

# The role of Nitric Oxide and the Renin-Angiotensin System in Salt-Restricted Dahl Rats

Hideo Kataoka, Fumio Otsuka, Toshio Ogura, Takayoshi Yamauchi, Masayuki Kishida, Masami Takahashi, Yukari Mimura, and Hirofumi Makino

To elucidate the role of nitric oxide (NO) and renin-angiotensin system (RAS) in the development of salt-sensitive hypertension, we investigated the pressor responses and renal histologic changes after long-term inhibition of endogenous NO synthesis in Dahl-Iwai salt-sensitive (DS) and salt-resistant (DR) rats under salt-restricted conditions that exaggerate RAS activation. Male DS and DR rats (6 weeks old) were fed with a low-salt (0.3%) diet for 5 weeks. *N*<sup>G</sup>-nitro-L-arginine (L-NA; dissolved in 60 mg/L deionized water), an arginine analogue acting as a NO-inhibitor, was also administered for 5 weeks. L-NA administration induced a gradual increase in systolic blood pressure (SBP) in both strains, and the pressor response in DS rats was apparently more enhanced relative to that in DR rats. Urinary nitrate plus nitrite (u-NO<sub>x</sub>) excretion was decreased by L-NA, with a significant negative correlation between SBP and u-NO<sub>x</sub> excretion in DS rats but not in DR rats. Plasma renin activity and urinary aldosterone level were significantly increased in L-NA-treated DS rats on week 5. Marked histologic changes with glomerular sclerosis and increased protein-

uria and urinary *N*-acetyl-β-glucosaminidase excretion were found in L-NA-treated DS rats but not DR rats. Competitive RT-PCR of mRNA extracted from the glomeruli revealed that angiotensin II type 1 receptor (AT<sub>1</sub>R) mRNA level was significantly lower in DS rats than in DR rats at week 2, and that L-NA administration significantly reduced glomerular AT<sub>1</sub>R level of DS rats at week 5, possibly because of downregulation. Our results showed that, even under sodium restriction, the pressor response and renal injury induced by chronic NO inhibition were markedly more enhanced in DS rats than in DR rats, which indicates that depletion of NO participates in both the development of hypertension and glomerular injury in DS rats through a potential activation of RAS irrespective of sodium loading. These data suggest that endogenous NO is an essential determinant of salt-sensitive hypertension in DS rats. *Am J Hypertens* 2001;14:276–285 © 2001 American Journal of Hypertension, Ltd.

**Key Words:** *N*<sup>G</sup>-nitro-L-arginine, AT<sub>1</sub>-receptors, glomerular injury, blood pressure.

**N**itric oxide (NO) is a diffusible vasorelaxing substance systematically released by vascular endothelial cells<sup>1</sup> and is an important vascular tone modulator.<sup>2</sup> Many studies have shown that NO is closely involved in the regulation of systemic blood pressure. To clarify the biology of NO, many studies have reported the use of various arginine analogues such as *N*<sup>G</sup>-nitro-L-arginine (L-NA), *N*<sup>G</sup>-nitro-L-arginine methyl ester (L-NAME), and *N*<sup>G</sup>-monomethyl-L-arginine (L-NMMA) *in vivo*.<sup>3</sup> These data showed that chronic inhibition of endogenous NO generation by arginine analogues leads to the progression of systemic hypertension in dose- and time-dependent manners.<sup>4</sup>

Dahl salt-sensitive (DS) rats have been used as a model of human salt-sensitive hypertension, because salt loading

exaggerates the development of hypertension in strains that are genetically predisposed to hypertension.<sup>5</sup> Several studies have demonstrated that impaired NO production during high salt intake is responsible for the salt sensitivity of DS rats.<sup>6–8</sup> The interrelationship between the development of hypertension and NO generation in DS rats has been extensively investigated under high-salt conditions. The results showed that the pathophysiology of hypertension in DS rats is attributable to reduced production of endogenous NO during high-salt loading. However, it remains unknown how the endogenous NO is involved in the development of hypertension and end-organ damage in DS rats under salt-restricted conditions.

The salt-restricted condition is well known to enhance the systemic renin-angiotensin system (RAS).<sup>9</sup> The RAS

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plays an important role in the control of systemic blood pressure, electrolyte balance, and extracellular fluid volume. Recent studies also have shown that RAS is potentially activated by inhibition of NO synthesis, which induces systemic hypertension in various experimental animals.<sup>10,11</sup> In contrast, angiotensin converting enzyme inhibitors (ACEI) exert their antihypertensive and organ-protective effects by reducing the production of angiotensin II (Ang II), which is the major molecule of the RAS, and by augmenting prostaglandins and NO synthesis. An alternative mechanism of the pathophysiology of hypertension in DS rats involves the renal system. The kidneys are possible determinants of salt sensitivity, as shown in previous renal cross-transplantation studies.<sup>12</sup> It is also known that salt-loaded DS rats progress to severe renal injury, including glomerulosclerosis and arteriosclerosis, after the development of systemic hypertension.<sup>13</sup> Although the mechanism underlying the development and progression of renal damage in DS rats remains to be elucidated, RAS activation is considered to be involved in this process.<sup>14</sup>

In the present study, we elucidated the contribution of RAS to the development of NO-inhibitory hypertension and accompanying renal injury of DS rats sequentially, under RAS-enhanced conditions by salt restriction. The purpose of this study was to examine the possible NO-RAS interaction under salt restriction in DS rats and evaluation of the renal injury associated with chronic NO-inhibition.

## Materials and Methods

### Materials

Five-week-old Dahl-Iwai salt-sensitive (DS;  $n = 24$ ) and salt-resistant (DR;  $n = 24$ ) rats were purchased from Japan SLC, Inc (Shizuoka, Japan). All rats were housed in climate-controlled metabolic cages with a 12-h light/12-h dark cycle. The rats were fed a low (0.3%) NaCl diet (MF, Oriental Yeast Co, Tokyo, Japan), with water provided ad libitum.  $N^G$ -nitro-L-arginine (L-NA) was purchased from Sigma-Aldrich Fine Chemicals (Tokyo). Oligonucleotides for RT-PCR were synthesized with a Model 380B DNA synthesizer (Applied Biosystems, Foster City, CA). The locations of oligonucleotides for primer pairs were as follows: AT<sub>1</sub>R, 131 to 152 and 736 to 759.<sup>15</sup> The experimental protocol was approved by the Animal Ethics Review Committee of Okayama University Medical School.

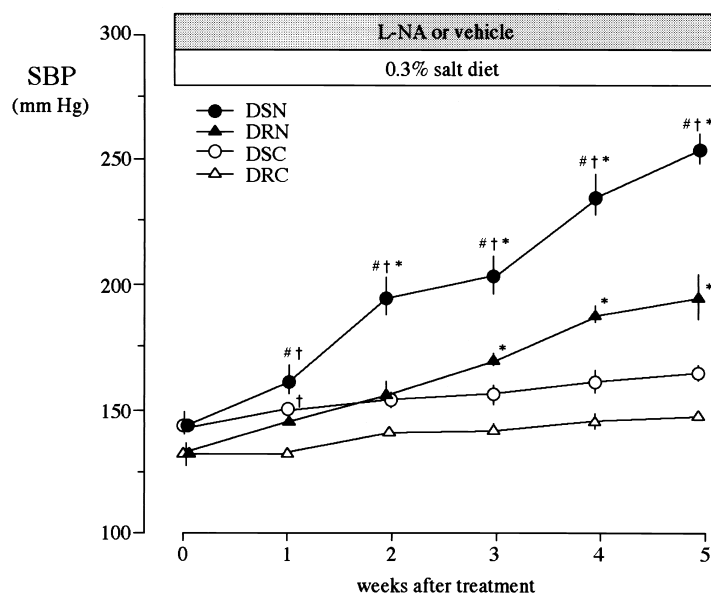
### Experimental Protocol

At 6 weeks of age, DS rats were divided at random into two groups, the first group was provided with deionized water containing L-NA (60 mg/L) (DSN) for 2 weeks ( $n = 6$ ) and 5 weeks ( $n = 6$ ), whereas rats in the second group were provided with the vehicle only (deionized water, DSC) for the same period of time (2 weeks;  $n = 6$ , 5 weeks;  $n = 6$ ). The DR rats were also divided at random

into two groups using a similar regimen (DRC;  $n = 12$ , DRN;  $n = 12$ ). All rats were maintained on a 0.3% NaCl diet throughout the experimental period. Systolic blood pressure (SBP) was measured weekly at 9 AM in conscious and restrained rats placed in a warm environment, using tail-cuff plethysmography (UR-5000, Ueda Seisakusyo, Tokyo). Changes in body weight (BW) and heart rate (HR) were recorded every week for 5 weeks. Once a week, urine was collected over a 24-h period and used for the measurement of urinary concentration of sodium urinary protein, *N*-acetyl- $\beta$ -glucosaminidase (NAG) activity, nitrate plus nitrite (u-NOx), and aldosterone (u-Aldo) excretion. Urinary concentration of sodium was measured by an autoanalyzer system. Urinary protein and NAG excretion were determined using the Bio-Rad protein assay kit (Bio-Rad Laboratories, Richmond, CA) and NAG test pack (Shionogi Pharmaceutical Co, Osaka, Japan), respectively. The u-NOx excretion was quantitated using NO<sup>2-</sup>/NO<sup>3-</sup>-assay kit-C (Dojindo, Kumamoto, Japan). The excretion of u-Aldo was determined using a SPAC-S-Aldosterone RIA kit (Daiichi Radioisotope, Tokyo). After 2 or 5 weeks of treatment, the rats were killed by decapitation, and the kidneys and hearts were removed quickly and weighed. The kidneys were then prepared for histologic examination. Trunk blood samples were collected and immediately stored at  $-30^{\circ}\text{C}$  until assayed for serum concentrations of sodium, potassium, creatinine, uric acid, and total protein by an autoanalyzer system. Plasma renin activity (PRA) was determined by RIA for ANG I (Dinabot Radioisotope, Tokyo).

### Histologic Examination

A portion of each kidney was fixed in 10% buffered paraformaldehyde, embedded in paraffin, sectioned into 4- $\mu\text{m}$  slices, and stained with periodic acid-Schiff (PAS) reagent. Histologic assessment included measurement of the diameter ( $\mu\text{m}$ ) of each of 200 to 300 randomly selected glomeruli in each experimental group using an objective micrometer (OB-M, Olympus Optical Co, Tokyo). The degree of glomerular sclerosis in each rat was assessed using the mesangial injury score described by Raij et al.<sup>16</sup> For this purpose, a minimum of 100 glomeruli in each specimen was examined, and the severity of the lesion was graded from 0 to 4+ according to the percentage of glomerular involvement. Thus, the involvement of 25% of glomeruli was scored as 1+ lesion, whereas the involvement of all glomeruli was scored as 4+ lesion. An injury score was calculated by multiplying the degree of damage (0 to 4+) by the percentage of the glomeruli with that type of injury (ie, increase in mesangial matrix material or glomerulosclerosis). The extent of injury in each tissue specimen was calculated by adding these scores. In addition, glomerular cellularity was determined by counting the total number of cells containing nuclei in each glomerulus (at least 100 glomeruli in each specimen).



**FIG. 1.** Serial changes in systolic blood pressure (SBP). Baseline SBP was similar in all groups but increased gradually in both the DSN and DRN groups. SBP in the DSN group was significantly higher than in DRN group 1 week after treatment with L-NA and remained high until the end of the study. The percent rise in SBP induced by NOS inhibition was higher in DS rats than in DR rats. The SBP of the DRN and DSN were  $117\% \pm 4\%$  and  $136\% \pm 7\%$  ( $P < .05$ ) at week 2, and  $134\% \pm 6\%$  and  $175\% \pm 8\%$  ( $P < .05$ ) at week 5, respectively. The SBP in the DSC group was higher than in the DRC group, albeit insignificantly, throughout the experimental period. Control Dahl salt-resistant rats (DRC;  $\Delta$ ), L-NA-treated Dahl salt-resistant rats (DRN;  $\blacktriangle$ ), control Dahl salt-sensitive rats (DSC;  $\circ$ ), and L-NA-treated Dahl salt-sensitive rats (DSN;  $\bullet$ ). L-NA, *N*<sup>G</sup>-nitro-L-arginine. Data represent the mean  $\pm$  SEM of six rats in each group. \* $P < .05$  versus the corresponding control group;  $\dagger P < .05$  v DRC; # $P < .05$  v DRN.

### Quantification of Glomerular RNA Expression by Competitive RT-PCR

The renal cortex was dissected out and minced in ice-cold PBS. Glomeruli were isolated using the graded sieving technique. Total RNA was extracted using RNeasy Midi Kits (Quiagen, Santa Clarita, CA). The RNA was quantified by measuring absorbance at 260 nm and stored at  $-20^{\circ}\text{C}$  until assay. Competitive RT-PCR for AT<sub>1</sub>R was performed as reported previously by our laboratory.<sup>13</sup> Briefly, after mutant cDNA for competitive PCR were generated using the PCR MIMIC Construction Kit (Clontech Laboratories, Palo Alto, CA), mutant fragments for G3PDH as internal controls and AT<sub>1</sub>R were reamplified with two sets of specific primers to determine their ability to act as competitors for the native mRNA. The obtained products were as follows: G3PDH, 596 bp; AT<sub>1</sub>R, 340 bp, which were purified using a CHROME SPIN Column (Clontech, Palo Alto, CA), and diluted to 100  $\mu\text{mol}/\mu\text{L}$  with 10  $\mu\text{g}/\mu\text{L}$  ultrapure glycogen. Extracted glomerular RNA was reverse transcribed using a Gene Amp RNA PCR kit (Perkin Elmer Cetus, Norwalk, CT). RT was carried out with 10 ng of RNA per reaction using random hexamer (2.5  $\mu\text{mol}/\text{L}$ ), reverse transcriptase (2.5 U/ $\mu\text{L}$ ), and deoxynucleotide triphosphate (dNTP; 1 mmol/L) at the following conditions:  $42^{\circ}\text{C}$  for 55 min,  $99^{\circ}\text{C}$  for 5 min, and  $4^{\circ}\text{C}$  for 5 min. The resulting cDNA was resuspended in 50  $\mu\text{L}$  deionized, autoclaved water for competitive PCR analysis. The linear portion of the relationship between native cDNAs and competitive mutant cDNAs was then

determined for G3PDH and AT<sub>1</sub>R. For a preliminary analysis, a fixed amount (0.5 ng) of cDNA derived from RNA of DSC was coamplified with 10-fold serial dilutions (1 to  $10^{-17}$ ) of competitive mutant cDNAs using the two primer sets and the preceding PCR kit. For fine-tuned competitive PCR, another reamplification was performed using twofold serial dilutions ( $2^{-1}$  to  $2^{-8}$ ) of one of the dilution steps of the preliminary PCR as a starting point as follows: AT<sub>1</sub>R,  $10^{-10}$ . The competitive PCR was performed using the preceding PCR kit and a thermal cycler (Perkin Elmer Cetus) under the following conditions; 36 cycles of denaturation at  $94^{\circ}\text{C}$  for 1 min, annealing at  $60^{\circ}\text{C}$  for 1 min, and extension at  $72^{\circ}\text{C}$  for 1 min. Aliquots of the PCR products of cDNA and competitive mutant cDNA were electrophoresed on 1.5% agarose gels, visualized by ethidium bromide staining, and photographed using an instant positive/negative film (337; Polaroid, Cambridge, MA). These negative photographs were analyzed by a scanning densitometer (Scanning Imager 300-SX, Molecular Dynamics, Sunnyvale, CA), and the relative integrated density of each band was calculated by taking the absorbance multiplied by the surface area. Finally, the ratios between the densitometric readings of the native cDNA- and mutant cDNA-PCR products were plotted linearly using a logarithmic scale, and cDNA from all individual samples were subjected to competitive PCR analysis according to the established working ranges in which linear relationship existed, using the AT<sub>1</sub>R primers. Control analysis was carried out using G3PDH primers.

**Table 1.** Body, kidney, and heart weights and body weight ratios after 2 and 5 weeks of treatment with L-NA

		DRC	DRN	DSC	DSN
BW (g)	2 wk	283.3 ± 6.1	283.3 ± 5.1	268.3 ± 7.6	275.8 ± 5.1
	5 wk	385.0 ± 4.7	376.3 ± 7.2	351.7 ± 3.7	301.3 ± 15.2*†‡
KW (g)	2 wk	1.01 ± 0.03	1.01 ± 0.01	1.07 ± 0.03	1.08 ± 0.03
	5 wk	1.22 ± 0.02	1.23 ± 0.03	1.32 ± 1.26	1.30 ± 0.04
HW (g)	2 wk	0.92 ± 0.02	0.90 ± 0.02	0.91 ± 0.02	0.91 ± 0.03
	5 wk	1.13 ± 0.01	1.23 ± 0.03	1.14 ± 0.02	1.30 ± 0.04*‡
KW/BW (%)	2 wk	0.35 ± 0.01	0.35 ± 0.01	0.39 ± 0.01#†	0.40 ± 0.01†‡
	5 wk	0.32 ± 0.06	0.32 ± 0.09	0.38 ± 0.07#†	0.42 ± 0.21*†‡
HW/BW (%)	2 wk	0.32 ± 0.01	0.32 ± 0.01	0.33 ± 0.01	0.33 ± 0.01
	5 wk	0.30 ± 0.04	0.33 ± 0.11	0.33 ± 0.04	0.44 ± 0.23*†‡

DRC = Dahl salt-resistant (DR) control rats received deionized water and treated with a low-salt diet; DRN = DR rats received deionized water containing L-NA and treated with a low-salt diet; DSC = Dahl salt-resistant (DS) control rats received deionized water and treated with a low-salt diet; DSN = DS rats received deionized water containing L-NA and treated with a low-salt diet.

Each value represents the mean ± SEM; n = 6 in each group.

\*  $P < .05$  v each control group; †  $P < .05$  v DRC; ‡  $P < .05$  v DRN.

## Statistical Analysis

Data were expressed as mean ± SEM. Differences between groups were analyzed for statistical significance using one-way analysis of variance (ANOVA) followed by post hoc Scheffé's test. Linear regression analysis was applied to analyze the correlation between u-NOx and SBP using data for the entire group (DS and DR rats). All statistical analyses were performed using the StatView software (Abacus Concepts, Berkeley, CA).  $P$  values < .05 were accepted as statistically significant.

## Results

### Serial Changes in SBP

Fig. 1 shows changes in SBP in the four groups of rats. Baseline SBP was similar in all groups; however, it increased gradually in both the DSN and DRN groups and reached  $256 \pm 6$  mm Hg and  $166 \pm 3$  mm Hg on week 5, respectively. The SBP in the DSN group was significantly higher than in the DRN group 1 week after treatment with L-NA and remained high until the end of study. The percent rise in SBP induced by NOS inhibition was higher in DS rats than in DR rats; namely, the SBP of the DRN and DSN were  $117\% \pm 4\%$  and  $136\% \pm 7\%$  ( $P < .05$ ) on week 2, and  $134\% \pm 6\%$  and  $175\% \pm 8\%$  ( $P < .05$ ) on week 5, respectively. The SBP in the DSC group was higher than in the DRC group, albeit insignificantly, throughout the experimental period.

### Body Weights, Organ Weights, and Body Weight Ratios

After 5 weeks of treatment with L-NA, the BW of the DSN group was significantly lower than that of other groups (Table 1). There were no significant differences in the kidney weights (KW) among the four groups either in 2-week or in 5-week treated animals. The mean heart weight (HW) of the DSN group after 5 weeks of treatment was significantly higher than that of the DSC and DRC groups. The KW/BW ratios were significantly higher in the DSC and DSN groups than in the DRC and DRN in both 2-week and 5-week treated animals. The KW/BW ratio of the DSN after 5 weeks of treatment was also significantly higher than that of the DSC. The HW/BW ratios were significantly higher in the DSN at week 5 compared with the other groups.

### Changes in Urine Volume, Urinary Sodium, Protein, and NAG Excretion

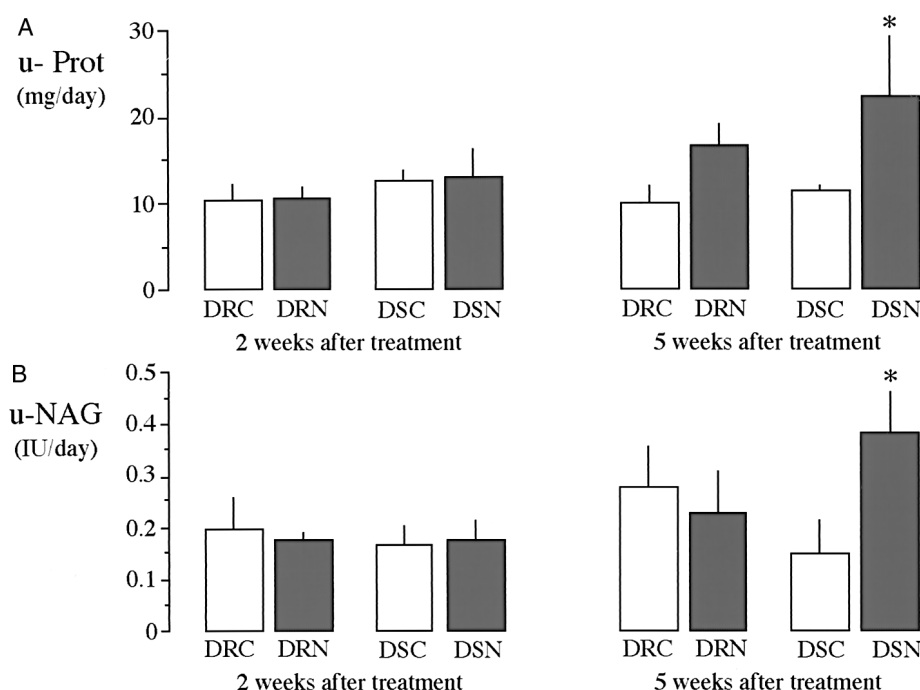
After 5 weeks of treatment with L-NA, 24-h urine excretion tended to increase in the DRN and DSN (Table 2). Urinary sodium excretion was not different among the groups. Urinary protein and NAG excretion in the DSN group were significantly higher than in the DSC group after 5 weeks of treatment, whereas no differences were observed in the groups treated for 2 weeks (Fig. 2).

**Table 2.** Urine volume and urinary sodium excretion after 2 and 5 weeks of treatment with L-NA

		DRC	DRN	DSC	DSN
Urine volume (mL)	2 wk	23.8 ± 2.6	26.1 ± 4.2	24.3 ± 2.8	30.7 ± 8.3
	5 wk	11.0 ± 2.5	20.1 ± 4.9	9.2 ± 0.6	22.7 ± 7.0
Urinary sodium excretion (mEq/day)	2 wk	1.0 ± 0.3	0.8 ± 0.1	0.8 ± 0.1	1.0 ± 0.2
	5 wk	0.3 ± 0.1	0.5 ± 0.1	0.4 ± 0.1	0.5 ± 0.1

For a description of the experimental groups, see Table 1. Each value represents the mean ± SEM; n = 6 in each group.





**FIG. 2.** Changes in urinary protein (u-Prot, **A**) and urinary *N*-acetyl- $\beta$ -glucosaminidase (u-NAG, **B**) excretion. u-Prot and u-NAG excretion in the DSN group were significantly higher than in the DSC group after 5 weeks of treatment, whereas no significant differences were observed in the groups at 2 weeks of treatment. For a description of the experimental groups, see Fig. 1. \* $P < .05$  v the corresponding control group.

### Serum Electrolytes, Renal Function, and RAS Components

Serum creatinine levels after 5 weeks of treatment with L-NA were significantly higher in the DSN group than in other groups (Table 3). In addition, there were no significant differences in serum concentrations of sodium or potassium in all experimental groups. As shown in Table 4, no significant differences were observed in the groups at week 2, either in PRA and in u-Aldo excretion. However, at week 5, the PRA level in the DSN group was significantly higher than in the DSC group, and the u-Aldo level significantly higher than in the other groups.

### Changes in u-NOx Excretion

In both strains, 2-week L-NA treatment tended to reduce u-NOx excretion (Fig. 3). At week 5, L-NA treatment

significantly reduced u-NOx excretion in both strains compared with the corresponding controls. The fall in excretion in DR rats (76% mean reduction from week 0 to 5) was similar to that in DS rats (77% mean reduction from week 0 to 5). The correlation between SBP and u-NOx excretion in DR rats was not significant ( $r = -0.201$ ,  $P = .145$ ,  $n = 60$ ). However, there was a significant negative correlation between SBP and u-NOx excretion in DS rats ( $r = -0.610$ ,  $P < .01$ ,  $n = 60$ ).

### Histologic Findings

The glomerular structure was compared in four groups after 5-week treatment using light microscopy (Fig. 4). The glomeruli, blood vessels, and tubules were normal in all groups at week 2 (data not shown) as well as in the DRC, DRN, and DSC groups at week 5. In contrast,

**Table 3.** Serum electrolytes, creatinine, and uric acid levels after 2 and 5 weeks of treatment with L-NA

		DRC	DRN	DSC	DSN
Sodium (mEq/L)	2 wk	137.8 $\pm$ 0.4	137.8 $\pm$ 0.3	139.8 $\pm$ 0.8	139.7 $\pm$ 0.9
	5 wk	139.0 $\pm$ 0.7	139.8 $\pm$ 0.5	137.0 $\pm$ 1.7	137.0 $\pm$ 1.1
Potassium (mEq/L)	2 wk	6.6 $\pm$ 0.4	6.5 $\pm$ 0.2	6.8 $\pm$ 0.1	7.3 $\pm$ 0.1
	5 wk	7.2 $\pm$ 0.2	7.3 $\pm$ 0.1	7.4 $\pm$ 0.2	8.1 $\pm$ 0.4
Creatinine (mg/dL)	2 wk	0.52 $\pm$ 0.02	0.50 $\pm$ 0.03	0.48 $\pm$ 0.03	0.52 $\pm$ 0.03
	5 wk	0.57 $\pm$ 0.04	0.57 $\pm$ 0.02	0.57 $\pm$ 0.04	0.83 $\pm$ 0.12*†‡
Uric acid (mg/dL)	2 wk	1.50 $\pm$ 0.33	1.35 $\pm$ 0.11	1.30 $\pm$ 0.08	1.32 $\pm$ 0.15
	5 wk	1.67 $\pm$ 0.06	1.63 $\pm$ 0.03	1.62 $\pm$ 0.06	1.92 $\pm$ 0.22

For a description of the experimental groups, see Table 1. Each value represents the mean  $\pm$  SEM;  $n = 6$  in each group.

\*  $P < .05$  v each control group; †  $P < .05$  v DRC; ‡  $P < .05$  v DRN.

**Table 4.** PRA levels, and u-Aldo excretion after 2 and 5 weeks of treatment with L-NA

		DRC	DRN	DSC	DSN
PRA (ng/mL/h)	2 wk	2.73 ± 0.67	1.84 ± 0.29	2.19 ± 0.15	1.68 ± 0.34
	5 wk	3.42 ± 1.44	4.20 ± 0.50	2.05 ± 0.23	7.11 ± 3.33*
u-Aldo (ng/day)	2 wk	15.9 ± 1.5	16.0 ± 2.0	16.0 ± 1.6	18.5 ± 4.8
	5 wk	11.5 ± 3.2	14.9 ± 3.2	7.7 ± 0.7	31.4 ± 6.0*†‡

For a description of the experimental groups, see Table 1. Each value represents the mean ± SEM; n = 6 in each group.

PRA = plasma renin activity; u-Aldo = urinary aldosterone excretion.

\*  $P < .05$  v each control group; †  $P < .05$  v DRC; ‡  $P < .05$  v DRN.

damaged glomeruli were noted in the DSN group at week 5. These were characterized by glomerular segmental sclerosis, collapse of the glomerular tuft, and thickening of the wall of small arteries and arterioles. Only minimal proliferative changes were present in the glomeruli. These findings were also quantitated by mesangial injury scoring and analysis of glomerular cellularity (Fig. 5). At week 5, the mesangial injury score was significantly higher in the DSN group compared with the other groups, whereas glomerular cellularity was similar among the four groups. At week 2, these two parameters were almost identical in the four groups.

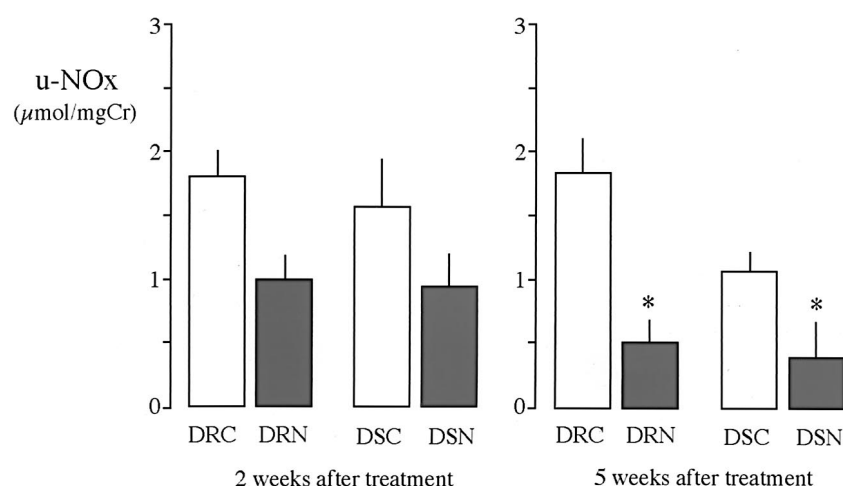
### Glomerular AT<sub>1</sub>R Expression

To quantify the level of glomerular expression of AT<sub>1</sub>R mRNA by competitive RT-PCR, we first determined the linear range of the ratios of coamplified mutant cDNAs and native cDNAs reverse transcribed from glomerular RNA, as described in Materials and Methods (Fig. 6A). For quantitation of native cDNA samples, we selected the

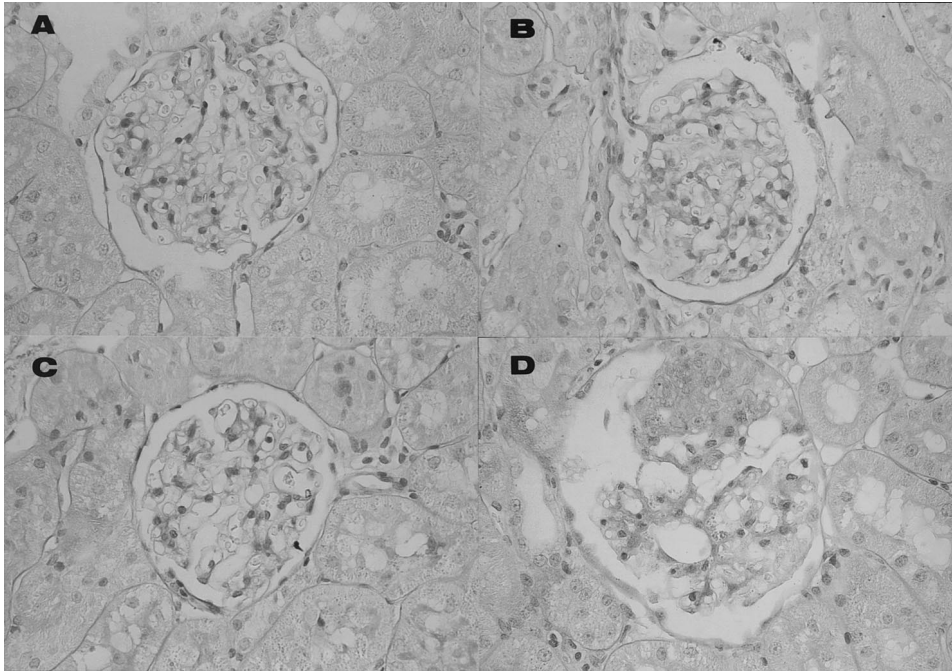
following logarithmic dilutions of mutant cDNA:  $2^{-4}$  ( $6.25 \times 10^{-10}$  amol/ $\mu$ L) for AT<sub>1</sub>R. For competitive PCR reactions, 2  $\mu$ L of these dilutions were added to 2  $\mu$ L of each cDNA (0.25 ng/ $\mu$ L). The resulting PCR products were quantified by densitometric scanning as described in Materials and Methods. At week 2, although AT<sub>1</sub>R mRNA levels were not significantly different between the L-NA-treatment groups and corresponding control groups either in DS or in DR rats, AT<sub>1</sub>R mRNA levels in DS rats were lower than in DR rats (Fig. 6B). At week 5, glomerular AT<sub>1</sub>R mRNA levels in DR rats were similar, whereas in DS rats alone L-NA-treatment markedly reduced glomerular AT<sub>1</sub>R mRNA level.

### Discussion

Our study was performed to elucidate the contribution of NO and RAS to hypertension and renal injury in DS rats. The salt-restrictive condition was intended to activate systemic RAS of DS rats as well as to reduce completely the



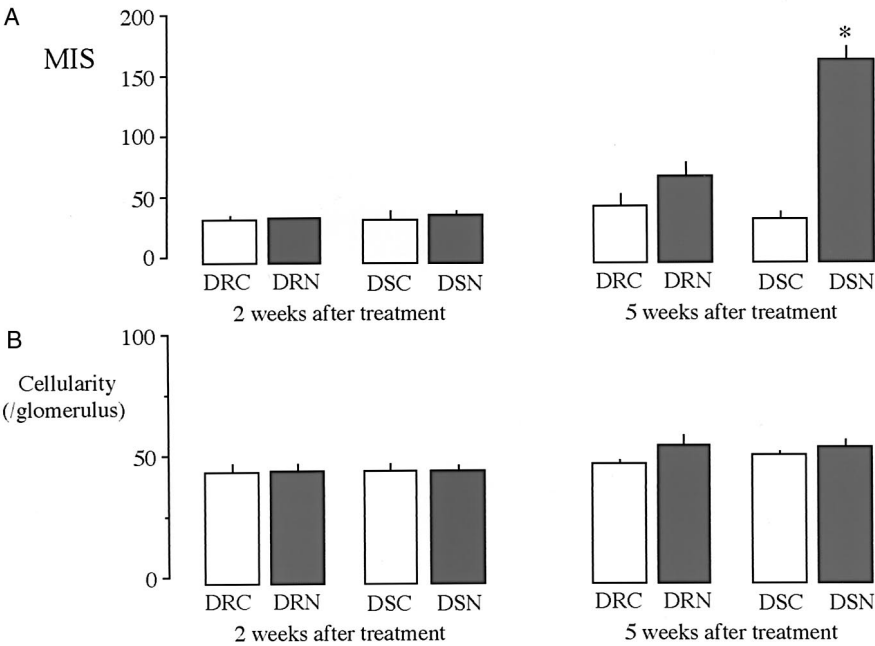
**FIG. 3.** Changes in urinary nitrate plus nitrite (u-NOx) excretion. The mean level of u-NOx excretion in the DSC group was less than in the DRC group throughout the experimental period. The u-NOx excretion in DS rats was lower than in DR rats, both at baseline and during treatment with L-NA. In both strains, u-NOx excretion at week 2 tended to decrease by L-NA treatment. At week 5, L-NA treatment significantly reduced u-NOx excretion in both strains compared with the corresponding controls. The fall in excretion in DR rats (76% mean reduction from week 0 to 5) was similar to that in DS rats (77% mean reduction from week 0 to 5). The correlation between SBP and u-NOx excretion in DR rats was not significant. However, there was a significant negative correlation between SBP and u-NOx excretion in DS rats ( $r = -0.610$ ,  $P < .01$ ). For a description of the experimental groups, see Fig. 1. Data are mean ± SEM of six rats. \* $P < .05$  v the corresponding control group.



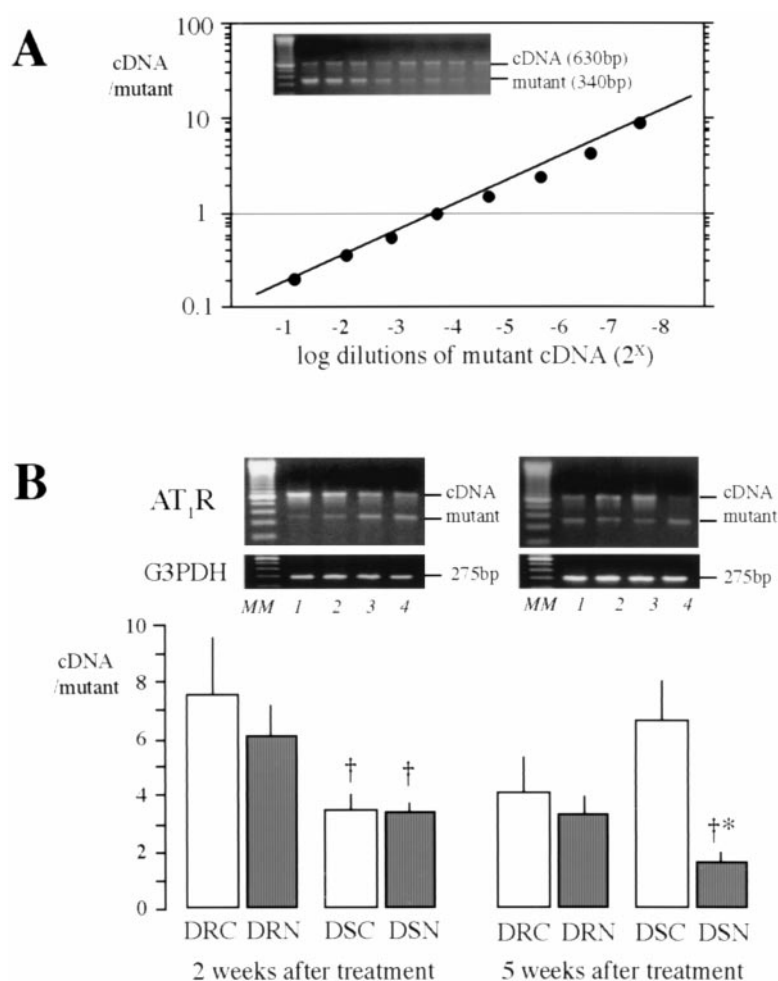
**FIG. 4.** Photomicrographs showing representative renal morphological changes in DRC (A), DRN (B), DSC (C), and DSN (D) groups after 5 weeks of treatment. Damaged glomeruli are noted in the DSN group. These are characterized by glomerular segmental sclerosis, collapse of the glomerular tuft, and thickening of the walls of small arteries and arterioles. In comparison, glomeruli, blood vessels and tubules were normal in all groups at week 2 (data not shown) as well as in DRC, DRN, and DSC groups at week 5. Periodic acid-Schiff staining, magnification  $\times 400$ .

direct action of salt loading such as salt retention, excessive glomerular hyperfiltration, and upregulation of  $AT_1R$ .<sup>17</sup> Our study showed three major findings: 1) the pressor response and glomerular injury to endogenous NO-inhibition were markedly enhanced in salt-restricted

DS rats compared to DR rats; 2) the response of circulating RAS to NO inhibition was enhanced at the late phase of salt-restricted DS rats; and 3) glomerular  $AT_1R$  mRNA levels of salt-restricted DS rats was significantly decreased by NO inhibition at the late phase.



**FIG. 5.** Mesangial injury score (A) and glomerular cellularity (B). At week 5, the mesangial injury score was significantly higher in the DSN group compared with the other groups, whereas glomerular cellularity was similar among the four groups. At week 2, these two parameters were almost identical in the four groups. For a description of the experimental groups, see Fig. 1. Data are mean  $\pm$  SEM of six rats in each group. \* $P < .05$  v other groups.



**FIG. 6.** Glomerular AT<sub>1</sub>R expression analyzed by competitive RT-PCR. **A**) Linear range for analysis of competitive RT-PCR and control. **B**) Quantitation of glomerular mRNA expression by competitive RT-PCR. The amounts of PCR products were determined by densitometric scanning. At week 2, although AT<sub>1</sub>R mRNA levels did not significantly differ between L-NA-treatment groups and the corresponding control groups either in DS or in DR rats, their levels in DS rats were lower than in DR rats. At week 5, glomerular AT<sub>1</sub>R mRNA levels in DR rats were similar, whereas in DS rats alone L-NA-treatment markedly reduced the glomerular AT<sub>1</sub>R mRNA level. Data are mean  $\pm$  SEM of six rats in each group. \* $P < .05$  v the corresponding control group; † $P < .05$  v DRC.

First, it is noteworthy that NO inhibition induced a significant rise in SBP in both DS and DR rats even under sodium restriction. The pressor effect was markedly enhanced in DS rats compared to DR rats, despite the fact that the NO reductive effect by L-NA was similar between DS and DR rats. Although a significant negative correlation between SBP and u-NOx excretion was observed in DS rats, the correlation was insignificant in DR rats. These findings indicate that endogenous NO production in DS rats is largely influenced by the arginine analogue of L-NA, suggesting that the NO system in DS rats may constitute an important factor as a determinant of systemic blood pressure. It is also presumed that DS rats may suffer from latent impairment of NO production independent of sodium handling, which may involve genetic susceptibility to hypertension and hypertensive organ injuries in DS rats. In other words, the difference in SBP could result from differences in the compensatory response to inhibition of NO synthesis, ie, the ability to increase other vasoactive

control systems such as sympathetic nervous system (SNS) activity, endothelin, and angiotensin levels. Previous studies have shown that persistent hypertension is sometimes associated with vascular damage, which is followed by diminished production of NO from the endothelium. Indeed, Luscher et al reported that long-term antihypertensive therapy prevented impairment of endothelium-dependent relaxation observed during the prehypertensive stage in DS rats.<sup>18</sup>

Inhibition of endogenous NO is considered to enhance the pressor response by vasoconstrictive substances such as angiotensin II or noradrenaline.<sup>19–21</sup> In this regard, Jover et al<sup>10</sup> reported that losartan, an AT<sub>1</sub>R antagonist, attenuated NO-inhibitory hypertension, suggesting that RAS is involved in the pathogenesis of this type of hypertension. In addition, our findings of increased levels of PRA and u-Aldo excretion in DS rats after 5 weeks of L-NA treatment suggest that RAS activation may be involved in the rise in blood pressure induced by chronic NO



inhibition in DS rats. NO is known to directly suppress renin secretion from the juxtaglomerular apparatus (JGA).<sup>22</sup> It is also reported that endogenous NO inhibition activates sympathetic nerves via the central nervous system,<sup>23,24</sup> resulting in an increase in PRA. Several studies have demonstrated the presence of glomerular sclerosis and fibrinoid necrosis after long-term NO inhibition.<sup>11,21,25,26</sup> However, to our knowledge, there are no reports documenting changes in glomeruli in sodium-restricted DS rats. In the present study, focal sclerotic lesions and mesangial expansion, quantitated by MIS scoring, were observed in the glomeruli of the DSN group. These histologic changes were accompanied by increased proteinuria and urinary NAG excretion. Glomerular lesions induced by chronic NO inhibition are considered to result from glomerular capillary hypertension<sup>25,26</sup> or from the suppression of platelet agglutination. Interestingly, the glomerular changes in the DSN group did not include glomerular hypercellularity. In DOCA salt hypertension (DOCA)<sup>27</sup> and high-salt loaded DS rats,<sup>13</sup> which develop high blood pressure equivalent to that in the DSN group in this study, glomerular cellularity is usually increased in parallel with the increase in MIS score.

Among the components of RAS, Ang II directly stimulates the growth of mesangial cells via AT<sub>1</sub>R. Overexpression of Ang II causes mesangial expansion or glomerulosclerosis rather than cell proliferation, because Ang II is involved in protein synthesis in mesangial cells.<sup>13,16</sup> The dissociation between MIS and glomerular cellularity in DSN group bears resemblance to the glomerular lesion caused by Ang II. To elucidate the contribution of Ang II to the glomerular lesion, the expression level of Ang II receptor was examined. Two major types of Ang II receptors, type 1 and type 2, have been identified.<sup>28,29</sup> The hemodynamic and nonhemodynamic effects of Ang II are mediated primarily by AT<sub>1</sub>R.<sup>30,31</sup> The inhibition of RAS using ACEI or AT<sub>1</sub>R antagonist was effective in inhibiting the cell-proliferative and sclerotic changes in glomeruli of hypertensive animals including DS rats.<sup>13</sup> As a parameter of RAS in the glomeruli of NO-inhibitory DS rats, we evaluated AT<sub>1</sub>R mRNA levels in glomeruli using competitive RT-PCR analysis. The glomerular AT<sub>1</sub>R level was lower in DS rats than in DR rats at week 2. This finding may reflect the difference in systemic blood pressure between DS and DR rats. On the other hand, at week 5, L-NA treatment markedly reduced the level of glomerular AT<sub>1</sub>R in DS rats compared to age-matched control DS rats, whereas such an alteration was not observed in DR rats. These findings imply that the reduction of glomerular AT<sub>1</sub>R in NO-inhibitory DS rats might act as a self-limiting effect toward progressive glomerular damage. In this regard, Amiri and Garcia<sup>32</sup> reported that AT<sub>1</sub>R levels of preglomerular vessels and glomeruli are differentially regulated during the development of hypertension of two-kidney, one-clip hypertensive rats (a model of hypertension) because of RAS activation, suggesting that the glomerular AT<sub>1</sub>R level is regulated by plasma Ang II level. Considering the increase in PRA and aldosterone

levels in DS rats treated with L-NA in our study, reduced levels of glomerular AT<sub>1</sub>R might become downregulated through the activation of systemic RAS. Although we postulated that the present glomerular changes are caused by the indirect enhancement of RAS because of NO inhibition in salt-restricted DS rats, we cannot exclude the possible involvement of prolonged systemic hypertension (from the lack of NO) in these glomerular changes.

In conclusion, we have shown that NO inhibition induces an enhanced pressor response and renal histologic injury in DS rats even under salt restriction conditions. This change was accompanied by augmentation of RAS and glomerular AT<sub>1</sub>R downregulation. Our findings suggest that endogenous NO synthesis is closely involved in the development of hypertension and renal injury in DS rats under salt-restricted conditions. Differences in the interaction between NO and RAS might partially determine the salt sensitivity of Dahl rats.

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