Chapter V: Renin Gene Expression in the AT1a Receptor Deficient Mice

Summary

Angiotensin type-1a (AT1a) receptor gene-knockout (AT1a-/-) mice exhibit chronic hypotension and renin overproduction. In the kidneys of AT1a-/- mice, the activity of neuronal type nitric oxide synthase (N-NOS) was histochemically detected by NADPH diaphorase (NADPHd) reaction combined with N-NOS immunohistochemistry. The localization of renin was detected by immunohistochemistry and the results were analyzed morphometrically. The levels of N-NOS and renin mRNA in the renal cortical tissue were determined by reverse transcription-PCR and Northern blot analysis, respectively. In the renal sections from wild-type mice, NADPHd activity and N-NOS immunoreactivity were localized to the discrete region of the macula densa in contact with the parent glomerulus. In contrast, N-NOS-positive macula densa cells were distributed beyond the original location of the macula densa, occasionally extending to the opposite side of the distal tubules. The mean number of N-NOS positive macula densa cells was significantly increased in AT1a-/- mice (186 per 100 glomeruli) compared with wild-type mice (65 per 100 glomeruli). AT1a-/- mice showed 1.4-times higher N-NOS mRNA levels in the renal cortical tissues than wild-type mice. The plasma renin activity was significantly higher in AT1a-/- mice (205.5 ± 26.1 ng/ml/h) than in wild-type mice (8.0 ± 0.2 ng/ml/h). The renin-positive areas per glomerulus and renal renin gene expression

were 12-times and 2.6-time higher in AT1a-/- mice than in wild-type mice, respectively. These abnormalities, however, were less remarkable in AT1a-/- mice compared with angiotensinogen-knockout mice. When AT1a-/- 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. Dietary salt loading produced a parallel decrease in plasma renin activity, renal renin-immunoreactive areas, and the levels of renin mRNA without affecting systemic blood pressure. These results provide evidence for the possible involvement of N-NOS at the macula densa in the increased renin production in AT1a-/- mice.

Introduction

Renin is the rate-limiting enzyme of the renin-angiotensin system (RAS). The synthesis and secretion of renin are thought to be regulated by physiologic parameters such as tubular sodium chloride delivery, renal perfusion pressure, and plasma level of angiotensin II [61]. The concentrations of sodium chloride at the distal tubules are monitored by the macula densa and the information is transferred to the juxtaglomerular renin-producing cells of the afferent arterioles [62-64]. Although the signal transduction mechanisms within this system are not yet well understood, it has recently been proposed that nitric oxide (NO) derived from the macula densa is an important candidate for the local intercellular mediator in the juxtaglomerular apparatus [65-72]. Histologic studies have demonstrated the localization of neuronal isoform of NO synthase (N-NOS) in the macula densa [73, 74]. When applied to the distal tubules, L-arginine enhances renin secretion from the microperfused glomerulus, and this effect is abolished by perfusing with a high-salt medium or by the blockade of NOS with NG-nitro-L-arginine [75]. In vivo studies have demonstrated a parallel increase in renal N-NOS and renin production by various experimental manipulations such as salt restriction and furosemide treatment [69, 76, 77]. Renin synthesis and secretion induced by these treatment are abolished by a selective inhibition of N-NOS by 7-nitroindazole [67, 71]. These lines of evidence suggest that NO derived from macula densa N-NOS is inversely regulated by sodium chloride delivery at the distal tubules and participates in the stimulatory control of renin, although this notion remains controversial [78, 79].

In our previous study, we generated angiotensin type-1a (AT1a) receptor gene-knockout mice by gene targeting to obtain a model for examining RAS-regulation mechanisms

[19]. Homozygous mutant (AT1a-/-) mice exhibit chronic hypotension and mild morphological abnormalities in the renal tissue [19, 81]. The levels of renal renin mRNA and renin immunoreactive areas are significantly higher in mutant mice than in wild-type mice[19, 81]. This increase in renal renin expression has been explained by a reduced renal perfusion pressure and/or a disruption of AT1a receptor-mediated negative feedback of angiotensin II on renin-producing cells [19]. On the other hand, it remains unknown whether macula densa mechanism participates in the renin overproduction in AT1a-/- mice.

We recently demonstrated that N-NOS and renin are upregulated in the juxtaglomerular apparatus of angiotensinogen gene-knockout (Atg-/-) mice, another type of RAS deficient model [82-86]. In Atg-/- mice, 7-nitroindazole significantly reduces the level of renal renin gene expression [83]. In addition, dietary salt loading produces a parallel decrease in the overexpressed N-NOS and renin [84]. The AT1 receptor antagonist losartan and angiotensin converting enzyme (ACE) inhibitor ramipril consistently produce a parallel increase in renal N-NOS and renin expression [68, 87]. Losartan- or ramipril-induced increases in renal renin gene expression and renin secretion rate are abolished by an inhibition of NOS activitiy with NG-nitro-L-arginine methyl ester (L-NAME) [68, 72]. These results suggest that NO-mediated macula densa mechanism contributes to the stimulus regulation of renin in various RAS-deficient conditions. The present study was designed to provide evidence for the possible involvement of macula densa N-NOS in the renin overproduction in AT1a-/- mice. We investigated whether the levels of renal N-NOS and renin expression are enhanced and if so, whether dietary salt loading produces a parallel decrease in these enzymes in AT1a-/- mice. For comparison, the levels of renal N-NOS and renin expression in Atg-/- mice were determined.

Materials and Methods

Experimental animals and protocols

AT1a-/- and Atg-/- mice were generated by gene targeting as described previously [19, 82]. In brief, TTS2 ES cells, derived from an F1 embryo between C57BL/6 and CBA mice, were grown on embryonic fibroblast feeder cells. After electroporation of the cells with a targeting vector for the AT1a receptor gene or angiotensinogen gene, homologous recombination was confirmed by PCR and Southern blot analyses. The ES clones positive for these analyses were injected into ICR 8-cell embryos to generate chimeric mice. Chimeric males were bred to ICR females. After confirmation of the germline transmission of the mutations either for AT1a receptor or angiotensinogen gene, the heterozygous mice were intercrossed to produce homozygous mutant. F1 offspring homozygous for the AT1a receptor gene disruption was backcrossed six times with C57BL/6 mice. Therefore, genetical background of AT1a-/- mice except for AT1a receptor gene was thought to be identical to that of C57BL/6 mice. Wild-type (C57BL/6), AT1a-/-, and Atg-/- mice, aged 10-12 weeks, were used in the following experiments.

The animals were housed under a 12 h: 12 h day-night cycle at a temperature of 25 °C. Standard chow (0.3% NaCl) and tap water were allowed ad libitum. To study the effects of salt loading, a separate group of AT1a-/- mice was fed a high salt diet containing 4.0% NaCl for 2 weeks [84]. Systolic blood pressure was measured by the tail-cuff method (BP-monitor MK-1100, Muromachi Kikai Co., Tokyo, Japan).

NADPH diaphorase reaction

The NADPH diaphorase (NADPHd) reaction was used as an index for tissue activity of N-NOS [76, 83, 84]. Animals were anesthetized with chloroform and perfused with 50 ml of saline, followed by 100 ml of 0.1 M phosphate buffer (pH 7.4) containing 4% paraformaldehyde and 0.2% picric acid [88]. Kidneys were postfixed with the same fixative for 48 hours. Sections of 8 μm were cut in a cryostat and thaw mounted on chrome gelatin-coated glass slides. The sections were incubated in phosphate buffer containing 0.02% nitro blue tetrazolium, 0.08% ß-NADPH, and 0.3% Triton X-100 for 60 min at 37 °C. In each experiment, tissues from wild-type, AT1a-/-, and Atg-/- mice were simultaneously subjected to the fixation and staining process.

The enzyme activity was evaluated by counting the number of macula densa cells where the signal intensity of the reaction products was above the visible level. The results were expressed as the mean total number of positive cells per 100 glomeruli [76, 83, 84].

Immunohistochemical staining

For immunohistochemical staining, slides were incubated with rabbit antibody directed against N-NOS (1:1000, Euro Diagnostica, Stockholm, Sweden) and then with fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (1:80, Cappel, Durham, NC). After washing, the slides were coverslipped with 30% glycerol containing 0.1% p-phenylenediamine and examined under a fluorescence microscopy.

Renin protein expression was quantified by a morphometric analysis. Sections were incubated with renin antibody (1:3000) [83], followed by goat anti-rabbit IgG (1:200, Cappel) and peroxidase-rabbit anti-peroxidase complex (1:250, Cappel). They were then reacted with diaminobenzidine (0.5 mg/ml) and 0.03% H2O2 in 50 mM Tris-HCl buffer (pH 7.6) for 5

min, dehydrated, cleared, and coverslipped. A total of 8 sections were evaluated from each animal. Photomicrographs of the sections were entered into a Macintosh computer (8500/180) with an image scanner (LS-4500AF, Nikon, Tokyo, Japan). Renin-immunoreactive areas were traced and the corresponding pixels were counted. The results were expressed as the sum of the renin-immunoreactive areas per glomerulus [68].

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from the renal cortex by the acid guanidinium thiocyanate-phenol-chloroform extraction method [89]. Semiquantitative RT-PCR was performed as described previously [90]. In brief, 5 μg of the sample RNA was mixed with 10 U/μl SuperScript IITM reverse transcriptase (Gibco BRL, Gaithersburg, MD), 25 ng/μl oligo(dT)12-18, 500 mM dNTPs, 2.5 mM MgCl2, 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 SuperMIXTM (Gibco BRL) containing 22 mM Tris-HCl (pH 8.4), 55 mM KCl, 1.65 mM MgCl2, 220 μM dNTPs, and 0.022 U/μl recombinant DNA polymerase, and 250 nM primers for N-NOS or ß-actin [83]. The initial denaturation step was conducted at 94°C for 3 min. The temperature profile of the PCR was 25-40 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]CTP-labeled probes for N-NOS [90] or ß-actin [82, 83],

1 M NaCl, 1% sodium dodecyl sulfate, 10% dextran sulfate, and 150 μg/ml denatured salmon sperm DNA at 65 °C for 18 h. After washing, the membranes were exposed to a BAS 2000 imaging plate and the relative radioactivities of the bands were quantified using a FUJIX BIO-Imaging analyzer (Fuji Film, Tokyo, Japan). The relative radioactivity of the DNA bands was increased linearly with the amount of sample RNA (1.25 to 10 μg) or PCR cycles (25 to 40) [83].

Northern blot analysis

Twenty μg of RNA isolated from the renal cortex were denatured with glyoxal, size fractionated by 1% agarose gel electrophoresis, and transferred to nylon membranes. The membranes were hybridized with [32P]CTP-random labeled probes for renin or 18S rRNA at 65 °C for 18 h [80, 82]. The radioactivity of the bands were measured with a FUJIX BIO-Imaging analyzer (Fuji Film).

Plasma renin activity

Blood samples from wilde-type and AT1a-/- mice were collected into a polypropylene tube containing EDTA and then centrifuged at 4 °C to isolate plasma fraction. Plasma renin activity was measured using a radioimmunoassay for generated angiotensin I as described previously [80]. Atg-/- mice were not used for the determination of plasma renin activity, because this parameter could be affected by the different genetic background (Ren-1 and Ren-2 genes), whereas renal renin expression is not [91-93].

Statistical analysis

Data are expressed as means ± SE. Statistical significance was determined by unpaired Student's t-test with P values <0.05 being deemed statistically significant.

Results

Measurement of systolic blood pressure

When mice were fed a normal salt diet, systolic blood pressure of AT1a-/- mice was significantly lower (90 ± 2 mmHg, n=10) than that of wild-type mice (110 ± 6 mmHg, n=10, $P<0.05$ vs AT1a-/- mice) and was higher than that of Atg-/- mice (72 ± 4 mmHg, n=7, $P<0.05$ vs AT1a-/- mice). Blood pressure of AT1a-/- mice showed a tendency to increase during a high-salt diet whereas the increase did not reach statistical significance (101 ± 5 mmHg, n=10).

Histologic examinations for N-NOS and renin

Fig. 10 illustrates the distribution of N-NOS and renin in the kidneys of wild-type and AT1a-/- mice. NADPHd-positive cells were sparsely distributed in the sections from wild-type mice. The enzyme activity was localized to the macula densa in contact with the parent glomerulus (Fig. 10 A). N-NOS immunoreactivity was located in the same macula densa cells which were positive for NADPHd activity (Fig. 10 B). In contrast, NADPHd-positive cells in AT1a-/- mice were distributed beyond the original location of the macula densa, occasionally extending to the opposite side of the distal tubules (Fig. 10 C). A double labeling method demonstrated the colocalization of the enzyme activity and N-NOS immunoreactivity in the same macula densa cells of AT1a-/- mice (Fig. 10 D). Atg-/- mice also have a marked increase in N-NOS activity in the macula densa. The most characteristic observation in Atg-/- mice was that N-NOS-positive cells often occupy the entire cross sectional profiles of the distal tubules (data not shown). This distribution pattern of N-NOS, however, was not observed in the kidneys of AT1a-/- mice. These findings were quantitatively evaluated by counting the number of NADPHd-positive macula densa cells. As shown in Fig. 11, the mean total number of

stained cells per 100 glomeruli was 3-times higher in AT1a-/- mice than in wild-type mice. On the other hand, the number of NADPHd-positive macula densa cells was significantly lower in AT1a-/- mice compared with Atg-/- mice.

In wild-type mice, renin-immunoreactive cells were located in the discrete region of the afferent arterioles (Fig. 10 E). In contrast, renin-positive cells were distributed along the afferent arterioles of AT1a-/- mice (Fig. 10 F and 12). Most of the interlobular arteries, were negatively stained, and therefore the increase in renin-immunoreactivity was less striking in AT1a-/- mice compared with Atg-/- mice, in which renin-positive cells were distributed from the afferent arterioles to the interlobular arteries (data not shown). These observations were assessed by a morphometric analysis (Fig. 12). The mean renin-immunoreactive area per glomerulus was 12-times higher in AT1a-/- mice than in wild-type mice, whereas the index was 50%-lower in AT1a-/- mice than in Atg-/- mice (Fig.13).

The effects of dietary salt loading on the levels of renal N-NOS activity and renin immunoreactivity were determined in AT1a-/- mice. As shown in Fig. 11, the number of NADPHd-stained macula densa cells was significantly decreased by 55% during the treatment. Fig. 12 shows renin staining and corresponding tracing in the sections from normal-salt and high-salt groups. Renin immunoreactivity is diminished in the renal vasculature of salt loaded AT1a-/- mice (Fig. 12 B). As shown in Fig. 13, the mean renin-immunoreactive areas per glomerulus was significantly decreased by 50% during the treatment.

N-NOS and renin gene expression in the renal cortex

The renal cortical expression of N-NOS mRNA was analyzed by RT-PCR. Fig. 14 shows representative ethidium bromide-stained agarose gels and the corresponding Southern

blots of the RT-PCR products for N-NOS and ß-actin. The levels of N-NOS gene expression were higher in AT1a-/- mice compared with wild-type mice, whereas those of ß-actin were not different between these mice. As a result, the mean level of N-NOS mRNA relative to ß-actin mRNA at the PCR cycle of 30 was 1.4-times higher in AT1a-/- mice than in wild-type mice (Fig. 14 C). On the other hand, the levels of N-NOS mRNA were lower in AT1a-/- mice compared with Atg-/- mice.

Renal renin gene expression was determined by Northern blot analysis. Fig. 15 shows a representative blot and the radioactivity of renin mRNA relative to 18S rRNA. The mean level of renal renin mRNA in AT1a-/- mice was 2.6-times higher than that in wild-type mice. On the other hand, the levels of renin gene expression were lower in AT1a-/- mice than in Atg-/- mice.

Chronic dietary salt loading produced a concomitant decrease in the levels of renal N-NOS and renin mRNA in AT1a-/- mice (Fig. 14 and 15). N-NOS mRNA expression relative to ß-actin in AT1a-/- mice was decreased to the levels comparative to those found in wild-type mice (Fig. 14 B). A high-salt diet also produced a significant decrease in renal renin gene expression by approximately 40% (Fig. 15 B).

Plasma renin activity

The plasma renin activity was extremely higher in AT1a-/- mice (205.5 ± 26.1 ng/ml/h) than in wild-type mice (8.0 ± 0.2 ng/ml/h) (Fig. 16). In AT1a-/- mice, a high-salt diet produced a significant decrease in this parameter.

Discussion

In the present study, we demonstrated that the number of NADPHd-positive macula densa cells and signal intensity of the reaction products were higher in AT1a-/- mice than in wild-type mice. The colocalization of NADPHd activity and N-NOS immunoreactivity in the same macula densa cells indicates the specificity of NADPHd staining as a marker for N-NOS protein [74]. NADPHd reaction is reported to be proportional to N-NOS activity in the fixed tissue [94]. Therefore, macula densa cells that have N-NOS activity above the detectability of the signal intensity are countable [76]. These results suggest that N-NOS activity is upregulated in the macula densa of AT1a-/- mice. Consistent with the results of the histologic study, the levels of N-NOS mRNA in the renal cortical tissues were significantly higher in AT1a-/- mice than in wild-type mice, thereby indicating that N-NOS activity is modulated at the levels of gene transcription and/or stability of the message.

The macula densa monitors tubular concentrations of sodium chloride and transfer the information to renin-producing cells in the afferent arteriole [62-64]. Recent physiologic studies have proposed that NO is an important candidate as the intercellular mediator derived from macula densa cells [65-72]. In the isolated perfused juxtaglomerular apparatus, L-arginine applied to the distal tubules 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 NG-nitro-L-arginine [75]. In anesthetized rats, the inhibition of tubular Na+-K+-2Cl- cotransporter by furosemide induces renin release and the effect is abolished by a selective blockade of N-NOS with 7-nitroindazole [7]. Chronic dietary salt restriction produces a parallel increase in renal N-NOS and renin

expression, whereas salt loading produces opposite effects on these enzymes [76, 77]. An inhibition of NOS activity by L-NAME or 7-nitroindazole attenuates the stimulatory effect of salt restriction on renin synthesis and secretion [69, 71]. These lines of evidence indicate that the activity of N-NOS in the macula densa is inversely regulated by the sodium chloride delivery at the macula densa and that NO-derived from macula densa cells stimulates renin synthesis and secretion.

The present study confirmed the increased levels of plasma renin activity, renal renin gene expression and renin immunoreactive areas in AT1a-/- mice. The previous studies have proposed that this increase in renin production is explained by reduced blood pressure and/or a disruption of negative feedback of angiotensin II on juxtaglomerular cells [19, 81]. On the other hand, it remains to be investigated whether macula densa mechanism participates in the renin overproduction in AT1a-/- mice. In this connection, it is noteworthy that the number of NADPHd-positive macula densa cells and the levels of renal N-NOS gene expression are increased in AT1a-/- mice. Furthermore, dietary salt loading produced a parallel decrease in the renal N-NOS expression, and the levels of plasma renin activity, renal renin gene expression, and renin immunoreactive areas. Systemic blood pressure did not show significant change during the treatment, and therefore altered renal perfusion pressure may not directly affect the renin expression. The present results are in good agreement with the idea that the N-NOS activity at the macula densa is functionally linked, at least in part, to the renin production in AT1a-/- mice.

In consistent with the present results for AT1a-/- mice, it has been demonstrated that a pharmacological blockade of AT1 receptor with losartan produces a parallel increase in the juxtaglomerular immunoreactivity of N-NOS and renin [68, 87]. The increased renin expression is completely abolished by L-NAME, suggesting a contribution of NO-mediated

system to the renin synthesis induced by losartan [68]. The present study confirmed that the expressions of renal N-NOS and renin are extremely increased in Atg-/- mice. A selective inhibition of N-NOS activity with 7-nitroindazole significantly reduces the enhancement of renal renin gene expression in Atg-/- mice [83]. In addition, the ACE inhibitor ramipril-induced renin synthesis and secretion in normal rats are abolished by L-NAME [68, 72]. The macula densa mechanism mediated by NO may be involved in the increased renal renin production in various RAS-deficient conditions.

Systemic blood pressure was significantly lower in AT1a-/- mice than in wild-type mice, thereby suggesting that a reduced renal perfusion pressure contributes to the increased renal renin expression. In the 2-kidney/1-clip Goldblatt hypertensive rats, the levels of N-NOS and renin expression are increased in the clipped kidney [95, 76]. 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 expression [66, 76, 95]. In support of this notion, the enhancement of renin expression in the clipped kidney is abolished by an inhibition of NOS activity with L-NAME [95]. The increased renal N-NOS and renin expression in AT1a-/- mice can be explained partially by a reduced renal perfusion pressure.

It could be argued that reduced renal perfusion pressure directly increases renin synthesis and secretion in AT1a-/- mice through the pressure-dependent mechanism. Indeed, the levels of renal renin expression and systemic blood pressure were inversely related in wild-type, AT1a-/-, and Atg-/- mice. Earlier studies have demonstrated a perfusion pressure-dependent release of renin from isolated afferent arterioles, thereby suggesting that vascular

mural pressure directly activates renin secretion via stretch receptors on the afferent arterioles [96-98]. However, hydrostatic pressure did not affect renin secretion from the afferent arteriole under a stop-flow condition. The results provide evidence against the presence of pressure mechanism for the control of renin production [99]. In vivo studies have demonstrated that losartan- or ramipril-induced renin gene expression is independent on blood pressure changes [68]. In the present study, decreases in renal N-NOS and renin expressions in AT1a-/- mice during a high-salt diet could not be explained by an increase in renal perfusion pressure, because systemic blood pressure was not significantly changed during the treatment.

The increased renin expression in AT1a-/- mice could be explained by a disruption of negative feedback loop between angiotensin II and renin-producing cells via AT1a receptors. In isolated perfused juxtaglomerular apparatus and cultured granular cells, angiotensin II suppresses renin secretion and this effect is inhibited by losartan [100, 80]. In vivo study demonstrated that a subpressor dose of angiotensin II attenuates renin gene expression induced by the ACE-inhibitor enalapril [56]. However, controversial results were recently obtained using chimeric mice carrying regional null mutation of the AT1a receptor [61]. In the kidneys of the regional AT1a receptor knockout mice, the expression of renin mRNA and renin immunoreactive areas are identical between normal and AT1a receptor-deficient juxtaglomerular cells, thereby providing evidence against a disruption of local feedback of angiotensin II on juxtaglomerular cells as the primary cause for the renin overexpression in AT1a-/- mice [61]. The pathophysiologic condition secondary to AT1a receptor-deficiency may be important for the increased renin production in these mice.

In summary, AT1a-/- mice have significantly larger number of NADPHd-positive macula densa cells and renin-immunoreactive areas in the afferent arteriole than wild-type mice.

The levels of N-NOS and renin gene expressions were also increased in the kidneys of AT1a-/- mice. Dietary salt loading produced a parallel decreases in these enzymes without affecting systemic blood pressure. These results suggest that N-NOS activity in the macula densa is upregulated at the levels of mRNA and protein. It could be speculated that macula densa-derived NO participates in the increased renin expression in AT1a-/- mice.

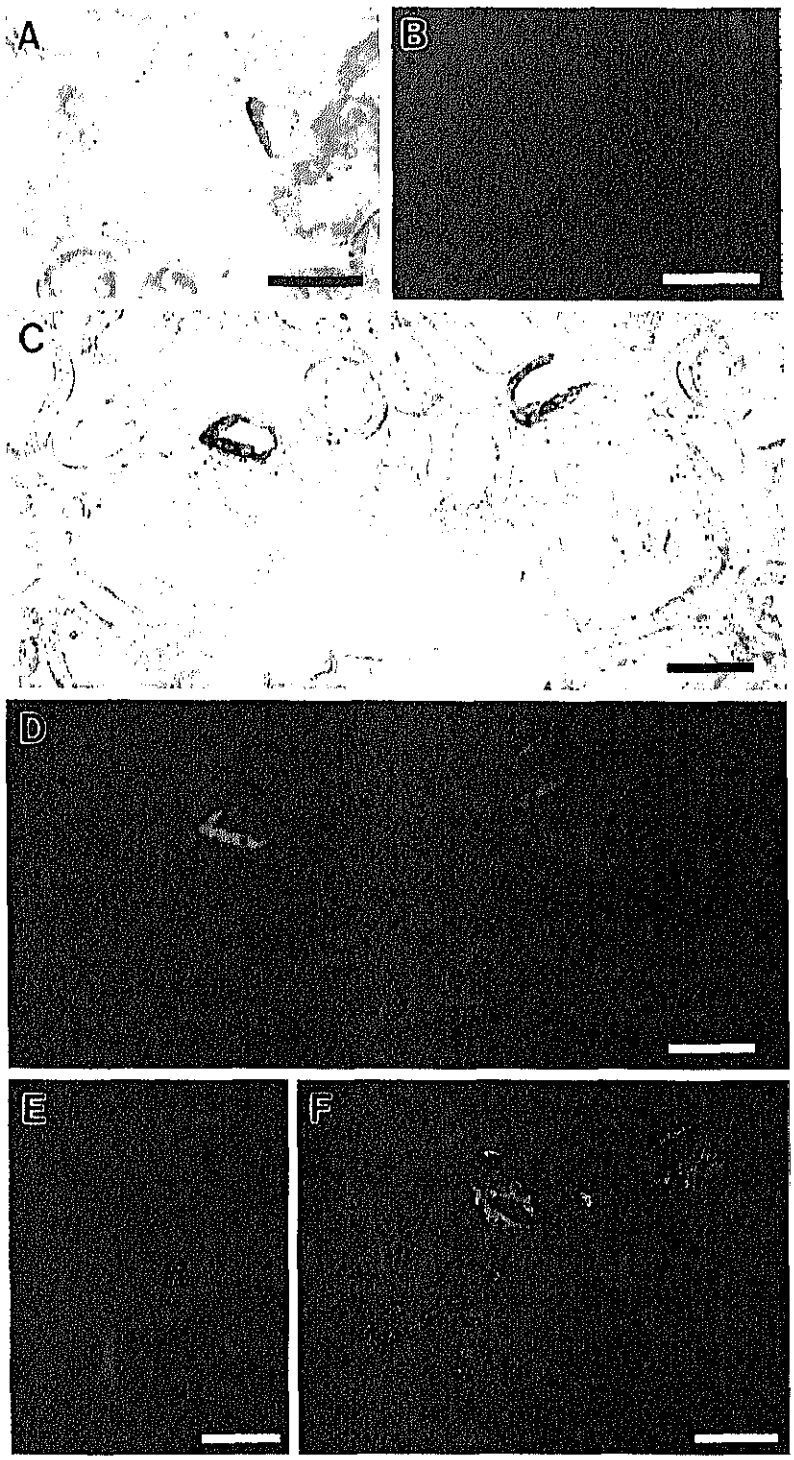

Fig. 10. **Histochemical and immunohistochemical localization of neuronal type nitric oxide synthase (N-NOS) and renin.** (A-D) NADPH diaphorase (NADPHd) activity (*A and C*) and N-NOS immunoreactivity (*B and D*) in the kidney sections from wild-type (A and B) and *AT1a*$^{-/-}$ mice (C and D). (*E and F*) Immunohistochemical localization of renin in the kidneys of wild-type (E) and *AT1a*$^{-/-}$ mice (F). Bars, 50 μm.

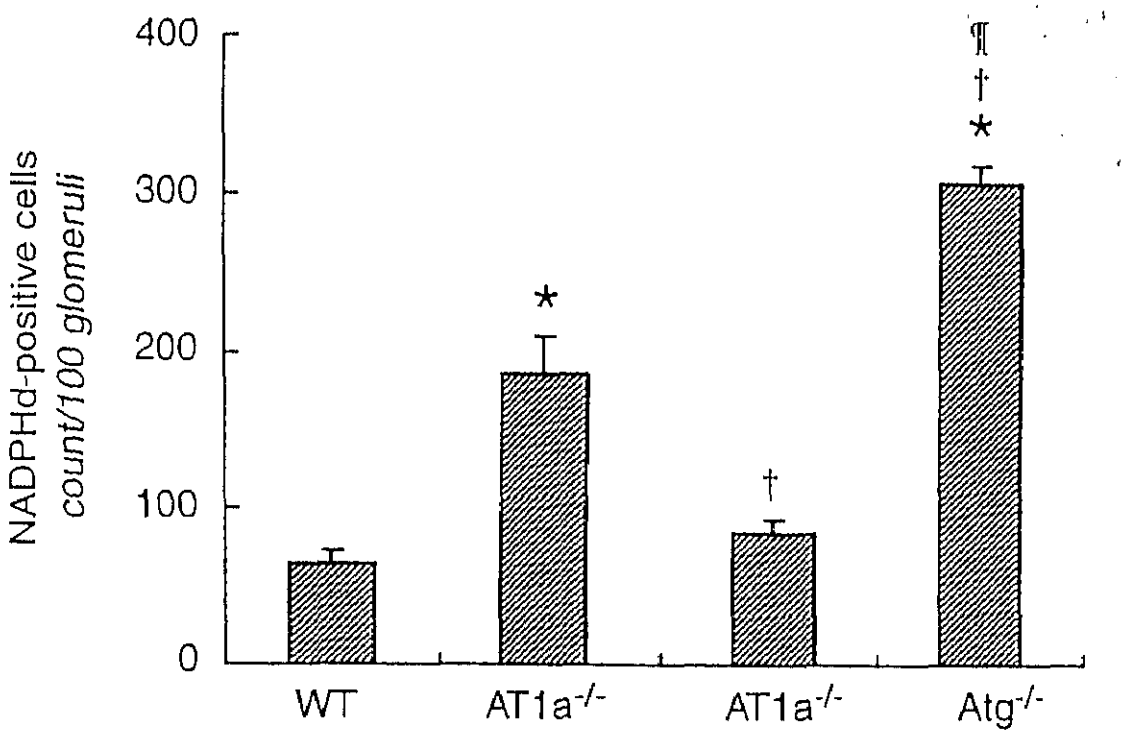

Fig. 11. **Quantification of NADPH diaphorase activity in the macula densa.** Values are expressed as the mean total number of NADPH diaphorase-positive macula densa cells per 100 glomeruli in the kidney sections from wild-type, $AT1a^{-/-}$, and $Atg^{-/-}$ mice. Data are means ± SE from each of four animals. *$P < 0.05$, versus wild-type (WT) mice; $P < 0.05$, versus $AT1a^{-/-}$ mice fed a normal-salt diet; $P < 0.05$, versus $AT1a^{-/-}$ mice fed on a high-salt diet (horizontal bar).

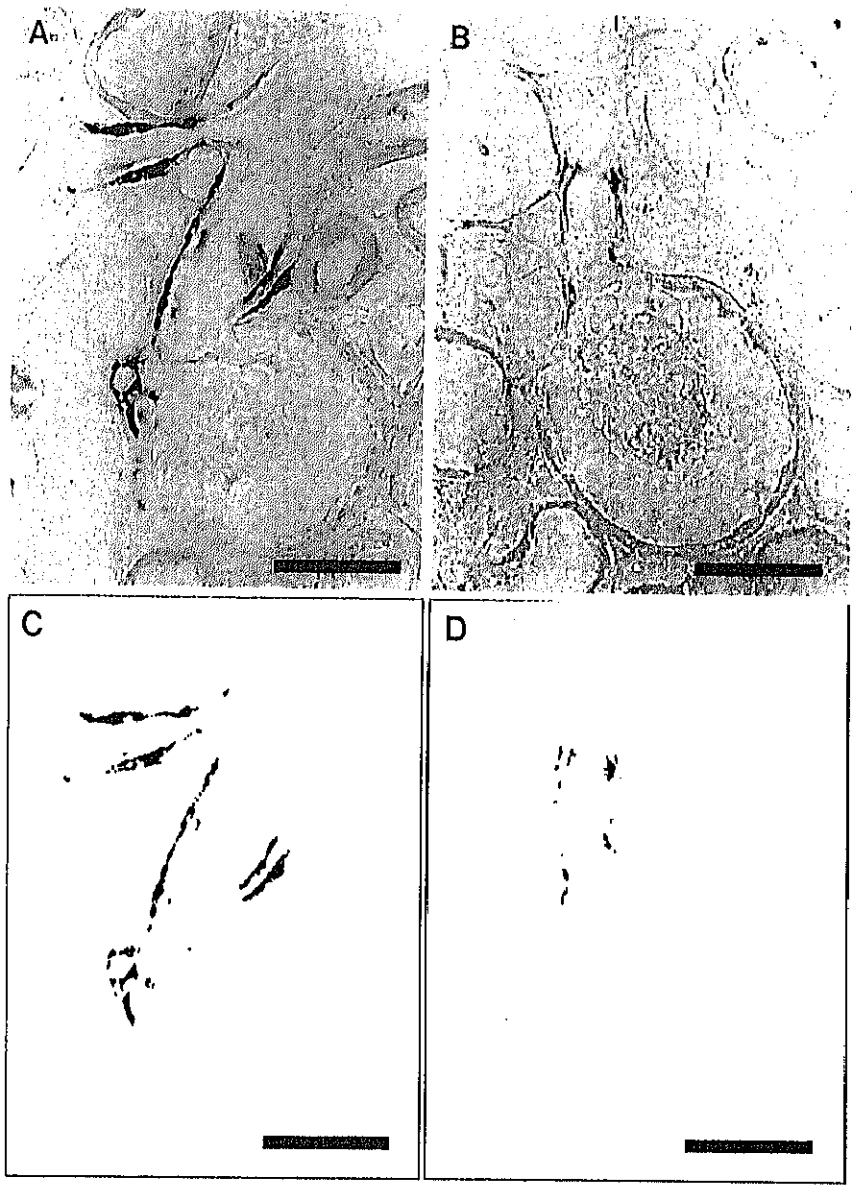

Fig. 12. **Immunohistochemical localization and corresponding tracing of renin.** (*A and C*) Kidney sections from $AT1a^{-/-}$ mice fed a normal-salt diet for two weeks. (*B and D*) Kidney sections from $AT1a^{-/-}$ mice fed a high-salt diet for two weeks. The sections were processed for immunohistochemical staining for renin with PAP method (A and B) and corresponding renin-stained areas were traced (C and D). Bars, 50 μm.

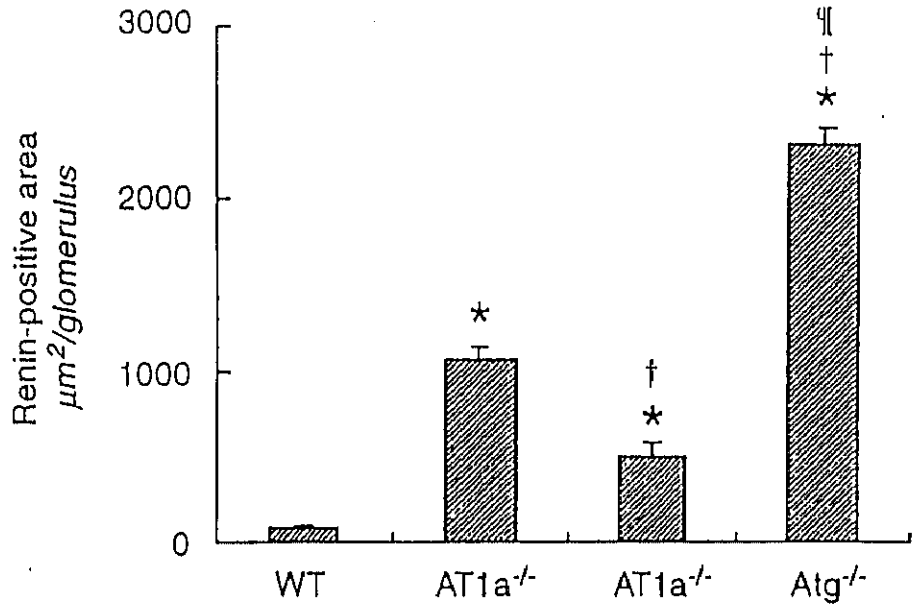

Fig. 13. **Morphometrical quantification of renin-immunoreactive areas.** Values are expressed as the sum of renin-positive areas per glomerulus. Data are means ± SE from each of four animals. $^{*}P < 0.05$, versus wild-type (WT) mice; $^{\dagger}P < 0.05$, versus $AT1a^{-/-}$ mice fed a normal-salt diet; $^{\P}P < 0.05$, versus $AT1a^{-/-}$ mice fed a high-salt diet (horizontal bar).

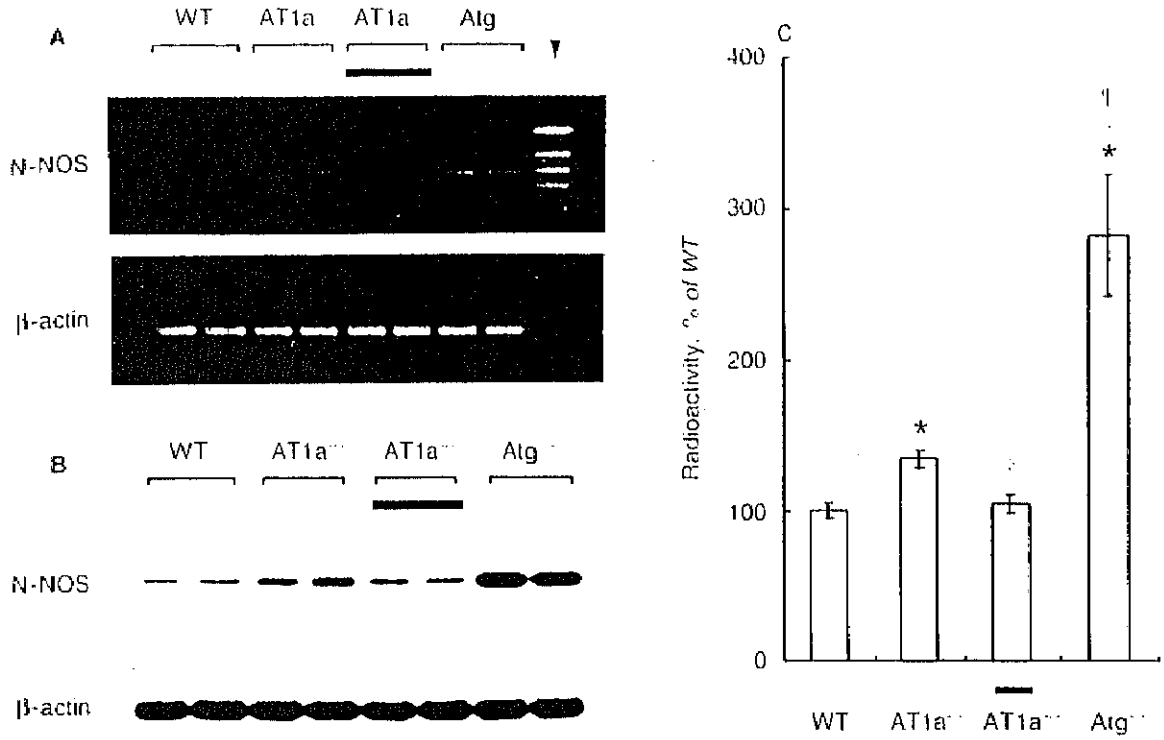

Fig. 14. Reverse transcribed-polymerase chain reaction (RT-PCR) analysis of neuronal type nitric oxide synthase (N-NOS) and ß-actin mRNA expression in renal cortical tissues. (*A*) Representative agarose gel stained with ethidium bromide. Arrowhead indicates a lane for size markers (1057,770,612,495,392 and 345 bp). (*B*) Corresponding Southern blots of RT-PCR products for N-NOS and ß-actin. (*C*) Radioactivity of N-NOS relative to ß-actin at the PCR cycle of 30. Data are means ± SE from four experiments. * $p< 0.05$ versus wild-type (WT) mice; † $p< 0.05$ versus AT1a -/- mice fed a normal-salt diet; ¶ $p< 0.05$ versus AT1a -/- mice fed a high-salt diet (horizontal bars).

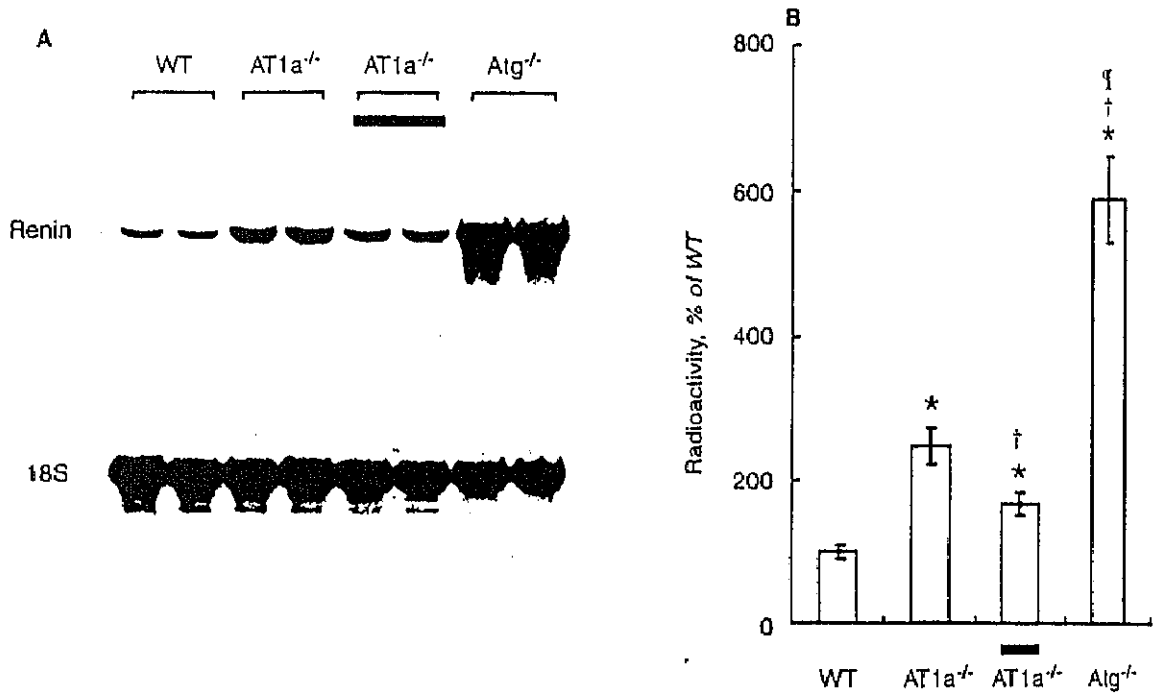

Fig. 15. Northern blot analysis of renal renin gene expression. (A) Representative blots for renin mRNA and 18S rRNA. (B) Renin mRNA expression relative to 18S rRNA. Data are means ± SE from four experiments. * $p< 0.05$ versus wild-type (WT) mice: † $p< 0.05$ versus AT1a -/- mice fed a normal-salt diet: ¶ $p< 0.05$ versus AT1a -/- mice fed a high-salt diet (horizontal bars).

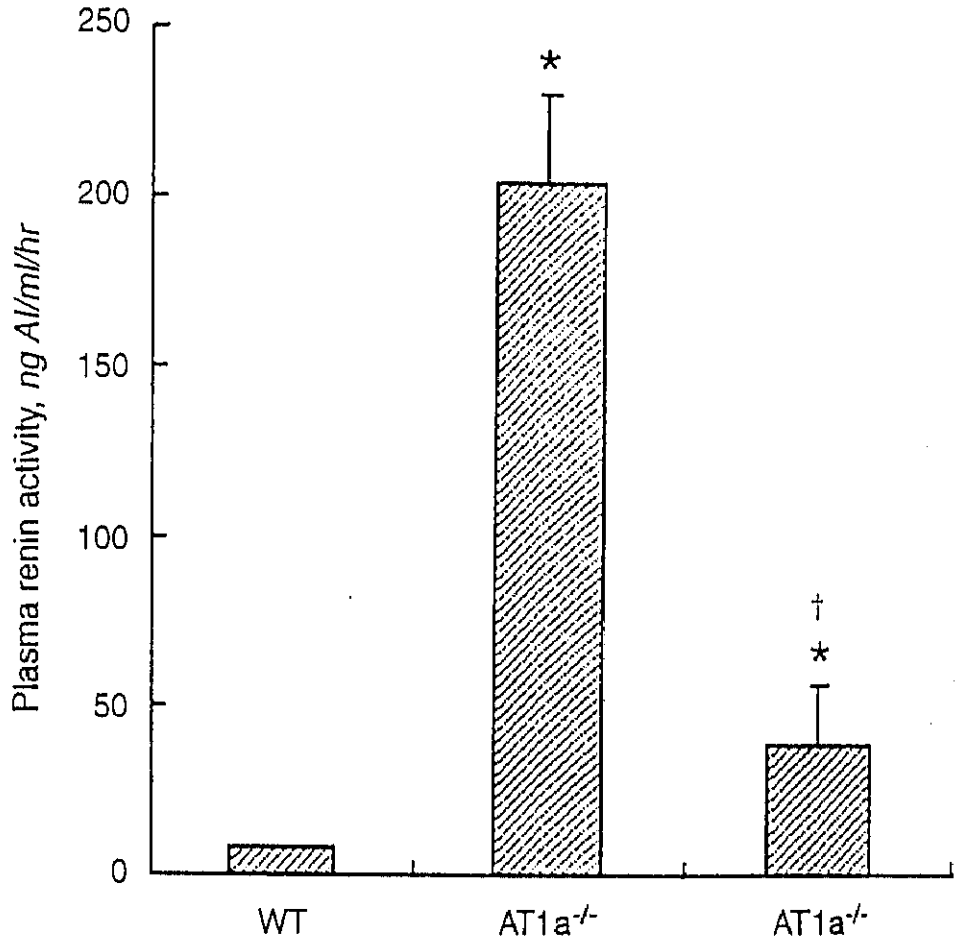

Fig. 16. **Plasma renin activity in wild-type and *AT1a*$^{-/-}$ *mice.*** Data are means ± SE from five to seven experiments. $^{*}P < 0.05$ versus wild-type (WT) mice; $^{\dagger}P < 0.05$ versus *AT1a*$^{-/-}$ mice fed a normal-salt diet. Horizontal bar, a high-salt diet.