Chapter II: Generation of the AT1a Receptor Deficient Mice

Summary

The renin angiotensin system plays a key role in cardiovascular homeostasis and metabolism of fluid and electrolytes. Pharmacological study has revealed that there are two different types of angitensin II (AII) receptors, AT1 and AT2, in mammalian, and that the major physiological responses of AII are mediated through AT1 receptors. In rodents, the two isoforms of AT1 receptors, AT1a and AT1b, are highly homologous and pharmacologically indistinguishable to each other. In order to elucidate the physiological role of AT1a receptor, we generated mutant mice in which the AT1a receptor gene was disrupted by replacing with the LacZ gene. In the heterozygous mutant mice, the strong LacZ staining was identified in the glomerulus and the juxtaglomerular apparatus of the renal cortex. The localization of the LacZ staining coincided with that of the signals by in situ hybridization using the cRNA probe for the AT1a specific region. Both the expression levels of renin mRNAs in the kidney and the plasma renin activities were markedly increased in homozygous mutant mice. The heterozygous and homozygous mutant mice exhibited chronic hypotension, with 10 and 22 mmHg lower systolic blood pressure, respectively, than that of wild-type littermates. These results demonstrated that an AT1a-mediated signal ransduction pathway is, at least in part, involved in the regulation of renin gene exprssion and blood pressure.

Introduction

The renin angiotensin system (RAS) plays a key role in cardiovascular homeostasis, and metabolism of fluid and electrolytes [1]. A potent vasoconstrictor octapeptide, angiotensin II (AII), also exerts numerous physiological responses including secretion of aldosterone from adrenal cortex, inhibition of renin release from the juxtaglomerular apparatus (JGA) of the kidney and development of cardiac hypertrophy and myocardial fibrosis [1]. Pharmacological study has revealed that there are two different types, AT1 and AT2, of AII receptors in mammalian, and that above responses are mainly mediated through AT1 receptors [2]. In rodents, AT1 receptors are situated as two isoforms in two different locus. Both types of recepters, designed AT1a and AT1b, are 94% identical at amino acid level and pharmacologically indistinguishable to each other [3].

Previous studies have reported that the overproduction of angiotensin II in transgenic mice carrying both the human renin and human angiotensinogen genes (Tsukuba Hypertensive Mice) leads to a sastained increase in blood pressure [4]. Furthermore, we have recently reported that a null mutation of the angiotensinogen gene displays chronic hypotension [5]. These observations indicate that the upstream components of RAS play an important role in the maintenance of blood pressure under physiological condition, which can't be compensated by other homeoststic systems such as autonomic nervous system.

In the present study, we generated mutant mice in which the AT1a receptor gene was disrupted by replacing with ß-galactosidase (LacZ) gene to clarify the cellar localization of the receptor, that is, one of downstream components of RAS. We demonstrated that the AT1a receptor localized in gromerulus and JGA of renal cortex and the homozygous mutant mice

display hyperreninemia and hypotension together.

Materials and Methods

Animals.

AT1a-KO mice were generated as previously described [19]. C57BL/6J control and CD-1 mice were obtained from CLEA Japan Co. Ltd., Tokyo and Charles River Japan Inc., Yokohama, respectively. Animals were used according to the regulation of "Standards for Human Care and Use of Laboratory Animals, University of Tsukuba".

Gene Targeting and Generation of Mutant Mice

A genomic DNA phage library from C57BL/6 mouse was screened with a 416-b of the human AT1 cDNA which corresponds to the nucleotide sequence encoding from the first methionine to the 3rd transmembrane region as a probe. After screening of $5x10^5$ phages, we isolated two clones encoding a complete open reading frame of the AT1a gene confirmed by sequencing [6]. To construct a targetting vector for the AT1a gene, an NcoI site was created around the nucleotide sequence including the translation initiation codon of the gene by site-directed mutagenesis method using polymerase chain reaction (PCR), then the NcoI and PmaCI fragment of the gene was replaced with the LacZ cassette [7]. The neomycin phosphotransferase (Neo) gene possesing the promoter and polyadenylation signal from pMC1neo (Stratagene) was placed downstream of the LacZ gene in the opposite orientation. The 810-b XhoI/NcoI fragment and the 4.0-Kb PmaCI/BamHI fragment of the AT1a gene were included upstream and downstream of the cassette, respectively. At the 3' terninus of the homologous region, the herpes simplex virus thymidine kinase (HSV-TK) gene was inserted to negatively select by Ganciclovir (GANC; Syntex) for random integrations (Figure 1A). The

TT2 ES cells, derived from an F1 embryo between C57BL/6 and CBA mice, were grown on embryonic fibroblast feeder cells as described elsewhere [8]. Following electroporation of cells with 20 μg/ml of linearized targeting vector (Bio-Rad gene pulser at settings of 400V and 125 μF), cells were selected in 225 ug/ml of G418 and 0.5 mg/ml GANC. Homologous recombination in ES cells were checked by PCR using the recombination-specific primer set. ES clones positive for PCR analysis were further analyzed by Southern blot analysis: after digestion of genomic DNA with BamHI, size separation in a 0.8 % agarose gel, and transfer to nylon membrane, generation of an 8.0-kb wild-type and a 6.8-kb mutant fragment was identified by probes A and B. Absence of additional random integrations of the targeting construct was confirmed by hybridization with a neo probe (probe C). Chimeric mice were generated by injecting the ES cells into ICR 8-cell embryos [8]. Chimeric males with greater than 50% agouti coat color were bred to ICR females, and germline transmission of the mutant allele was identified by Southern blot analysis of tail DNA from F1 offspring with agouti coat color. The heterozygous mice were interbred to obtain mice homozygous for the AT1a gene disruption.

Histological analysis

For in situ hybridization, samples were immediately frozen at -60°C and cut into 15 μm cryostat sections. The 490-b SacI/PmaCI fragment and the 520-b SacI/SpeI fragment which correspond to 3' untranslated regions of AT1a and AT1b, respectively were used as their specific probes to prevent the cross-hybridization. The AT1a and AT1b specific regions were subcloned into pBluescript II (Stratagene), respectively. Antisense and sense probes were made by in vitro transcription in the presence of digoxigenin-labeled dUTP. In situ

hybridization using the digoxigenin-labeled probes was performed as discribed previously [9]. For LacZ staining, samples were fixed in a solution containing 2% paraformaldehyde, 0.2% glutaraldehyde and 0.02% NP-40 in PBS for 60 min at room temperature and treated in 30% sucrose for 12 hr at 4°C. 10μm frozen sections were stained at 37°C for 1-3 hr in a solution containing 0.4 mg/ml of Bluo-Gal (GIBCO BRL), 3mM K3Fe(CN)6, 3mM K4Fe(CN)6 and 1mM MgCl2 in PBS.

RNA Isolation amd Northern Blot Analysis

Total RNA was isolated from the kidneys of 6 independent age-matched mice using ISOGEN (NipponGene) based on the acid guanidium thiocyanate-phenol-chloroform extraction method [10]. Ten micrograms of RNA was denatured with formamide, separated by electrophoresis, and transferred to a nylon membrane. 490-b SacI/PmaCI fragment which correspond to 3' untranslated regions of AT1a was used as the AT1a receptor specific probe. Probes for mouse renin and GAPDH were previously described[11].

Measurement of Plasma Renin Activities

Blood samples were withdrawn from wild-type (+/+), heterozygous (+/-), and homozygous mutant (-/-) mice (88-94 days). Blood was collected into ice-cold microcentrifuge tubes containing EDTA which were then immediately centrifuged in order to isolate the plasma fraction. The plasma renin activities was measured by radioimmunoassay (RIA). The concentration of inactive renin was determined by subtraction of angiotensin I cleavaged by the plasma renin with or without the treatment of proteinase-activations as described previously [12].

Measurement of Blood Pressure

Wild-type (+/+), heterozygous (+/-), and homozygous mutant (-/-) mice at 56 to 62 days were used for blood pressure measurement. The systolic and diastolic blood pressure were measured by a programmable sphygmomanometer (BP-200; Softron, Japan) using the tail-cuff method as described previously [13]. Statistical analysis for comparison of blood pressure was performed by using Student's test. A value of $p<0.05$ was considered significant. Results are expressed as mean ± S.E.M.

Results

Generation of The AT1a receptor Deficient Mice

Homologous recombination at the target loci in TT2 ES cell, were confirmed by PCR and Southern blot hybridization analysis (Fig 1B). Three independent cell lines out of 330 G418 and GANC-resistant cells had undergone homologous recombination in the AT1a locus. These clones were injected into ICR 8-cell embryos to generate chimeric mice and two clones gave rise to germline transmission.

To generate a null mutation, we designed a targeting vector that would replace the complete AT1a coding region with the promoterless LacZ gene. In these clones, to confirm that the LacZ gene would be inserted in-frame strarting from the translational initiation codon of the AT1a gene and expressed under the control of regulatory element of that gene, we compared the LacZ staining and the signals of specified expression of AT1a mRNA . In the heterozygous mice, the strong lacZ staining was identified in the glomerulus and the JGA of the renal cortex (Fig.2A and E). The localization of the LacZ staining in the heterozygous mice coincided with that of the signals obtained by in situ hybridization using the antisense cRNA probe for the AT1a specific region in the wild-type (Fig.2B) and the heterozygous mice (data not shown). Any signals using the antisense probe for the AT1b specific region (Fig.2C) or the sense probe for the AT1a specific region (Fig.2D) were not detected in the heterozygous mice.

After confirmation of the transmission of the mutations, the heterozygous mice were intercrossed to produce homozygous offspring. The mutated AT1a receptor gene was detected by genomic Southern analysis of tail DNA (Fig. 1C). Of the 396 offspring analyzed, 82 (21%) were homozygous for the disrupted allele, and 121 (31%) were wild type, indicating normal

embryonic development of homozygous mutant mice. In the following study, to make equivalent the effects of other gene background except for the AT1a gene, we have taken these intercrossed littermates of heterozygous mice into the physiological experiments.

Measurement of Blood Pressure

Significant decrease in bothThe systolic and diastolic blood pressure were observed in heterozygous and homozygous mutant mice (Fig.3). These mice exhibited chronic hypotension, with 10 and 22 mmHg lower systolic blood pressure, respectively, than that of wild-type littermates. The systolic blood pressure in AT1a receptor homozygous mutant mice decreased to 77% of that in wild-type mice. We have reported that the systolic blood pressure in angiotensinogen deficient mice decreased to 66% of that in wild-type mice. Thus, concerning blood pressur reguration, it can be said that the approximate 2/3 of the ligand disruption effect in RAS is linked to the AT1a receptor and that the remaining effect will be caused by other AII receptor.

RNA Analysis

We analysed the expression levels of AT1a receptor and renin mRNAs in the kidney from wild-type, heterozygous and homozygous mice by Northern blotting (Fig. 4A). The hybridized signals appeared in heterozygous mice at approximately half the levels present in wild-type , whereas homozygous mice had no detectable message. A duplicate blot was analyzed with a GAPDH probe to confirm that the RNA sample was intact. These results indicate that the AT1a receptor transcript was completely absent from homozygous mutant mice.

Plasma Renin Activities

To determine whether the expression levels of renin mRNAs in the kidney from wild-type, heterozygous and homozygous come up to the plasma renin activities, blood samples were collected from wild type (+/+), heterozygous mutant (+/-), and homozygous mutant (-/-) mice and analyzed by RIA (Fig.4B). Plasma renin activities were almost detected as an active form. The plasma concentration of active renin increased 7-8 fold in homozygous mutant mice compared with that in heterozygous mutant and wild-type mice. These results indicate that the homozygous mice clearly display hyperreninemia.

Discussion

In the present study, we generated mutant mice in which the AT1a receptor gene was disrupted by replacing with ß-galactosidase (LacZ) gene to clarify the cellar localization of the receptor, that is, one of downstream components of RAS. We demonstrated that the AT1a receptor localized in gromerulus and JGA of renal cortex and the homozygous mutant mice display hyperreninemia and hypotension together.

These mice exhibited chronic hypotension, with 10 and 22 mmHg lower systolic blood pressure, respectively, than that of wild-type littermates. The systolic blood pressure in AT1a receptor homozygous mutant mice decreased to 77% of that in wild-type mice. We have reported that the systolic blood pressure in angiotensinogen deficient mice decreased to 66% of that in wild-type mice. Thus, concerning blood pressur reguration, it can be said that the approximate 2/3 of the ligand disruption effect in RAS is linked to the AT1a receptor and that the remaining effect will be caused by other AII receptor.

It has been known that AII exerts a negative feedback effect on renin gene expression [14,16-20] and that this effect may be mediated via the AT1 receptor because DuP753 (AT1 antagonist) treatment in rats increases renin gene expression in the kidney [15]. To know whether expression of the renin gene was affected by a null mutation of the AT1a receptor, we performed Northern blot analysis of kidney RNA from wild-type, heterozygous, and homozygous mutant mice. The level of renin mRNA markedly increased in homozygous mutant mice compared with that in heterozygous mutant and wild-type mice. These results strongly suggest that AII supresses renin gene expression through the AT1a receptor.

In conclusion, we generated mutant mice in which the AT1a receptor gene was

disrupted by replacing with LacZ gene to elucidate the physiological role of AT1a. In the heterozygous mutant mice, the strong lacZ staining was identified in the glomerulus and the JGA of the renal cortex. The localization of the LacZ staining coincided with that of the signals by in situ hybridization using the cRNA probe for the AT1a specific region in wild-type mice. Both the expression levels of renin mRNAs in the kidney and the plasma renin activities were not significantly different between heterozygous mutant and wild-type mice, but were markedly increased in homozygous mutant mice. On the contrary, significant phased decreases in blood pressure were observed in heterozygous and homozygous mutant mice. These results indicated that the homozygous mice display hyperreninemia and suggested that the function of AT1a is domnant regarding the regulation of renin gene expression because AII can suppress it even in the heterozygouse mice. Further histological and physiological experiments using these mutant mice may clarify the specific function of AT1a receptor.

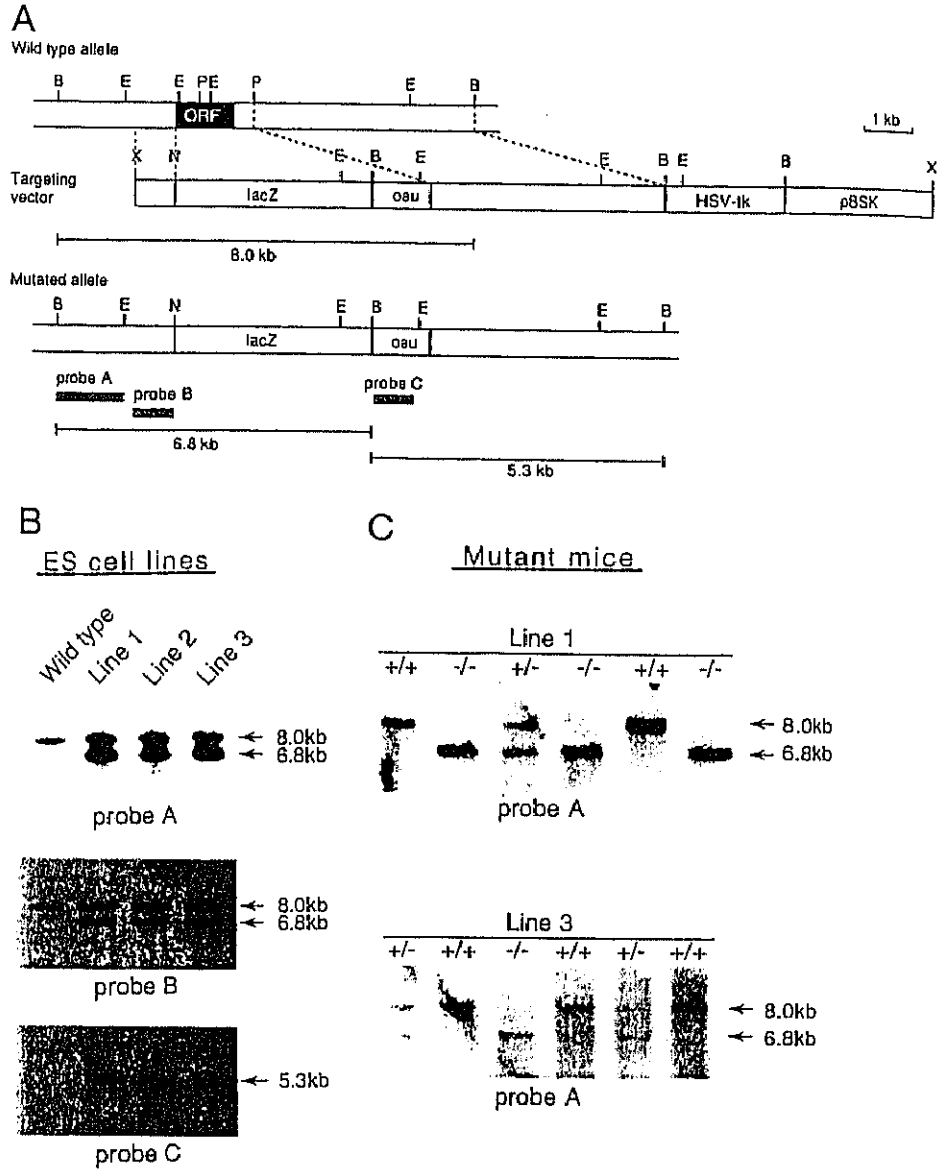

FIG. 1. **Targeted disruption of the AT1a gene by homologous recombination in ES cells and mice.** *A*, structure of the targeting vector and partial restriction map of the AT1a gene locus before and after targeting event. The intronless open reading frame (*ORF*) is shown as a *closed box*, and *lacZ* (β-galactosidase gene), *neo* (neomycin phosphotransferase gene), *HSV-tk* (herpes simplex virus-thymidine kinase gene), and *pBSK* (Bluescript KS(−)) are shown as *open boxes*, respectively. To construct the targeting vector, a 1.4-kb fragment including the translation initiation codon of the AT1a gene was replaced with *lacZ* and *neo* cassettes. *neo* was placed in the opposite orientation downstream of *lacZ*. The position of the probes used for Southern blot analysis (*closed bar*) and expected fragment sizes after *Bam*HI digests of genomic DNA are also shown. The restriction sites used are: *B*, *Bam*HI; *E*, *Eco*RI; *P*, *Pma*CI; *X*, *Xho*I; and *N*, *Nco*I. *B*, Southern blot analysis of ES cell DNA. Genomic DNAs extracted from wild-type and targeted ES cell clones were digested with *Bam*HI, electrophoresed, and blotted. The hybridization probes were: *A* and *B*, probes located outside and inside the targeting vector, respectively; and *C*, *neo* probe. Other restriction enzymes were used to confirm the homologous nature of the recombination (data not shown). *C*, Southern blot analysis of representative litter derived from a heterozygous intercross. Genomic DNAs isolated from tails of wild-type (+/+), heterozygous (+/−), and homozygous (−/−) mutant mice were digested with *Bam*HI, electrophoresed, and blotted. Fragments obtained from wild-type (8.0 kb) and targeted alleles (6.8 kb) were detected by probe A in two lines.

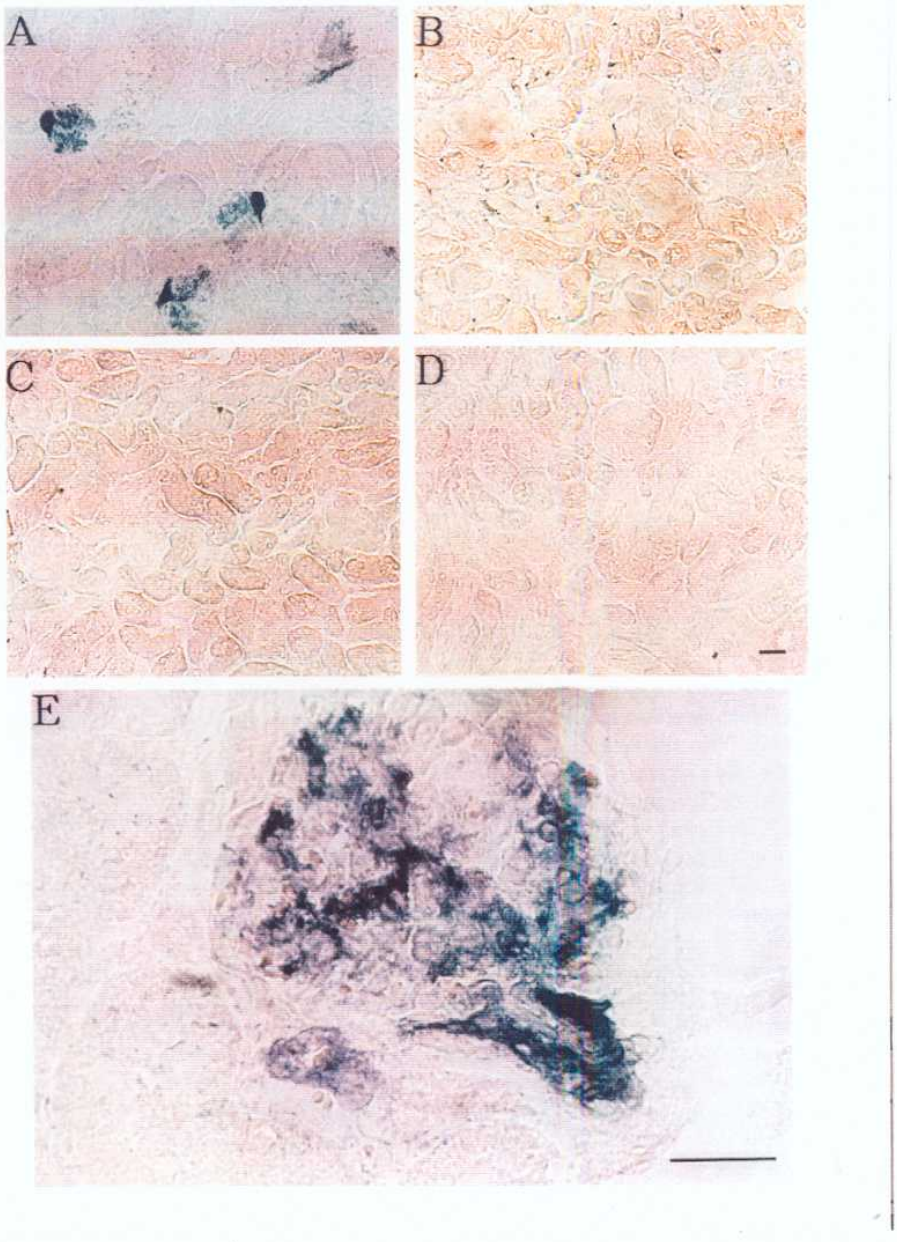

Fig. 2. **LacZ staining and in situ hybridization of AT1a receptor in renal cortex from wild-type and heterozygous mutant mice.** The LacZ staining in heterozygous mutant mice was obserbed in glomeruli, juxtaglomerular apparatus and proximal tubules (A). Espscially, the strong signals were obtained in mesangical cells and afferent arteriole (E). The localization of the LacZ staining in the heterozygous mice coincided with that of the signals obtained by in situ hybridization using the antisense cRNA probe for the AT1a specific region in the wild-type (B) and the heterozygous mice (data not shown). No signal using the antisense probe for the AT1b specific region (C) or the sense probe for the AT1a specific region (D) was detected in glomeruli of the heterozygous mice. Bars=25μm.

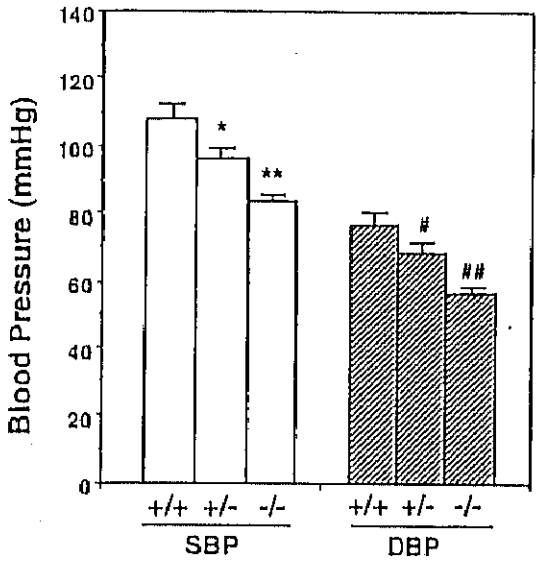

FIG. 3. **Comparison of blood pressures between mutant mice.** Statistical differences were analyzed by Student's *t* test. Symbols: *, $p = 0.0551$ and **, $p = 0.0002$ *versus* +/+ (systolic blood pressure (*SBP*)); #, $p = 0.1914$ and ##, $p = 0.0029$ *versus* +/+ (diastolic blood pressure (*DBP*)). +/+, wild-type mice; +/−, heterozygous mutant mice; −/−, homozygous mutant mice. Values shown are mean ($n = 7$) ± S.E.

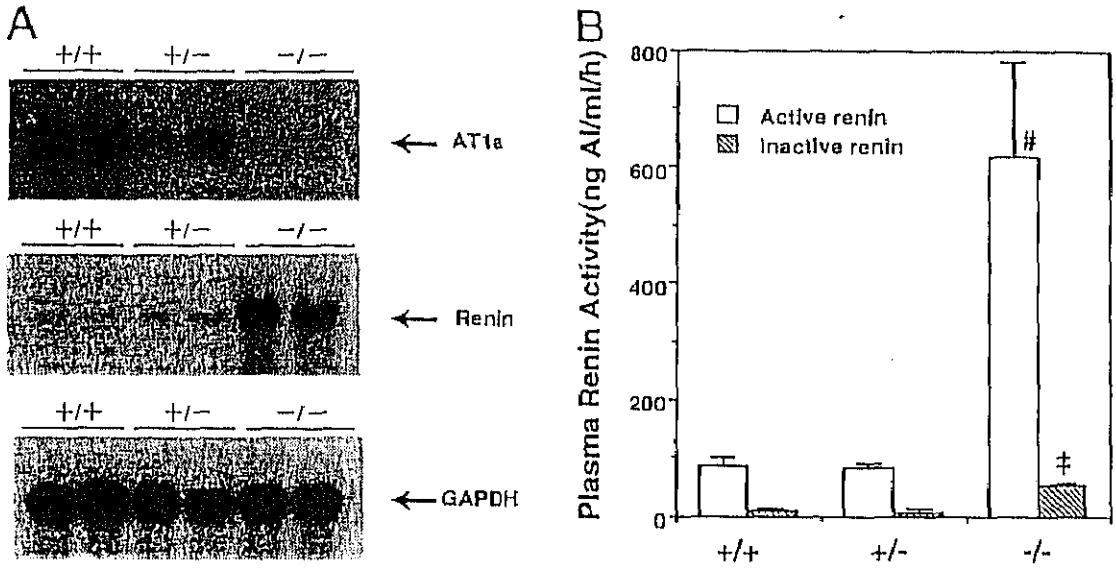

FIG. 4. **Northern blot analysis (*A*) and plasma renin activity (*B*) of the mutant mice.** *A*, 10 μg of total RNA, isolated from the kidneys of six independent age-matched adult mutant mice, were hybridized with either a 3′-untranslated AT1a-specific probe, renin probe, or glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) probe. *B*, plasma renin activity. Statistical differences were analyzed by Student's *t* test. Values shown are mean (n = 5) ± S.E. *Symbols*: #, p = 0.0135 *versus* +/+ (active renin); ‡, p = 0.0019 *versus* +/+ (inactive renin); +/+, wild-type mice; +/−, heterozygous mutant mice; −/−, homozygous mutant mice.