Renin plays a key role in controlling blood pressure through its specific cleavage of angiotensinogen to generate angiotensin I (AI). Although possible existence of the other angiotensin forming enzymes has been discussed to date, its in vivo function remains to be elucidated. To address the contribution of renin, we generated renin knockout mice. Homozygous mutant mice show neither detectable levels of plasma renin activity nor plasma AI, lowered blood pressure 20–30 mm Hg less than normal, increased urine and drinking volume, and altered renal morphology as those observed in angiotensinogen-deficient mice. We recently found the decreased density in granular layer cells of hippocampus and the impaired blood-brain barrier function in angiotensinogen-deficient mice. Surprisingly, however, such brain phenotypes were not observed in renin-deficient mice. Our results demonstrate an indispensable role for renin in the circulating angiotensin generation and in the maintenance of blood pressure, but suggest a dispensable role for renin in the blood-brain barrier function.

Renin (EC 3.4.23.15) catalyzes the specific cleavage of angiotensinogen to decapeptide (amino acids 1–10) angiotensin I (AI),1 the first and rate-limiting step in the renin-angiotensin (RA) system. The biologically inactive decapeptide AI is then converted by angiotensin-converting enzyme (ACE) to the effector molecule angiotensin II (AII). The RA system plays a key role in the control of blood pressure and electrolyte homeostasis as well as the pathogenesis of several diseases, including hypertension (1). Renin is synthesized principally in the kidney juxtaglomerular cells, are in close contact with the macula densa that signals to the renal arterioles to regulate glomerular filtration rate and the secretion of renin (2). This system was originally believed to be exclusively a circulating system, but because components of the RA system have been demonstrated in a variety of tissues, the local RA system has been proposed as a paracrine function with a physiological importance to the tissues in which it is expressed (3).

The brain is of particular interest with regard to the local RA system, because the blood-brain barrier (BBB) prevents the penetration of all components and products of this system in the circulation. It is well established that the injection of AII into the brain causes significant physiological responses (4). Moreover, we recently reported the importance of angiotensin fragments in maintaining the structure of hippocampus and the BBB function by analyzing angiotensinogen-deficient (Agt−/−) mice (5, 6). Although all the components are present for the RA system in the brain, the mismatch distribution of each component can be observed. The major gap in such evidence is the role of renin. The brain expresses mRNA for renin but the levels are very low and the distribution is dissimilar to the distribution of angiotensinogen mRNA and angiotensin immunoreactivity (7). There are several explanations for the lack of abundant renin in the brain, but the true answer remains to be defined.

We and other groups reported that Agt−/− mice exhibit several abnormal phenotypes (5, 6, 8, 12). These findings establish the notion that the RA system is essential for normal blood pressure maintenance, architecture of kidney and brain, and BBB function. In recent years, Sharp et al. (13) and Clark et al. (14) generated mouse strains that carry null mutated renin genes. Of note, these mouse strains have duplicated renin genes, Ren-1D and Ren-2, encoding highly homologous proteins with approximately 97% similarity at the amino acid level. It is conceivable that the inactivation of a specific renin leads to activation of the others in a compensatory manner, thereby minimizing manifestation of abnormalities. Therefore, the role of renin in the RA system remains to be elucidated. To address this question, we generated complete renin-deficient mice and suggest dispensable and indispensable roles for renin in vivo.

EXPERIMENTAL PROCEDURES

Generation of Renin-deficient Mice—The Ren-1C DNA was cloned from strain-C57BL6J genomic library. An NcoI site was created at the translation initiation codon of the gene and then the NcoI/KpnI fragment of the gene was replaced with the lacZ cassette (15). The neomycin phosphotransferase (neo) gene cassette derived from pMC1neo (Stratagene) was placed downstream of the lacZ gene. The 7.5-kb KpnI/NcoI fragment and the 4.5-kb KpnI/XhoI fragment of the Ren-1C gene were

1 The abbreviations used are: AI and AII, angiotensin I and II, respectively; RA, renin-angiotensin; ACE, angiotensin-converting enzyme; BBB, blood-brain barrier; kb, kilobase pair(s).
included upstream and downstream of these cassettes, respectively. The negative selection with the DT-A cassette are described elsewhere (16). The targeted TT2 ES cells were grown on feeder cells as described elsewhere (17). Homologous recombination was detected by Southern blotting. Chimeric mice were generated by injecting the ES cells into ICR eight-cell embryos.

**Measurement of PRA, Angiotensinogen, and AI—**Blood samples were withdrawn from mice (32–36 days) under pentobarbital anesthesia and collected into ice-cold tubes containing EDTA, which were immediately centrifuged. Plasma renin activity was estimated by measuring the rate of AI formation with the subsequently generated AI being quantitated by radioimmunoassay (18). The concentration of angiotensinogen was determined by measurement of the amount of angiotensin I released by use of an excess of mouse submandibular gland renin.

**Measurement of Blood Pressure—**The systolic, mean, and diastolic blood pressure were measured by a programmable sphygmomanometer (BP-98A; Softron, Japan) using the tail-cuff method as described previously (8). Statistical analysis for comparison of blood pressure was performed by using Student’s t test.

**Measurement of Urine Volume and Drinking Water—**Urine collection and measurement of drinking water was performed for 5 days. Statistical analysis used unpaired Student’s t test. Results are expressed as mean ± S.E.

**Immunohistochemistry—**Two micrometer serial paraffin sections prepared by a routine procedure were stained using anti-mouse submandibular gland renin antiserum (Sigma), anti-lacZ (Caltag Lab. Inc., South San Francisco, CA), or anti-PGP9.5 (UltraClone Limited, UK). Control sections were incubated with preabsorbed antisera or nonimmunized rabbit serum substituted for the primary antisera.

**Electron Microscopy—**Ultrathin sections were stained with uranyl acetate and lead citrate and observed with a JEM-1210 electron microscope (JEOL, Japan) at 100 kV.

**Cold Injury—**Cold injury was conducted as described previously (5).

## Results

### Generation of Renin-deficient Mice—An inbred mouse strain, C57BL/6, has the single renin gene, termed Ren-1C, whereas CBA possesses the two renin genes, termed Ren-1D and Ren-2. We generated renin-deficient mice with targeted replacement of the Ren-1C loci by the lacZ gene in the TT2 ES cells derived from an F1 embryo between C57BL/6 and CBA mice (17) (Fig. 1A). Male chimeras derived from the stem cells were mated with C57BL/6 females to obtain heterozygous F1 hybrids (Ren-1C/−), and heterozygous progeny were intercrossed to produce renin −/−. Our previous study showed that almost Agt −/− mice died of renal failure, but most saline-treated animals lived over a year (12). To prevent neonatal death, the resulting offsprings were subcutaneously injected dorsally with saline (0.05 ml/g/day) in the 7 days following birth. Without such treatments, renin −/− mice also died within a week after birth (data not shown). The mutated Ren-1C gene was confirmed by genomic Southern and Northern blot analyses (Fig. 1, B and C).

**Plasma Angiotensin Measurements—**To test angiotensin generation in the plasma, blood samples were analyzed by a radioimmunoassay (Table I). Although there was no significant difference between renin1C+/− and renin1C−/− mice, the plasma renin activity and the plasma content of AI decreased to below normal limits in renin−/− mice.

### Table I

| Study                        | Renin1C+/− | Renin1C−/− | Renin−/− |
|------------------------------|------------|------------|----------|
|                              | δ ± ϑ      | δ ± ϑ      | δ ± ϑ    |
| Plasma renin activity (ng AL/ml/h) | 18.34 ± 2.55 | 18.03 ± 4.39 | 18.92 ± 3.58 |
|                              | (n = 5)    | (n = 4)    | (n = 5)  |
| Angiotensinogen (ng/ml)      | 7.12 ± 0.43 | 7.03 ± 0.96 | 8.21 ± 1.22 |
|                              | (n = 5)    | (n = 4)    | (n = 5)  |
| Angiotensin I (ng/ml)        | 10.9 ± 1.68 | 19.07 ± 2.95 | 9.90 ± 2.34 |
|                              | (n = 5)    | (n = 4)    | (n = 5)  |
| Systolic blood pressure (mm Hg) | 105.88 ± 4.24 | 110.00 ± 4.23 | 99.47 ± 2.64 |
|                              | (n = 6)    | (n = 5)    | (n = 6)  |
| Mean blood pressure (mm Hg)  | 86.18 ± 3.38 | 87.44 ± 3.67 | 82.83 ± 1.51 |
|                              | (n = 6)    | (n = 5)    | (n = 6)  |
| Diastolic blood pressure (mm Hg) | 76.48 ± 3.18 | 76.32 ± 3.60 | 74.55 ± 0.99 |
|                              | (n = 6)    | (n = 5)    | (n = 6)  |
| Urine volume (ml/day 10 g)   | 0.53 ± 0.10 | 0.60 ± 0.05 | 0.67 ± 0.09 |
|                              | (n = 6)    | (n = 5)    | (n = 6)  |
| Drinking water (ml/day 10 g) | 2.45 ± 0.17 | 2.62 ± 0.11 | 2.50 ± 0.12 |
|                              | (n = 6)    | (n = 5)    | (n = 6)  |

* ND, not determined.

* p < 0.01 versus other groups.
detectable levels, but the plasma angiotensinogen concentration increased in the renin \(^{-/-}\) mice.

**Blood Pressure Homeostasis**—We measured the systolic, mean, and diastolic blood pressures of mice at 8 weeks after birth (Table I). The blood pressures between renin\(^{1C/1C}\) and renin\(^{2/2}\) mice were indistinguishable, whereas a significant difference in blood pressures was observed in the renin\(^{-/-}\) mice, the decreased levels of which were similar to ones of Agt\(^{-/-}\) mice (8, 9).

**Renal Examination**—Renin\(^{-/-}\) mice demonstrated major morphological changes in the kidney, i.e. dilated renal pelvis and atrophy of renal parenchyma from the papilla to the outer strip, indicating hydrenephrosis (Fig. 2B), as compared with those of renin\(^{1C/1C}\) mice (Fig. 2A). In renin\(^{-/-}\) mice, macula densa morphology, showing simple plaque of distal tubule cells, was similar to that in wild-type mice (Fig. 2C and data not shown). Macula densa in renin\(^{-/-}\) mice was characterized by the increase in macula densa number (Fig. 2D) or by the marked accumulation of nuclei like as pseudostratified epithelium (E), although the renin\(^{1C/1C}\) ones show simple plaque of distal tubule cells (C). Scale bars, 25 μm. Electron microscopy revealed that juxtaglomerular cells of the renin\(^{-/-}\) mice were completely devoid of the storage/secretory granules (G) typically present in wild-type controls (F). Scale bars, 0.25 μm.

![Kidney morphology](image)

**Fig. 2. Kidney morphology.** Cross-section of the kidney of renin\(^{1C/1C}\) (A) and renin\(^{-/-}\) (B) mice. Hydrenephrosis with atrophic papilae is prominent in the kidneys of the renin\(^{-/-}\) mice. Scale bars, 1 mm. Morphology of the cells of the macula densa. Macula densa in renin\(^{-/-}\) mice was characterized by the increase of macula densa number (D) or by the marked accumulation of nuclei like as pseudostratified epithelium (E), although the renin\(^{1C/1C}\) ones show simple plaque of distal tubule cells (C). Scale bars, 25 μm. Electron microscopy revealed that juxtaglomerular cells of the renin\(^{-/-}\) mice were completely devoid of the storage/secretory granules (G) typically present in wild-type controls (F). Scale bars, 0.25 μm.

![Immunohistochemical analysis](image)

**Fig. 3. A, immunohistochemical analysis.** In the renin\(^{1C/1C}\) and renin\(^{2/2}\) kidney, vascular poles of the glomeruli were immunologically stained for renin (renin), but those were negative in the renin\(^{-/-}\) ones at all. Renin\(^{-/-}\) mice expressed β-galactosidase in association with thickening of the vascular wall, whereas in renin\(^{2/2}\) mice, LacZ positive cells were limited to cells of the juxtaglomerular cell apparatus. For α-smooth muscle actin (α-SMA), the vascular smooth muscle cells of renin\(^{-/-}\) mice were stained weakly, though those of the renin\(^{1C/1C}\) and renin\(^{2/2}\) ones were stained in a diffuse and fine granular manner. **Scale bar, 50 μm.** B, immunohistochemical analysis using anti-PGP9.5 antiserum. In the kidney of renin\(^{1C/1C}\) and renin\(^{2/2}\) mice, a few nerve fibers constituting the varicosity were scattered along the adventitia of arteriolar vessels. Whereas in the kidney of renin\(^{-/-}\) mice, many nerve fibers were noted to distribute along the hypertrophied vessel walls and to localize at the juxtaglomerular apparatus. **Scale bar, 50 μm.**

Correctly regulated lacZ expression under control of the endogenous renin promoter. In the renin\(^{-/-}\) kidney, lacZ signals were observed in hyperplastic vascular smooth muscle cells. For α-SMA, the vascular smooth muscle cells of renin\(^{-/-}\) mice were stained weakly, although those of the renin\(^{1C/1C}\) and renin\(^{2/2}\) ones were stained in a diffuse and fine granular manner.

To examine the innervation in association with the juxtaglomerular apparatus, the immunohistochemical analysis using an anti-PGP9.5 antiserum, a marker of the nervous and neuroendocrine system, was performed (19) (Fig. 3B). In the kidney of renin\(^{1C/1C}\) and renin\(^{2/2}\) mice, a few nerve fibers constituting the varicosity were scattered along the adventitia of arteriolar vessels. Whereas in the kidney of renin\(^{-/-}\) mice, many nerve fibers were noted to distribute along the hypertrophied vessel walls and to localize at the juxtaglomerular apparatus.

To study the renal function, we examined drinking intake and urine output (Table I). Drinking and urination were significantly higher in renin\(^{-/-}\) mice than the other control mice, indicating that renin\(^{-/-}\) mice were polydipsia and polyuria.

**Brain Examinations**—Recently, we reported the decreased density in the granular layer cells of hippocampus and the impaired BBB function in Agt\(^{-/-}\) mice with a C57BL/6J genetic background (5, 6). To make a comparative study, we re-examined the density in the granular layer cells of the initial line of Agt\(^{-/-}\) mice, because Agt\(^{-/-}\) mice, like renin\(^{-/-}\) mice, were initially produced with a hybrid genetic background using TT2 ES cells (8). As shown in Fig. 4A, hybrid Agt\(^{-/-}\) mice revealed a characteristic appearance of granular layer cells, whose nuclei are larger and round shaped with coarse chromatin. The cellular density decreased with less layers, compared with that of wild-type. Moreover, we compared the disruption of the BBB.
in hybrid Agt−/− mice with that of wild-type mice, using cold injury followed by intraperitoneal injection of Evans blue (Fig. 4B). Exudation of this dye was evident in the hybrid Agt−/− brain by day 5 after cold injury, indicating that the abnormalities observed in congenic Agt−/− mice are not affected by the genetic background. Surprisingly, however, renin−/− mice were indistinguishable from wild-type mice in these phenotypes.

**DISCUSSION**

We tried to analyze Agt-processing cascade by generating the complete renin-deficient mice with the Ren-1C null mutation in the present study (Fig. 1). Because some mouse strains have duplicated renin genes, phenotypic changes are often obscured by the genetic redundancy when gene knockout technique is applied to this case. As expected, our Ren-1C null mutants considerably differ from the previously reported Ren-1D or Ren-2 null mutants (13, 14). First, the plasma renin activity and the plasma content of immunoreactive AI decreased below detectable level (Table I). Second, the mutant mice showed lowered blood pressure, 20–30 mm Hg less than normal in both sexes (Table I). These observations are identical to those observed in Agt−/− mice (8–12). Therefore, we conclude that the renin-dependent classical pathway plays an essential role in generating plasma AI and maintaining blood pressure.

Renin has been generally considered to be the only enzyme processing angiotensinogen in vivo. We demonstrated, however, a dispensable role for renin in the maintenance of density in granular layer cells of hippocampus and the blood-brain barrier function (Fig. 4), but renin is indispensable in the circulating RA system. Although the lack of abundant renin in the brain has been discussed for a long time, these renin-independent brain functions suggest a possible existence of other angiotensinogen processing enzymes. This notion drives us to the question what other enzyme(s) would contribute to the production of angiotensin fragments in the brain. Hackenthal et al. (20) purified isorenin from the rat brain that is distinct from renal renin but indistinguishable from cathepsin D. In addition to the classical pathway of AI formation via renin or cathepsin D in association with ACE, cathepsin G and tonin have been shown to generate AI directly from angiotensinogen, suggesting a contribution to AI generation in brain regions without detectable ACE (21–22). Even if these enzymes contribute to angiotensin production, there remains a second question: where is angiotensinogen processed? As these aspartyl proteases have activities only in the acidic condition, the processing event should occur in the cytosolic vesicles (23). Interestingly, Moffett et al. (24) proposed a model of intracellular AI formation in the brain. Further study will define the cascade(s) which are involved in angiotensin generation pathway in the brain. Our renin-deficient mouse line should be an excellent model for understanding a renin-independent pathway(s) in vivo.

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