used to generate the S714D mutation were the following: 5′-CCCAGGCCGAGCTGCCTAGACTTCAA-CAAAGC-3′ and 5′-GCTTTGTTGAGGTTGCTAGAGCT-CTGGGCTCTGGG-3′. Primers used to generate the S714T mutation were the following: 5′-CCCAGGCCGAGCTGCCTAGACTTCAA-CAAAGC-3′ and 5′-GCTTTGTTGAGGTTGCTAGAGCT-CTGGGCTCTGGG-3′. The entire 523-bp fragment was sequenced (ABI Prism), and the desired mutations were confirmed. The fragments were then ligated into NOs2 cDNA (from pRc/CMV2 (Invitrogen)).

Transient Transfection of COS-7 Cells

COS-7 cells (CRL-1651, SV40-transformed African green monkey kidney cells, American Type Culture Collection) were transfected with mixtures of pRc/CMV2 containing 12 μg of cDNA under study (wild-type, S714P, and S714A mutations), 50 μL transfection reagent (GenePORTER reagent, Gene Therapy Systems), and 5 mL serum-free medium for 5 hours. FBS (20%; Intergen) was then added, and assays were performed as described below.

Northern and Immunoblot Analyses of NOs2 Expression

Northern analysis was performed as described previously,30,36,37 using 15 μg of total RNA. Membranes were hybridized with a 32P-labeled, ∼1-kb cDNA fragment of NOs2, followed by rehybridization with 32P-labeled human GAPDH probe obtained through American Type Culture Collection. Bands were quantified using densitometry (model 620 video densitometer, Bio-Rad).

To examine protein expression, transfected COS-7 cells were lysed in buffer that contained 0.3 mol/L sucrose; 1.0% NP40 (vol/vol); 50 μmol/L PMSF; in mmol/L, HEPES 10, EDTA 0.1, DTT 1, and Tris/HCl (pH 7.6) 50; and 10 μg/mL each of aprotinin, leupeptin, and trypsin inhibitor (all from Sigma). Cytosolic extracts were obtained by centrifugation of the lysates at 100,000g for 1 hour. Protein concentration was determined using a kit (Micro BCA Protein Assay Reagent Kit, Pierce). Western blotting was performed in standard fashion,30,36,37 using 10 μg of total protein. NOs2 was identified using a mouse anti-NOS2 monoclonal antibody (Transduction Laboratories), 1:500 dilution, followed by goat anti-mouse IgG conjugated to peroxidase (Bio-Rad), 1:20,000 dilution.

Determination of Production of NO Metabolites

 Twenty-four hours after initiation of simultaneous transfections of plates of COS-7 cells with plasmids containing the wild-type and S714P and S714A mutants, medium was aspirated and replaced with 10 mL of DMEM that contained varying concentrations (0 to 200 μmol/L) of L-arginine. After an additional 48 hours, medium was harvested and assayed for nitrite in standard fashion using Griess reagent, as described previously.23,30 Each experiment was performed in duplicate at each concentration of L-arginine and was averaged. Five separate experiments were performed.

NOs2 Enzyme Activity Assay

NOs2 was purified from COS-7 cells 72 hours after transfection, using standard biochemical purification procedures that had been proven successful in purification of NOs3.38–41 Briefly, NOs2 was isolated from cytosolic extracts (∼25 mg total protein) using a 2′,5′-ADP-Sepharose 4B (Pharmacia) column, followed by a calmodulin affinity column. Arginine was left out of all buffers beginning with the calmodulin column. Purity of the preparations was demonstrated by SDS-PAGE along with Western blotting to confirm identity of the bands as NOs2. Concentration of protein in each sample was determined using a kit (Micro BCA Protein Assay Reagent Kit).

NOs2 activity was determined by the rate of formation of [3H]-citrulline from [3H]-L-arginine as described,42 with modifications as follows. Enzyme assays contained 2 μg NOs2 in mmol/L, NADPH 0.5, CuCl2 1.25, and HEPES (pH 7.6) 50; in mmol/L, FAD 10, FMN 0.5, calmodulin 7.5, and (6R)-5,6,7,8-tetrahydro-L-biotinphenyl dihydrochloride (BH4) 15; and 1 μCi [3H]-L-arginine (NEN/DuPont). Except for BH4, which was obtained from Schirks Laboratories, all reagents came from Sigma. The concentration of L-arginine was varied in these experiments between 0 and 100 μmol/L. In preliminary studies, production of [3H]-citrulline was linear over 30 minutes of observation. The kinetics experiments were therefore terminated at 20 minutes. The reaction was performed at 37°C and was stopped by adding 2 mL of ice-cold 20 mmol/L HEPES (pH 5.5) containing 5 mmol/L EDTA. The sample was applied onto a Dowex AG 50W-X8 column that had been prequillibrated with 20 mmol/L HEPES (pH 5.5). Activity of eluate, which contained [3H]-citrulline, was quantified by liquid scintillation spectrometry.

Metabolic Labeling Study to Determine NOs2 Half-Life

Metabolic labeling of NOs2 was performed in standard fashion, following published methods.43,44 Seventy-two hours after transfection, COS-7 cells were incubated for 20 minutes at 37°C in MEM (MEM Select-Amine Kit, GibcoBRL), which did not contain cysteine, methionine, or serum. Cells were pulse-labeled for 30 minutes in 150 μL of MEM supplemented with [35S]cysteine and [35S]methionine (Tran35S-label, ICN Biochemicals), 300 μCi/mL. Pulse-labeled cells were incubated for 0, 6, 12, 18, and 24 hours at 37°C in complete medium. Cell lysates (n=4 in each group) were prepared in standard fashion using radioimmunoprecipitation assay buffer containing protease inhibitors (Complete, Boehringer Mannheim, Ly-
sates (100 μg total protein per 200 μL) were incubated with 4 μL mouse anti-NOS2 monoclonal antibody (Transduction Laboratories), 1:50 dilution in Tris-buffered saline, overnight at 4°C, and then incubated with 20 μL of protein A–Sepharose beads (CL-4B, Amersham Pharmacia Biotech) for 2 hours at 4°C with gentle rocking. The beads were washed four times with 500 μL of lysis buffer, and 35S activity was quantified by liquid scintillation spectrometry. In other experiments, 400 μg of total protein lysates from pulse-labeled cells were incubated with 8 μL anti-NOS2 monoclonal antibody, 1:50 dilution, and then protein A–Sepharose beads. The immunoprecipitated proteins were solubilized in loading buffer and resolved using SDS-PAGE. After exposure to x-ray film, density of each band was quantified.

**Role of the Proteasome in NOS2 Degradation**

Forty-eight hours after simultaneous transfections of plates of COS-7 cells with plasmids containing the wild-type and S714P and S714A mutants, the medium was aspirated and replaced with DMEM supplemented with 20 μmol/L cycloheximide (an inhibitor of protein synthesis), and either 10 μmol/L clasto-lactacystin β-lactone (the active metabolite of lactacystin45–47 or 0.1% DMSO, which served as a vehicle control. All of these chemicals came from Sigma. COS-7 cells were harvested at 0, 6, 12, 18, and 24 hours after the change of the medium. After washing with ice-cold PBS, cells were lysed in three volumes of lysis buffer (in mmol/L, HEPES [pH 7.4] 10, DTT 1, EDTA 2, and Na3 VO4 10; 0.3 mol/L sucrose; 50 μmol/L PMSE; 5 μg/mL, leupeptin 10, aprotinin 2, and trypsin inhibitor 10; and 1% NP40). The lysates were centrifuged at 16 000g for 10 minutes, and the supernatant was harvested. The protein concentration was determined using a kit (Micro BCA Protein Assay Reagent Kit). Fifteen micrograms of total protein was subjected to electrophoresis using 8% SDS-polyacrylamide gels and then transferred onto nitrocellulose membranes. The membranes were probed with an anti-NOS2 polyclonal antibody (Transduction Laboratories), 1:1000 dilution. In other experiments, 400 μg of total protein lysates from pulse-labeled cells were incubated with 8 μL anti-NOS2 monoclonal antibody, 1:50 dilution, and then protein A–Sepharose beads. The immunoprecipitated proteins were solubilized in loading buffer and resolved using SDS-PAGE. After exposure to x-ray film, density of each band was quantified.

Figure 1. Northern analysis (gel at top) showed that steady-state mRNA levels did not differ among the three groups expressing wild-type NOS2 and S714P and S714A mutations (n=4 in each group). Immunoblot analysis demonstrated single bands migrating at the expected ~130 000 size in all three groups. NOS2 protein expression was diminished in S714P group. M. indicates molecular weight.

**Statistical Analysis**

All data are mean±SE. Statistical differences were determined using either the unpaired t test or 1-way ANOVA with standard post hoc testing (Statview, version 5.0, SAS Institute, Inc). A P value of <0.05 assigned significance.

**Results**

S714P and S714A mutations of NOS2 were created, and activities of these mutant NOS2 enzymes were compared with wild-type NOS2. The alanine mutation was generated to determine whether serine phosphorylation or alteration in structure produced by replacement of serine with proline was the cause of altered NOS2 activity. Northern analysis showed that steady-state mRNA levels of the three NOS2 enzymes by the transfected COS-7 cells did not differ (Figure 1). Using a monoclonal anti-NOS2 antibody, however, Western blotting demonstrated a decrease in protein expression of the S714P mutant (Figure 1). This disparity prompted an analysis of the biological half-life of the S714P mutant. To address this hypothesis, metabolic labeling of NOS2 was performed 72 hours after transfection of COS-7 cells. The amount of protein labeled at the initial time point did not differ among the three groups, suggesting that a posttranslational mechanism was involved in altered expression of the S714P mutant (Figure 2). Degradation rates of wild-type NOS2 and S714A did not differ, but were greater (P<0.05) at every time point tested from 6 to 24 hours for the S714P mutant.

To determine whether the proteasome degraded NOS2, clasto-lactacystin β-lactone was added along with cycloheximide to the medium of cells expressing NOS2, S714P, and S714A. Cells treated with cycloheximide alone showed findings that were consistent with the metabolic labeling experiments, with the S714P mutant disappearing from the cytoplasm faster than wild-type and S714A mutant (Figure 3). Addition of clasto-lactacystin β-lactone reduced degradation rates of the S714P mutant to levels comparable with those of the other two proteins.

Nitrite production rates by cells transfected with the S714P mutation were lower (P<0.05) than nitrite production rates by cells transfected with either the wild-type NOS2 or the S714A mutation (Figure 4). Initial kinetic analysis of the S714P mutant using cytoplasmic preparations demonstrated

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no change in $K_m$ but a decrease in $V_{\text{max}}$, compared with the wild-type NOS2 and the S714A mutant (data not shown). These findings were consistent with a decrease in cytoplasmic protein concentration of the mutant. Kinetic analysis was therefore performed using purified NOS2 enzymes to determine whether an intrinsic abnormality in enzyme function was present. Under these conditions, neither $K_m$ (in $\mu$mol/L, 26.1±4.5 for wild-type NOS2; 27.1±4.7 for S714P; and 30.9±3.2 for S714A) nor $V_{\text{max}}$ (in nmol of citrulline/min per mg NOS2, 1283±407 for wild-type NOS2, 1370±373 for S714P, and 1529±421 for S714A) differed among the three enzymes. Under comparable experimental conditions, Yui et al. observed the similar $K_m$ (32.2 $\mu$mol/L) and $V_{\text{max}}$ (1052 nmol/min per mg protein) for purified rat NOS2.

### Discussion

The purpose of the present study was to determine whether an S714P mutation identified in Nos2 of S rats altered the function of the enzyme. Expression of this mutation, along with wild-type NOS2 and an S14A mutant, was performed using COS-7 cells. The initial observation was that the amount of NO produced, as determined by nitrite accumulation in the medium, did not differ between wild-type NOS2 and the S714A mutant but was less with the S714P mutant. Subsequent kinetic experiments showed an unexpected decrease in $V_{\text{max}}$, but no change in $K_m$ of the S714P mutant. To determine the cause of the altered expression, Northern analysis was performed and demonstrated no alteration in steady-state levels of mRNA among the three groups. Western analysis, however, showed a decrease in protein expression of the S714P mutant, compared with the wild-type NOS2 and S714A mutant. To address the mechanism of altered expression, standard pulse-chase labeling experiments were performed. These experiments showed that the amount of protein labeled initially did not differ among the three groups. Thus, a posttranslational event was responsible for the altered expression of the S714P mutant. The subsequent “chase” portion of the experiment confirmed that the biological half-life of this mutant was reduced, compared with the other two groups. Addition of cycloheximide to the medium produced similar results. These data were supported by experiments that examined enzyme kinetics after purification of NOS2. In these studies that used purified NOS2 and not cytoplasmic extracts, differences in $V_{\text{max}}$ or $K_m$ were not observed.

To determine the mechanism of degradation of NOS2, clasto-lactacystin $\beta$-lactone, a cell-permeable and irreversible inhibitor of the proteasome, was added to the medium along with cycloheximide. These experiments demonstrated that clasto-lactacystin $\beta$-lactone, the active metabolite of lactocystin, inhibited the degradation of wild-type NOS2 and the S714P and S714A mutants. Thus, the data confirmed a previous study that suggested that NOS2 was degraded by the proteasome and further showed that the S714P mutant was removed more rapidly from the cytoplasm by the proteasome. The mechanism by which the S714P mutant was sensitized to degradation by the proteasome remained unclear but was not related to a serine phosphorylation event, because mutating the serine to alanine did not alter enzyme function or half-life, compared with wild-type NOS2.

Several laboratories have demonstrated increased NO production in vivo in response to dietary salt. Physiological studies have shown that NOS2 is involved in the increase in NO synthesis and blood pressure response to dietary salt. In addition, an alteration in NOS2 function has been suggested in S rats. NO production does not increase...
when dietary salt is increased in S rats, which manifest salt-sensitive hypertension. These animals are unique in that administration of L-arginine “cures” the salt sensitivity.19,25–27 NO production is particularly important in the kidney, where it serves an important natriuretic function.12–16 R rats responded to an increase in dietary salt by increasing renal production of NO through calcium-independent NOS activity. In contrast, renal calcium-independent NOS activity was impaired in kidneys of S rats.33 The importance of NOS2 in blood pressure regulation as dietary salt is increased was further shown by studies that showed R rats develop salt-sensitive hypertension with administration of highly selective inhibitors of NOS2.21 NO production by NOS2 from primary cultures of vascular smooth muscle cells from S rats is less than NO production by cells from Sprague-Dawley and R rats.26 In addition, a point mutation in NOS2 has been found in S rats.26 Thus, abundant evidence supports a role for NOS2 in the increase in NO production in response to dietary salt and further suggests defective NOS2 function in generation of salt-sensitive hypertension. Although it is attractive to consider NOS2 as a candidate gene in salt-sensitive hypertension, genetic analyses to determine the role of Nos2 in blood pressure regulation in humans and rats provided conflicting results. Using sibling-pair analysis, a susceptibility locus for human hypertension was identified on human chromosome 17, which is syntenic with the region of rat chromosome 10 that contains Nos2.49 In a recent study, however, association or linkage of two polymorphic markers at the Nos2a locus with hypertension was not confirmed specifically in an Australian Anglo-Caucasian population of subjects.50 The phenotype of salt sensitivity was not examined, and none of the population in this study was identified as being of African descent. Dukhanina et al51 showed that a strong quantitative trait locus near Nos2 on chromosome 10 of the rat was linked to blood pressure control, but, using congenic strains of rats, Nos2 did not appear to be involved in hypertension in S rats.51 Thus, both genetic analyses50,51 conflict with physiological data, suggesting Nos2 as a gene involved in salt-sensitive hypertension.

In summary, the present study demonstrated that the S714P mutation of NOS2 did not alter the Km of the enzyme for the substrate, L-arginine, but within the physiological concentration range of L-arginine27 did decrease the amount of NO produced, compared with wild-type NOS2. The findings confirmed that NOS2 was removed from the cytoplasm by the proteasome. Furthermore, accelerated degradation of the S714P mutant through this posttranslational event modulated NOS2 function, suggesting a biochemical explanation for diminished NO production in the S rat in response to an increase in salt intake.19,25,26,33

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References


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