Alterations in Renal Endothelial Nitric Oxide Synthase Expression by Salt Diet in Angiotensin Type-1a Receptor Gene Knockout Mice

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Abstract. The effects of altered dietary salt intake and/or hydralazine-induced hypotension on renal endothelial nitric oxide synthase (eNOS) expression were determined in angiotensin type-1a receptor gene knockout (At1a−/−) and wild-type (At1a+/+) mice. In At1a−/− mice, the levels of renal cortical eNOS mRNA and protein were 5 times and 3.5 times higher, respectively, in the high-salt (4% NaCl) group than in the low-salt group (0.3% NaCl). Systemic BP of the high-salt group (105 ± 4.4 mmHg) was significantly higher than that of the low-salt group (77.0 ± 4.7 mmHg). When hydralazine was administered to the mutant mice fed a high-salt diet, BP was reduced to 72.5 ± 1.3 mmHg, with decreases in the levels of renal eNOS mRNA and protein expression to about half of those found in nontreated group. Consistent with the results for eNOS mRNA and protein expression, nicotinamide adenine dinucleotide phosphate (NADPH) diaphorase activity and eNOS immunoreactivity localized in the endothelium of the renal vasculature changed parallel with the amount of salt intake. In contrast to mutant mice, At1a+/+ mice did not show any changes in renal eNOS expression during the manipulation of salt intake and/or hydralazine-induced hypotension. These results suggest that At1a receptor-mediated inputs play critical roles in maintaining renal vascular eNOS expression and activity during changes in salt-water balance and systemic BP.

The endothelial nitric oxide synthase (eNOS) is distributed throughout the renal vasculature, from the renal arterial branches to the glomerular capillaries. NO synthesized by eNOS in response to renal perfusion pressure and the tubuloglomerular feedback system is thought to be an important modulator of vascular tone. Renal perfusion pressure has been shown to increase urinary NO2/NO3 excretion as well as output current from an electrode inserted into the renal cortex (1).

eNOS is a constitutively expressed enzyme responsible for the generation of basal NO release from endothelial cells. The major stimuli of enzyme activity include shear stress and/or pressure stretch. In the kidney, however, the regulation of eNOS activity under various physiologic conditions is not well understood. Previous experiments testing the effects of altered salt intake or BP on renal eNOS activity have yielded inconsistent results (2–8). While there is little information on the endogenous factors that regulate eNOS expression within the kidney, angiotensin II (AngII) is regarded as an important candidate for the stimulatory regulator of renal vascular eNOS expression (9,10).

In a previous study, we described the generation of angiotensin type-1a (At1a−/−) receptor gene knockout mice by gene targeting (11). We have used these mice as a model system to study the regulation of the renin angiotensin system (RAS) and related systems (11). We have found that homozygous mutant mice exhibit chronic hypotension and morphologic abnormalities in the renal vasculature (11,12). Moreover, we recently demonstrated that intrarenal arteries of At1a−/− mice show irregular arrangements of vascular smooth muscle cells and that these cells have a proliferative phenotype (13). Although At1a receptor deficiency in these mice had no effect on the regulation of RPF or GFR (14), physiologic experiments have shown that the tubuloglomerular feedback (TGF) response is impaired (15).

The association between AngII and eNOS suggested that the absence of the At1a gene might be associated with aberrant expression of eNOS. We therefore assayed eNOS expression in the renal vasculature of At1a−/− mice. We also tested the effects of salt loading or salt restriction on renal eNOS expression in these mice to demonstrate whether interruption of the AngII-mediated feedback system alters the regulation of eNOS activity.

Materials and Methods

Animals and Experimental Protocols
At1a−/− mice were generated by gene targeting as described previously (11). F1 offspring homozygous for the At1a receptor

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disruption were backcrossed six times with wild-type (At1a+/+)
C57BL/6J mice. Mice were maintained at the Yokohama City
University School of Medicine Laboratory Animal Center at 25°C under
a 12-h day/night cycle. All At1a+/+ mice and At1a+/− mice used in
these experiments were 8- to 10-wk-old. All experiments were per-
formed under the guidelines for animal experiments set by the Animal
Experiment Committee of Yokohama City University School of
Medicine.

Animals were fed a low-salt (LS, 0.04% NaCl), normal-salt (NS,
0.3% NaCl), or high-salt (HS, 4.0% NaCl) diet for 10 d. In some
cases, At1a+/− mice fed the HS diet and At1a+/+ mice fed the NS
diet also received hydralazine, at doses of 64 mg/kg per d and 80
mg/kg per d, respectively, for 7 d. Systolic BP was measured by a
tail-cuff method (BP-monitor MK-1100; Muromachi Kikai Co.,
Tokyo, Japan). Following experimental treatment, the mice were anes-
ethetized with chloroform, and the tissues were removed into liquid
nitrogen or fixative.

Real-Time Quantitative Reverse Transcription-PCR

Real-time quantitative reverse transcription (RT)-PCR was per-
determined to eNOS expression in the renal cortex. Total RNA
was isolated from the renal cortex by the acid guanidinium thiocya-
nate-phenol-chloroform extraction method (16); 0.3 µg of the sample
RNA was mixed with 10 U/µl SuperScript III reverse transcriptase
(Invitrogen, Ontario, Canada), 25 ng/ml oligo(dT)12-18, 0.5 mM
dNTP, and 5 mM dithiothreitol. The mixture was incubated at 50°C
for 50 min, heated to 70°C for 15 min to terminate the RT reaction,
and then incubated with 0.1 U/µl RNase H at 37°C for 20 min. PCR
amplification was performed by incubating 2 µl of the RT product
with 25 µl of Universal PCR Master Mix (Applied Biosystems)
and the 250 nM designed TaqMan probe for eNOS and 18S at a 50-
% annealing temperature. Following an optimal UNG enzyme
activity at 50°C for 2 min and to activate AmpliTaq Gold enzyme at
95°C for 10 min, and then the amplification protocol consisted of 40
cycles of denaturation at 95°C for 15 sec, annealing at 60°C for 60
sec. Expression levels of eNOS mRNA were normalized with 18S
ribosomal RNA levels. Relative eNOS expression levels were ex-
pressed by comparative threshold cycle (Ct) methods.

Western Blotting

Each kidney cortex sample was homogenized in buffer containing
50 mM Tris-HCl (pH 8.0), 2 mM EGTA, 10 mM phenylmethylsul-
fonyl fluoride, 20 mg/ml aprotonin, 10 mg/ml leupeptin, and 0.1%
SDS using a Teflon glass homogenizer. The total protein concentra-
tion of each sample was measured, and the volume of each sample
was adjusted to allow loading of 30 µg of protein into each well.
Samples were boiled in SDS-Laemmli buffer for 10 min and loaded
onto 7.5% SDS-polyacrylamide gels. After gel electrophoresis, the
proteins were transferred to nitrocellulose membranes, which were
subsequently washed in 50 mM Tris-HCl (pH 7.4), 0.9% NaCl, 0.2%
Triton-X100 (TBST), blocked overnight with 5% nonfat milk and
BSA in TBST, and incubated for 2 h with a 1:2500 dilution of mouse
monoclonal antibody against eNOS (Transduction Laboratories). The
membranes were rinsed 3 times with TBST and incubated for 30 min
with a 1:5000 dilution of horseradish peroxidase–labeled rabbit anti-
mouse IgG in TBST. After rinsing with TBST, the bound antibody
was detected using a SuperSignal substrate kit (Pierce).

NADPH Diaphorase Reaction

The nicotinamide adenine dinucleotide phosphate (NADPH) diaph-
orase (NADPHd) histochemical reaction was used as an index of
tissue activity of eNOS. Kidneys were fixed in 0.1 M phosphate buffer
(pH 7.4) containing 4% paraformaldehyde and 0.2% picric acid for
24 h, as described previously (12,17,18). The samples were cryopro-
tected with phosphate buffer containing 30% sucrose for 24 h and then
frozen in isopentane cooled by liquid nitrogen. Sections of 8 µm
were cut in a cryostat and thaw-mounted on chrome gelatin-coated glass
slides. The sections were washed twice with TBST and incubated for
60 min at 37°C in 50 mM Tris-HCl (pH 7.6), 0.02% nitroblue
tetrazolium, 0.08% b-NADPH, 0.3% Triton X-100. In each experiment,
tissues from At1a+/+ mice and At1a+/− mice were simulta-
neously subjected to the fixation and staining process. The reaction
times were identical for all groups. The staining intensity of NADPHd
was quantified by a morphometric analysis. The endothelial areas
in the interlobular arteries, from 30 to 40 µm in diameter (19), were
traced, and mean density was determined using Scion Image (Scion
Corporation). In each experimental group, data from the cross-sectioned
areas of the 30 interlobular arteries were integrated, and then
statistical analysis was determined by unpaired t test using Statview
5.0 statistical software application. Data are expressed as mean ±
SEM.

Immunohistochemical Staining

Fixed kidneys were dehydrated, embedded in paraffin, sectioned at
4 µm thickness, and mounted on glass slides. The sections were
processed using the labeled streptavidin-biotin complex (LSAB-II)
technique (DAKO, catalyzed signal amplification system). Briefly,
sections were dewaxed, rehydrated, and incubated with 3% hydrogen
peroxide for 5 min. After rinsing in TBST, the sections were pre-
incubated in 5% normal goat serum for 60 min and incubated for 1 h
with a primary polyclonal antibody against eNOS (dilution 1:100; Transduction Laboratories). The sections were rinsed with TBST
and subjected to sequential 15 min incubations with biotinylated second-
ary antibody and the streptavidin-biotin-peroxidase complex. Staining
was completed by a 5 min incubation with 3,3’-diaminobenzidine
tetrahydrochloride (DAB), and the sections were counterstained with
hematoxylin. In each experiment, tissues from At1a+/+ mice and
At1a+/− mice were simultaneously subjected to the fixation and
staining process. The reaction times were identical for all groups.

Statistical Analyses

Data are expressed as mean ± SEM. Statistical significance was
determined by unpaired t test, with P < 0.05 being deemed statisti-
cally significant.

Results

Effects of Salt Intake on Systolic BP and Body Weight

At1a+/− mice fed a NS diet had significantly lower systolic
BP (88.9 ± 2.4 mmHg, n = 12) than At1a+/+ mice fed the
same diet (110 ± 6 mmHg, n = 10, P < 0.05). In the At1a+/− mice,
the HS diet significantly increased systolic BP to 105.0
± 4.4 mmHg (P < 0.01 versus NS group, n = 12), and the LS
diet significantly decreased systolic BP to 77.0 ± 4.7 mmHg
(P < 0.05 versus NS group, n = 6). In contrast, altered dietary
salt intake did not affect systolic BP of the At1a+/+ mice.

Treatment with hydralazine decreased the systolic BP of the
At1a+/+ mice fed the NS diet to 71.3 ± 2.0 mmHg (n = 6),
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and it also decreased the systolic BP of At1a−/− mice fed the HS diet to 72.5 ± 1.3 mmHg (n = 8).

Neither altered salt intake nor hydralazine had any significant effect on body weight of At1a+/+ mice. In contrast, ingestion of a HS diet significantly increased body weight of At1a−/− mice from 27.5 ± 0.23 g (n = 6) in the NS group to 29.6 ± 0.2 g (n = 6) in the HS group (P < 0.01 versus NS group), and ingestion of a LS diet significantly decreased body weight to 23.7 ± 0.6 g (n = 6) in the LS group (P < 0.01 versus NS group, n = 6). Hydralazine treatment, however, did not affect the body weight of At1a−/− mice.

Expression of eNOS mRNA and Protein
The amounts of eNOS mRNA in the renal cortex were analyzed by real-time quantitative RT-PCR. There was no significant difference in eNOS mRNA expression between At1a+/+ mice and At1a−/− mice fed the NS diet (data not shown). In wild-type mice, altered salt intake did not affect the levels of eNOS gene expression in the renal cortex (Figure 1A). In the At1a−/− mice, however, eNOS mRNA levels were significantly higher in the HS group than in the NS group and significantly lower in the LS group than in the NS group. When we compared eNOS mRNA levels in the renal cortices of mice fed the LS and HS diets, we found that eNOS mRNA levels were 5.0 times higher in the HS group than in the LS group (Figure 2A). In other organs, including the liver and the heart, the levels of eNOS gene expression did not differ between the wild-type (data not shown) and mutant mice (Figure 3A).

We also assayed eNOS protein expression in the renal cortices of wild-type and mutant mice by Western blotting. At1a+/+ mice and At1a−/− mice fed the NS diet had the same level of expression of eNOS protein (data not shown). In the wild-type mice, salt level had no effect on eNOS protein expression (Figure 1B). In the mutant mice, however, renal expression of eNOS protein significantly increased during salt loading (HS diet) and decreased during salt restriction (LS diet), with eNOS protein levels 3.5 times higher in the HS group than in the LS group (Figure 2B). eNOS protein expression in the heart and liver did not change during altered salt intake in either At1a+/+ (data not shown) or At1a−/− mice (Figure 3B).
Effect of Dietary Salt on NADPHd Histochemistry

NOS activity in renal tissues was detected by NADPHd histochemical reaction. In agreement with previous findings (17), NADPHd-positive structures were distributed in the endothelium of the renal vessels, the macula densa, and the nerve fibers. Renal tubular cells were also positive for NADPHd, although the staining intensity was low. The distribution pattern and staining intensity did not differ between At1a+/+ and At1a−/− mice (data not shown).

When we assayed NADPHd activity in the renal vasculature of At1a−/− mice, we found it localized to the endothelial cells throughout the afferent arterioles to the renal arteries (Figure 4). When we compared mutant mice fed LS diet with those fed HS diet, we detected 3.0 times higher NADPHd staining intensity in the latter of interlobular arteries. This difference was especially pronounced in the afferent arterioles and interlobular arteries, but less so in the other renal vasculature, including the arcuate, interlobar, and renal arteries. In the At1a+/+ mice, we detected no differences in endothelial NADPHd staining intensity between the LS and HS groups.

Effect of Dietary Salt on eNOS Immunohistochemistry

The localization of eNOS protein in the renal tissues was assessed by immunohistochemistry. In both the At1a+/+ and At1a−/− mice, eNOS-positive structures were observed in the endothelium of the renal vasculature, primarily throughout the renal arterial branches and glomerular capillaries. The distribution pattern of eNOS was similar to that of NADPHd. In At1a−/− mice fed the HS diet, eNOS immunoreactivity was enhanced in the renal resistant vessels, including the afferent arterioles and interlobular arteries, whereas eNOS immunoreactivity was almost completely absent in mutant mice fed the LS diet (Figure 5). In contrast, altered salt intake had no effect on eNOS immunoreactivity in renal tissues from At1a+/+ mice.

Effect of Hydralazine on eNOS Expression

To determine whether altered BP affects eNOS expression, we evaluated the effects of hydralazine treatment on the expression and activity of eNOS in At1a+/+ and At1a−/− mice. In At1a+/+ mice, renal expression of eNOS mRNA and protein maintained constant levels during hydralazine treatment. In the At1a−/− mice, however, hydralazine-induced hypotension significantly reduced eNOS mRNA and protein expression (Figure 6).

We also tested the effect of hydralazine on NADPHd activity in the kidneys of At1a−/− mice (Figure 7). Hydralazine treatment significantly reduced the activity of this enzyme in the interlobular arteries of At1a−/− mice fed the HS diet. The signal intensity was comparable to that found in mutant mice fed the LS diet. In contrast, altered BP induced by hydralazine did not affect enzyme activity in the At1a+/+ mice.

Discussion

We have shown here that the level of renal cortical eNOS expression is increased by a HS diet and decreased by a LS diet in At1a−/− mice. Using the NADPHd histochemical reaction as an index of NOS activity, we found that, in mutant mice, the intensity of NADPHd staining in the endothelium of small arteries were dependent on the amount of dietary salt intake. These findings were not observed in larger renal vessels.
significantly alteration in renal eNOS expression or vascular NADPHd activity when dietary salt intake was altered.

The expression of vascular eNOS is primarily regulated by vascular shear stress and/or pressure stretch (20). In the kidney, renal blood flow (RBF) and intrarenal arterial pressure would affect eNOS expression. There is a positive correlation between renal perfusion pressure and urinary NO2/NO3 excretion, as well as between perfusion pressure and the output current from a NO-sensitive electrode inserted in the renal cortex, thereby suggesting that the amount of NO release from endothelium reflects renal vascular flow (1). Under normal conditions, however, renal circulation is adjusted within limited ranges by autoregulation, which covers wide ranges of salt intake and systemic BP. Earlier studies on normal rats have reported relatively constant renal eNOS expression during altered salt intake. Moreover, HS diet containing 4% NaCl did not affect eNOS expression in the renal cortex (3), and unaltered renal eNOS expression has been demonstrated during salt loading or salt restriction (5,7). The findings reported here on wild-type mice are thus in good agreement with these lines of evidence. Precisely controlled microcirculation by the renal autoregulation mechanism may help to fix the levels of eNOS expression within a limited range. In contrast, depressed renal cortical eNOS expression and increased BP have been reported during salt loading (2). The discrepancy between these findings may be due to the different experimental methods employed and/or by the different animal strains used.

The autoregulation mechanism of the renal vasculature consists of the myogenic response and TGF system, which are the major functions of the juxtaglomerular apparatus (21,22). The myogenic response consists of pressure-induced constriction of the renal resistant vessels, including the afferent arterioles and interlobular arteries (23). TGF secretion is part of the vasoconstrictor response of the afferent arterioles brought about by signals induced by NaCl delivery to the macula densa. These mechanisms regulate the vascular tone of the afferent arterioles and GFR. The juxtaglomerular apparatus also participates in regulating the synthesis and release of renin, the rate-limiting enzyme for the production of AngII. This peptide is known to play an important role in resetting myogenic responses and TGF (20,24,25). During salt restriction, increased AngII enhances the sensitivity of the myogenic response (25) and shifts the TGF curve to the right, thereby preventing further loss of NaCl. During salt loading, the sensitivity of the myogenic response is diminished, and the TGF curve shifts to the right, with a decrease in AngII production.

The autoregulation mechanisms of the intrarenal circulation may thus be disturbed in animals that lack the RAS. For example, At1a−/− mice have an impaired TGF response (15). Moreover, Atg−/− mice have been shown to have pathophysiologic conditions associated with increased diuresis and sodium/water loss (26). Ichihara et al. (27) demonstrated that the attenuation of the TGF response observed in At1a−/− mice involved enhanced counteracting modulation by the influence of nNOS possibly derived from macula densa. On the other hand, Patzak et al. (28) demonstrated that mainly endothelium-derived NO counteracts AngII-induced vasoconstriction of iso-

including the interlobar, arcuate, and renal arteries. These histochemical findings were confirmed by direct eNOS immunohistochemistry, suggesting that the changes in eNOS expression in the renal cortex may reflect changes in endothelial eNOS expression in the renal resistant vessels of At1a−/− mice. In contrast to mutant mice, At1a+/+ mice showed no...
lated mouseAf. Thus, the AngII–NO interaction is necessary for the intact TGF response. Myogenic response exhibited a 31% contribution to the autoregulation of renal circulation (29). Just et al. (25) demonstrated that AngII augments not only TGF, but also myogenic response. In At1a/H11002/H11002 mice, an intact myogenic response was reported as for the afferent arterioles (30), whereas such response has not yet been investigated in the interlobular arteries. Therefore it can be speculated that renal autoregulation of flow is incomplete state in At1a/H11002/H11002 mice and RBF cannot be maintain constant compared with At1a/H11001/H11001 mice. The experiments reported here on At1a/H11002/H11002 mice show that these animals experience dietary salt-dependent changes in body weight and systemic BP, suggesting that dietary NaCl intake can influence the balance of body fluid and circulating blood volume in mutant mice. This finding agree with prior studies that the absence of At1a receptors for AngII produces a state of sodium sensitivity in which alterations in sodium intake cause marked fluctuations in BP and At1a receptors expressed on renal vasculature and/or renal epithelia may play a critical role in sodium and volume homeostasis (31). Moreover, in agreement with this notion, whole kidney GFR and the size of glomerular tufts of Atg/H11002/mice were found to change markedly in response to altered salt intake (32).

The results reported here suggest that At1a/H11002/mice cannot maintain constant renal blood flow during conditions of volume expansion or depletion. In this context, it is noteworthy that altered dietary salt intake had profound effects on renal small arteries, such as the interlobular arteries and afferent arterioles, but did not affect the large renal vessels, such as the arcuate, interlobar, and renal arteries. This further suggests that the level of eNOS expression in the renal cortices of At1a/H11002/mice may reflect changes in renal perfusion pressure, leading to altered endothelial shear stress/pressure stretch in the small arteries.

In contrast to the mutant mice, At1a/H11002/mice, which possess an intact RAS, showed no significant changes in renal eNOS expression during altered salt intake or hydralazine treatment. The renal autoregulation mechanism mentioned above may be important in maintaining RBF and eNOS expression when either systemic BP or sodium/water balance is altered. In vitro studies have provided evidence for AT1 receptor-mediated stimulation of eNOS expression and activity in cultured endothelial cells (33–35). Moreover, in vivo experiments have shown that acute and chronic AngII infusion stimulates renal eNOS expression and Cai-dependent NOS activity (36). These lines of evidence support the idea that activation of the RAS by salt restriction or hydralazine treatment plays an important role in maintaining renal vascular eNOS activities.

It could be argued that altered salt intake itself directly affects renal eNOS expression in At1a/H11002/mice. Our finding, showing that renal eNOS expression stimulated by a HS diet was markedly reduced by hydralazine treatment, renders this unlikely. We found that hydralazine decreased the systolic BP of salt-loaded mutant mice to levels similar to those found in salt-restricted mutant mice. Moreover, hydralazine reduced NADPHd staining in the small arteries to negligible levels in salt-loaded mutant mice. Taken together, these results support the notion that renal perfusion pressure contributes substantially to eNOS expression in At1a/H11002/mice.

Under normal conditions, eNOS expression in At1a/H11002/mice was almost equal, suggesting that, despite the lower BP observed in mutant mice, renal blood flow was
maintained. This is in agreement with findings that the GFR, renal plasma flow, absolute sodium excretion, and fractional sodium excretion were not significantly altered in At1a−/− mice compared with wild-type mice (14). In the absence of AT1A receptors, AT1B receptors may partially assume their functional properties (37).

Although dietary manipulations altered eNOS expression in the renal cortex, salt intake had no effect on enzyme levels in other organs, including the liver, heart, lung, spleen, and quadriceps femoris muscle. This is in agreement with other studies on the tissue specificity of AngII-mediated regulation of eNOS. For example, AngII was shown to induce eNOS expression and nitrite release in cultured bovine pulmonary artery endothelial cells, but not in cultured coronary artery endothelial cells (34). In addition, chronic AngII infusion was shown to induce organ-specific changes in eNOS expression or Ca2+-dependent eNOS activity (36). Thus the eNOS activity of renal resistant vessels, such as the interlobular arteries and afferent arterioles, may be under stronger control of AngII than are other vessels.

In summary, we have shown that in At1a−/− mice renal cortical eNOS expression, as well as systemic BP and body weight, are altered by dietary salt intake and that lowering of the BP in the At1a−/− mice on HS diet by hydralazine blocked the elevation of renal eNOS expression. Changes in NOS enzymatic activity were observed along the endothelium of the interlobular arteries and afferent arterioles. In contrast, eNOS expression in other organs was not altered by salt intake or hydralazine treatment. In At1a+/+ mice, neither salt nor hydralazine produced significant alterations in renal eNOS expression. These results suggest that AT1a receptor-mediated inputs play essential roles in maintaining eNOS expression when renal perfusion pressure fluctuates. This regulation mechanism may be unique to the renal small arteries.

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