Abstract. The present study investigates whether neuronal type nitric oxide synthase (N-NOS) in the macula densa participates in the regulation of renal renin expression during altered dietary salt intake in angiotensinogen gene-knockout (Atg<sup>−/−</sup>) mice. Wild-type (Atg<sup>+/+</sup>) and Atg<sup>−/−</sup> mice were fed a low-salt (0.04% NaCl), normal-salt (0.3% NaCl), or high-salt (4% NaCl) diet for 2 wk. Histochemical staining for NADPH diaphorase (NADPHd) and renin were analyzed morphometrically. Levels of N-NOS and renin mRNA in renal cortical tissues were determined by reverse transcription-PCR and Northern blot analysis, respectively. In animals fed a normal-salt diet, the renal expressions of N-NOS and renin were markedly increased in Atg<sup>−/−</sup> mice compared with Atg<sup>+/+</sup> mice. When mutant mice were fed a high-salt diet, the signal intensity of the NADPHd reaction and the number of positively stained macula densa cells were significantly decreased. The levels of renal cortical N-NOS mRNA were also suppressed by the treatment. These changes were paralleled by decreases in renal renin-immunoreactive areas and the levels of renin mRNA. On the other hand, salt restriction did not produce further significant increases in the renal N-NOS and renin expressions in mutant mice, whereas a parallel inverse relationship was observed between these enzyme expressions and the levels of salt intake in wild-type mice. These results suggest that the N-NOS expression in the macula densa is inversely regulated by salt intake and that the enzyme activity is functionally linked to renal renin production. Salt-modulated renal N-NOS and renin expressions are independent on angiotensin formation in Atg<sup>−/−</sup> mice. (J Am Soc Nephrol 9: 355-362, 1998)

Renin is the rate-limiting enzyme for generating angiotensin II, a biologically active end product of the renin-angiotensin system (RAS). Renin synthesis and secretion are regulated by several factors, such as sodium chloride delivery at the distal tubules, renal perfusion pressure, sympathetic nerve tone, and the plasma level of angiotensin II (1,2). Tubular sodium chloride delivery affects renin production through a macula densa mechanism. The macula densa, a specialized cell cluster of the distal tubule, monitors the sodium chloride concentrations at the site and transfers the information to juxtaglomerular granular cells of the afferent arteriole (3–5). Although the signal transduction mechanism within this system is not yet well understood, it has recently been proposed that nitric oxide (NO) is an important candidate for the local intercellular mediator in the juxtaglomerular apparatus (6–8). Physiologic experiments have suggested that NO release from the macula densa is inversely regulated by tubular sodium chloride delivery and participates in the stimulatory control of renin synthesis and secretion (3,5–7,9,10). Histologic studies have demonstrated the presence of a neuronal isoform of NO synthase (N-NOS) in the macula densa (11–13). These results suggest that NO synthesized by the macula densa N-NOS serves as the negative feedback loop between dietary salt intake and the levels of renin production (14). This notion, however, is still a matter of controversy (15–18).

We previously generated angiotensinogen gene-knockout (Atg<sup>−/−</sup>) mice to obtain a model for examining RAS regulation mechanism (19). Mutant mice have no detectable plasma angiotensinogen or angiotensin peptides, and therefore lack functional RAS. The mean level of renal renin gene expression is sevenfold higher in mutant mice than in wild-type (Atg<sup>+/+</sup>) mice (19–21). This increase in renal renin expression has been explained by reduced renal perfusion pressure and/or complete disruption of the negative feedback of angiotensin II on renin-producing cells; however, the precise mechanisms remain unknown. Recently, we demonstrated that N-NOS is upregulated in the macula densa of Atg<sup>−/−</sup> mice, suggesting that increased N-NOS activity is involved in the overexpression of renin in the kidneys of mutant mice (22). The aim of the present study was to investigate whether altered tubular sodium chloride delivery affects renal renin...
production without affecting angiotensin formation and, if so, whether the N-NOS-mediated macula densa mechanism is functionally linked to the renin regulation. We determined the effects of altered dietary salt intake on the activity of N-NOS at the macula densa and renal renin expression at the levels of mRNA and protein in Atg⁻/⁻ mice.

Materials and Methods

Animals and Experimental Protocols

Homozygous mutant mice were generated by gene targeting as described previously (19). Atg⁺/+ and Atg⁻/⁻ mice, 10 to 12 wk old, were used. The animals were housed under a 12-h day/night cycle at a temperature of 25°C and fed either a low-salt (0.04% NaCl) or high-salt (4.0% NaCl) diet for 2 wk. Tap water was provided ad libitum. Before and after the treatment, systolic BP was measured by a tail-cuff method.

NADPH Diaphorase Reaction

Kidneys were fixed in vivo by perfusion as described previously (23,24). In brief, animals were perfused with 50 ml of saline containing 10 U/ml heparin, followed by 100 ml of 0.1 M phosphate buffer (PB; pH 7.4) containing 4% paraformaldehyde and 0.2% picric acid. Kidneys were post-fixed with the same fixative for 24 h, cryoprotected with 30% sucrose, and then frozen by liquid nitrogen. Sections of 8 μm were cut in a cryostat and thaw-mounted on chrome gelatin-coated glass slides.

The NADPH diaphorase (NADPHd) reaction was used as an index for tissue activity of NOS (25). Renal sections were incubated in 0.1 M PB containing 0.02% nitro blue tetrazolium, 0.08% -NADPH, and 0.3% Triton X-100 for 30 mm at 37°C, then washed with ice-cold PB. In each experiment, tissues from Atg⁺/+ and Atg⁻/⁻ mice were simultaneously subjected to the fixation and staining process. Enzyme activity was evaluated by counting the number of macula densa cells in which the signal intensity of the reaction products was above the visible level (26). The results were expressed as the number of stained cells per 100 glomeruli (13,22,26).

Immunohistochemical Staining

For immunohistochemical staining, slides were preincubated in 10% normal goat serum and 0.2% bovine serum albumin for 24 h. Slides were then incubated with rabbit antibody directed against N-NOS (1:1000, Euro Diagnostica, Stockholm, Sweden) (27) or renin (1:3000) (28) for 24 h at 4°C, followed by FITC-conjugated goat anti-rabbit IgG (1:80, Cappel, Durham, NC) for 2 h at room temperature. After washing, the slides were coverslipped and examined under a fluorescence microscope.

Renin protein expression was quantified by morphometric analysis. A total of 8 to 12 sections was evaluated from each animal. Photomicrographs of the sections stained with renin antibody were entered into a Macintosh 8500/180 computer with an image scanner (LS-4500AF, Nikon, Tokyo, Japan). Renin-immunoreactive areas were traced, and the corresponding pixels were counted after calibrating for a given area, using a videomicrometer (VM-60, Olympus, Tokyo, Japan). The results are expressed as the sum of the renin-immunoreactive areas per glomerulus (8).

Reverse Transcription-PCR

Total RNA was isolated from the renal cortex by the acid guanidinium thiocyanate-phenol-chloroform extraction method (29). Semi-quantitative reverse transcription (RT)-PCR was performed as described previously (22). In brief, 5 μg of the sample RNA was mixed with 10 U/μl SuperScript II™ reverse transcriptase (Life Technologies, Gaithersburg, MD), 25 ng/μl oligo(dT)$_{22-28}$, 500 mM dNTP, 2.5 mM MgCl$_2$, and 10 mM dithiothreitol. The mixture was incubated at 42°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 was performed by incubating 10 μl of the RT product with 45 μl of PCR SuperMIX™ (Life Technologies) containing 2 mM Tris-HCl (pH 8.4), 55 mM KCl, 1.65 mM MgCl$_2$, 220 μM dNTP, and 0.022 U/μl recombinant DNA polymerase, and 250-nM primers for N-NOS or β-actin, as described previously (22). The initial denaturation step was conducted at 94°C for 3 min. The temperature profile of the PCR was 25 to 40 cycles of 94°C for 1 min, 55°C for 2 min, and 72°C for 3 min, followed by the final extension step at 72°C for 7 min.

The PCR products were size-fractionated by agarose gel electrophoresis, stained with ethidium bromide, and transferred to nylon membranes. The membranes were incubated with the blotting solution containing [³²P]dCTP-labeled probes for N-NOS (30) or β-actin (31), 1 M NaCl, 1% sodium dodecyl sulfate, 10% dextran sulfate, and 100 μg/ml denatured salmon sperm DNA at 65°C for 18 h. After washing,
Figure 2. Double-labeling method demonstrating the colocalization of NADPHd activity (A) and neuronal type nitric oxide synthase (N-NOS) immunoreactivity (B) in the same macula densa cells of Atg" mice. Bars, 50 μm.

Northern Blot Analysis

Twenty micrograms of RNA isolated from the renal cortex was denatured with glyoxal, size-fractionated by agarose gel electrophoresis, and transferred to nylon membranes. The membranes were hybridized with [32P]dCTP random-labeled probes for renin or β-actin at 65°C for 24 h (19,32). The radioactivity of the bands was measured with a FUJIX BIO-Imaging analyzer (Fuji Film).

Statistical Analyses

Data are expressed as mean ± SEM. Statistical significance was determined between Atg" and Atg" mice, or between low-salt, normal-salt, and high-salt groups, by unpaired t test, with a P value of 0.05 deemed statistically significant.

Results

Systolic BP

When the mice were fed a normal-salt diet, Atg" mice exhibited a significantly lower systolic BP (68 ± 3 mmHg, n =
Atg

Distinct regions of the macula densa of N-NOS Expression changed during salt restriction in both types of mice. In mutant mice, BP was significantly increased by a high-salt diet (Figure 3). When the animals were fed a high-salt diet for 2 wk, the signal intensity of the NADPHd reaction and the number of positively stained macula densa cells were markedly decreased (Figure 1).

These observations were quantitatively evaluated (Figure 3). In animals on a normal-salt diet, the number of NADPHd-stained macula densa cells per 100 glomeruli was significantly larger in Atg<sup>−/−</sup> mice than in Atg<sup>+/+</sup> mice. A high-salt diet produced a significant decrease in this parameter in both types of mice; the number of NADPHd-positive cells in mice on a high-salt diet was 50 to 70% less than that in mice on a normal-salt diet. In contrast, salt restriction increased the number of stained cells by approximately 40% in Atg<sup>+/+</sup> mice, whereas this treatment did not affect the parameter in Atg<sup>−/−</sup> mice.

The expression of N-NOS mRNA in renal cortical tissues was analyzed by semiquantitative RT-PCR. As shown in Figure 4, N-NOS mRNA expression was much higher in Atg<sup>−/−</sup> mice compared with Atg<sup>+/+</sup> mice. In mutant mice, a high-salt diet produced a marked decrease in N-NOS mRNA levels; the radioactivity of N-NOS relative to β-actin was decreased by approximately 40% during the treatment. Salt restriction, however, did not affect N-NOS mRNA levels in mutant mice. In Atg<sup>+/+</sup> mice, there was no significant difference in renal N-NOS mRNA levels between the normal-salt and altered-salt groups.

**Renin Expression**

Figure 5 illustrates the immunohistochemical localization and corresponding tracing of renin in the kidneys. In the sections from Atg<sup>+/+</sup> mice, renin-positive structures were localized to the afferent arteriole just proximal to the parent glomerulus. In contrast, renin-immunoreactive areas were markedly increased in the kidneys of Atg<sup>−/−</sup> mice, distributing from the afferent arterioles to the interlobular arteries. When animals were placed on a high-salt diet, the renin-immunoreactive areas were decreased in both types of mice. A relatively sparse distribution of renin immunoreactivity was observed in the renal vasculatures of salt-loaded mutant mice.

Figure 6 summarizes the results of morphometric analysis. In animals fed a normal-salt diet, the mean value of renin-positive areas per glomerulus was 29 times higher in Atg<sup>−/−</sup> mice than in Atg<sup>+/+</sup> mice. Dietary salt loading led to a decrease in the index to approximately 50% of the values in the respective normal-salt groups for wild-type and mutant mice. A low-salt diet produced a significant increase in the renin-positive area in Atg<sup>+/+</sup> mice, but it did not affect the parameter in Atg<sup>−/−</sup> mice.

The expression of renin mRNA in renal cortical tissues was assessed by Northern blot analysis. As shown in Figure 7, Atg<sup>−/−</sup> mice have sevenfold higher levels of renal renin gene expression than Atg<sup>+/+</sup> mice. In mutant mice, a high-salt diet produced a significant decrease in renin mRNA levels. The

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7) than Atg<sup>+/+</sup> mice (93 ± 7 mmHg, n = 7, P < 0.05). In mutant mice, BP was significantly increased by a high-salt diet (96 ± 6 mmHg, n = 7, P < 0.05 versus before salt loading), whereas the treatment did not affect this parameter in wild-type mice (109 ± 5 mmHg, n = 7). BP was not significantly changed during salt restriction in both types of mice.

**N-NOS Expression**

Figure 1 shows the distribution of NADPHd activity in the renal sections. Normally, enzyme activity is localized to the distinct regions of the macula densa of Atg<sup>+/+</sup> mice. In contrast, densely stained cells are distributed beyond the original location of the macula densa in Atg<sup>−/−</sup> mice, often extending to the cross-sectional profiles of the distal tubules. Double-labeling demonstrated the colocalization of NADPHd activity and N-NOS immunoreactivity in the same macula densa cells (Figure 2). When the animals were fed a high-salt diet for 2 wk, the signal intensity of the NADPHd reaction and the number of positively stained macula densa cells were markedly decreased (Figure 1).

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radioactivity of renal renin mRNA relative to β-actin in the high-salt group was approximately 70% less than that in the normal-salt group. A low-salt diet, however, produced no significant change in the renin gene expression in mutant mice. In Atg<sup>+/+</sup> mice, renin mRNA levels showed an inverse relationship to the levels of salt intake.

**Discussion**

The present study demonstrated that chronic dietary salt loading decreases the number of NADPHd-positive macula densa cells in the kidneys of Atg<sup>+/+</sup> and Atg<sup>−/−</sup> mice. The NADPHd reaction is reported to be proportional to N-NOS activity in fixed tissue (25). Therefore, macula densa cells that have N-NOS activity above the detectability of the signal intensity are countable (26). Colocalization of the NADPHd reaction and N-NOS immunoreactivity in the same macula densa cells indicates the specificity of NADPHd staining as a marker for N-NOS protein under our experimental conditions (33). These results suggest that N-NOS activity in the macula densa was suppressed by dietary salt loading in Atg<sup>+/+</sup> and Atg<sup>−/−</sup> mice. In contrast, a low-salt diet significantly increased the number of NADPHd-positive macula densa cells in Atg<sup>+/+</sup> mice. Although Atg<sup>−/−</sup> mice showed an increase in the number of stained cells during salt restriction, the increase did not reach statistical significance. Consistent with the results of NADPHd staining, salt loading decreased the levels of N-NOS mRNA in the renal cortical tissues of Atg<sup>−/−</sup> mice, whereas salt restriction did not affect N-NOS gene expression. Although N-NOS is a constitutive isoform of NOS and the enzyme activity is thought to be regulated acutely by a calcium/calmodulin system, our results indicate that dietary salt loading affects N-NOS expression at the levels of gene transcription and/or stability of the message in mutant mice (34). In Atg<sup>+/+</sup> mice, altered salt intake did not produce a significant change in N-NOS gene expression. This may be due to a technical limitation of a semiquantitative RT-PCR. More sensitive methods such as RNase protection assay or quantitative RT-PCR will be needed to detect changes in the trace amount of N-NOS mRNA in the kidneys of wild-type mice (35,36).

A high-salt diet significantly decreased the renin-immunoreactive areas and the levels of renin mRNA in the kidneys of Atg<sup>−/−</sup> mice, indicating that salt loading affects renin production through mechanisms other than negative feedback of angiotensin II on renin-producing cells. In earlier studies on normal rats, the macula densa mechanism, an elevation of BP, and a decrease in sympathetic nerve tone have been suggested to be involved in the effects of dietary salt loading on renin synthesis and secretion (2,3,37). The present observation of concomitant decreases in N-NOS and renin expression suggest the functional link between the macula densa mechanism mediated by N-NOS and juxtaglomerular renin production in Atg<sup>−/−</sup> mice. In support of this idea, our previous study has demonstrated that a selective inhibition of N-NOS activity with 7-nitroindazole decreases the levels of renal renin gene expression in mutant mice (23).

Macula densa cells monitor the change in sodium chloride...
concentration at the site and transfer the information to renin-producing cells of the afferent arteriole (2–5). Among several locally acting substances, NO has recently been considered an important candidate as the intercellular mediator of the juxtaglomerular apparatus. In the isolated perfused juxtaglomerular apparatus, L-arginine increases the renin secretion rate when the preparation is maintained with low luminal sodium chloride concentrations, whereas this effect is abolished by perfusing with a high-salt medium or by the blockade of NOS with \( \text{NO}^- \)-nitro-L-arginine (9). In anesthetized rats, the inhibition of tubular Na\(^+\)-K\(^+\)-2Cl\(^-\) transport by furosemide induces renin release, and the effect was abolished by a selective blockade of N-NOS by 7-nitroindazole (7). Chronic dietary salt loading produces concomitant decreases in renal N-NOS and renin gene expressions, whereas salt restriction produces opposite effects on these enzymes (8,27,36). The present study confirmed a parallel change in NADPHd activity in the macula densa and renal renin expression during altered salt intake in wild-type mice. It has been demonstrated that a blockade of NOS activity by \( \text{NO}^- \)-nitro-L-arginine methyl ester (L-NAME) attenuates the stimulatory effect of salt restriction on renal renin gene expression (38). These results indicate that the levels of dietary salt intake inversely regulate N-NOS activity in the macula densa through long-term as well as short-term mechanisms and that macula densa-derived NO stimulates renin production at the levels of mRNA and protein.

In Atg\(^{-/-}\) mice, dietary salt loading produced a significant increase in BP, suggesting that an increased renal perfusion pressure participated in the reduction of renin expression. In the 2 kidney-1 clip Goldblatt hypertensive model, increases in N-NOS and renin expressions in the clipped kidney and decreases in the expression of these enzymes in the contralateral kidney were demonstrated with an elevation of systemic BP (27,35). The results may be explained by an NO-mediated macula densa mechanism, such that hypoperfusion in the clipped kidney decreases sodium chloride delivery at the macula densa by an enhanced reabsorption at the thick ascending limb of Henle’s loop, thereby leading to an activation of N-NOS and renin expressions. In contrast, an elevated renal perfusion pressure in the nonclipped kidney increases tubular sodium chloride delivery, which leads to an inhibition of N-NOS activity in the macula densa (6,27). The enhancement of renin expression in the clipped kidney is attenuated by an inhibition of NOS activity with L-NAME, further indicating the involvement of an NO-mediated system in perfusion pressure-dependent renin production (39,40). Salt-induced decreases in renal N-NOS and renin expression in Atg\(^{-/-}\) mice may be explained in part by increased renal perfusion pressure. On the other hand, a recent in vivo study in sodium-restricted rats demonstrated an inhibitory effect of 7-nitroindazole on renin secretion without any measurable changes in BP, suggesting that renin regulation by N-NOS is independent of renal perfusion pressure (41).

It could be argued that the salt-induced reduction of renal renin expression in Atg\(^{-/-}\) mice was due to a direct effect of increased renal perfusion pressure on renin-producing cells. Earlier studies demonstrated the secretion of renin from isolated afferent arterioles in response to a change in perfusion pressure, thereby suggesting that vascular mural pressure directly activates renin-secreting cells via stretch receptors on the

| Renin-positive area (\(\mu\text{m}^2/\text{glomerulus}\)) |
|-----------------|-----------------|-----------------|
| \(\text{LS}\)   | \(\text{NS}\)   | \(\text{HS}\)   |
| 180             | 120             | 60              |

\(\ast\) \(< 0.05\); n.s., not significant.

Figure 6. Morphometric quantification of renin-immunoreactivities in renal sections from Atg\(^{+/+}\) and Atg\(^{-/-}\) mice. Values are expressed as the sum of renin-immunoreactive areas per glomerulus. Data are mean ± SEM from four to six animals in each group. LS, low-salt; NS, normal-salt; HS, high-salt.
Atg’ mice have extremely higher levels of renal N-NOS and renin expression than Atg’ mice on a normal-salt diet. In this condition, the levels of N-NOS or renin expression in the kidneys of Atg’ mice were in good agreement with this idea.

In summary, dietary salt loading significantly attenuates the N-NOS and renin overexpressions in the juxtaglomerular apparatus of Atg’ mice. Salt restriction did not produce further increases in the renal N-NOS or renin expression in mutant mice, whereas a parallel inverse relationship was observed between these enzyme expressions and the levels of salt intake in Atg’ mice. These results suggest that N-NOS activity in the macula densa is inversely regulated by salt intake at the levels of mRNA and protein and that macula densa-derived NO is functionally linked to renal renin production. Salt-modulated renal N-NOS and renin expressions are independent of angiotensin formation in Atg’ mice.

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**References**


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**Figure 7.** Effect of dietary salt loading on renin gene expression in renal cortical tissues of Atg’/+ and Atg’/− mice. (A) Representative Northern blots for renin and β-actin. (B) Summary of renin mRNA levels normalized for β-actin. Values are mean ± SEM from six to eight experiments in each group. LS, low-salt; NS, normal-salt; HS, high-salt. *p < 0.05; n.s., not significant.


