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FULL PAPER

# Upregulation of renal renin-angiotensin system in mouse diabetic nephropathy

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Abstract

The aim of this study was to clarify the role of the renal renin-angiotensin system (RAS) in diabetic nephropathy (DN), which was induced by injection of streptozotocin (STZ). Male CBA/N and CBA/J mice were compared in this study. The former possesses a single renin gene, *Ren1*, whereas the latter carries two renin genes, *Ren1* and *Ren2*. To examine the molecular dynamics of renal RAS, including renin, angiotensinogen (*Agt*), angiotensin-converting enzyme (*Ace*), angiotensin type 1 (*Agtr1*) and type 2 (*Agtr2*) receptors in experimental DN, we performed laser-microdissection (LMD) followed by reverse transcriptase nested polymerase chain reaction using each specific primer pairs and immunohistochemistry for renin and angiotensin II.

CBA/N mice had a higher response after injection of STZ than CBA/J mice, showing a significant increase of the kidney/body weight ratio, although there was no significant difference between the two strains for the blood glucose level or pancreatic β-cell response. The onset of renal pathological changes associated with DN was earlier and more severe in CBA/N mice than in CBA/J mice. Distinct immunoreactivities for renin and angiotensin Ⅱ were newly distributed on the flattened epithelial cells in the dilated distal tubules in the cortex as well as the collecting ducts in the cortex and medulla, and were demonstrated more intensely in CBA/N mice than in CBA/J mice. Microdissectional analysis in both DN models revealed a higher incidence of RAS-related gene expression in CBA/J, *Ren 1 Ren 2* mice than in CBA/N, *Ren 1* mice.

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These findings suggest that intrarenal RAS plays an important role in the onset of renal pathological changes associated with DN. Additionally, *Ren 1* mice have more severe histopathological nephropathy than *Ren1 Ren2* mice, followed by marked production of angiotensin II.

Key words : diabetes, kidney, microdissection, renin-angiotensin system, streptozotocin

## Introduction

The renin-angiotensin system (RAS) directly regulates blood pressure by its vasopressor action, as well as indirectly integrating blood electrolytes via its effect on central and peripheral mechanisms. In the classical RAS, the precursor angiotensinogen (Agt), which is produced in hepatocytes and released into the bloodstream, is changed to angiotensin I by the action of renin, which is synthesized in the juxtaglomerular apparatus of the kidney. Angiotensin I is then cleaved by the action of the angiotensin-converting enzyme (Ace), a ubiquitous enzyme in endothelial cells, into the main effector, angiotensin II. Angiotensin II binds angiotensin type 1 (Agtr 1) and type 2 (Agtr 2) receptors to induce vasoconstriction, aldosterone production and cell proliferation[16].

It has been reported that all or some of the components in the RAS are synthesized and secreted outside of classical RAS organs or tissues[8]. This local RAS was demonstrated in many tissues, including endocrine glands[18,22], the reproductive system[19,33,40], central nervous system[3], and cardiovascular system[14]. The angiotensin series detected in these tissues are frequently originated from the activity of local tissue RAS, acting via paracrine/autocrine mechanisms.

The availability of specific immunohistochemical and *in situ* hybridization methods has permitted the demonstration of the localization of renin[37], Ace[36], angiotensin I, angiotensin II[5], Agtr 1[28], Agtr[29] and Agt[7] in kidney tissue. These findings support assumptions about angiotensin II generation and its possible function by intrarenal RAS.

Diabetes mellitus characterized by hyperglycemia is a long-term developing disease, including retinal, neuronal, renal and cardiovascular disorders. In these, diabetic nephropathy (DN) associated with the highest mortality in humans, is characterized initially by hypertrophy of glomerular and tubular epithelial cells and then the thickening of their basement membranes, which are found by clinical tests concerning hyperfiltration and microalbuminuria[6]. It finally progresses to glomerulosclerosis with accumulation of extracellular matrix proteins in the glomerular mesangium. The unignorable contribution of intrarenal RAS has been reported in more than 80% of patients with type 1 diabetes mellitus[12]. Those studies have indicated that the activation of intrarenal RAS is associated with the development of DN.

A single renin locus has been identified in many animals, including humans and rats. In mice, certain strains (for example, C57BL/6J) that produce low amounts of renin in the submandibular gland have a single renin locus (*Ren1*), whereas other strains (for example, DBA/2) that contain high submandibular gland renin activity have a duplication of the renin structural gene (*Ren1*, *Ren2*)[29,34]. However, the relationship between the strain difference of renin genes and the pathological dynamics of DN have not yet been elucidated.

The advent of microdissection techniques has made it possible to isolate distinct cells or tissues from histological sections[1,2]. This enables isolation of populations of glomerular, proximal and distal tubular epithelial cells from renal tissues and usage of the isolated tissue samples for subsequent analyses expression of various genes.

The aim of this study is to clarify the roles of intrarenal RAS in DN induced by an injection of streptozotocin (STZ). Male CBA/N and CBA/J mice were compared. The former possesses a single renin gene, *Ren1*, whereas the latter carries two renin genes, *Ren1* and *Ren2*. To examine the molecular dynamics of the renal RAS, including renin (*Ren1, Ren2*), *Agt, Ace, Agtr1* and *Agtr2* in experimental DN, we performed laser-microdissection (LMD) followed by reverse transcriptase nested polymerase chain reaction (RT-nested PCR) using specific primer pairs and immunohistochemistry for renin and angiotensin II.

## Materials and Methods

*Animals*

Inbred strains, including A/J, AKR/N, BALB/c, C3H/He, C57BL/6J, CBA/N, DBA/2, MRL/MpJ−+/+, MRL/MpJ-*lpr/lpr* ((Japan SLC, Inc., Hamamatsu, Japan) and CBA/J (Japan Charles River, Yokohama, Japan) were purchased from animal breeding companies, and maintained in a pathogen-free and climate-controlled environment with 12-hour light/dark cycle. In the experimental animal care and handling, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals, Hokkaido University, Graduate School of Veterinary Medicine." In the preliminary studies, genotypes were estimated with *Ren1* and *Ren2* specific primer pairs (primer pairs : *Ren1* forward5'-AGCTGGAA ACGCTGGGAG-3';*Ren2* forward 5'-GACTG CTCTTCCAAAGGTG-3';*Ren1* and *Ren2* reverse 5'-TGCAAGGACTCCAGAGCAA-3') using tail DNA samples from each animal strain. We found that A/J, BALB/c, C3H/He, C57BL/6J, CBA/N were the *Ren1* type, whereas the other strains, including CBA/J and DBA/2, were the *Ren1 Ren2* type (data are not shown). We chose CBA/N (*Ren1*) and CBA/J (*Ren1 Ren2*) mice for the following experiment to create the DN model.

*Injection of STZ*

Preliminarily, several doses of STZ were injected intraperitoneally into C57BL/6J, CBA/N, CBA/J and DBA/2 mice, showing a strain difference of STZ-sensitivity as reported like in a previous study[32]. In the present study, adequate diabetes was induced in 9-week-old CBA/N and CBA/J mice by a single injection of 300mg/kg of body weight of STZ (Calbiochem, La Jolla, USA) dissolved in sterile sodium citrate buffer (50mM, pH4.5). As sham controls, animals of both strains were injected with sodium citrate buffer before sampling at 56 days after administration. To estimate clinical symptoms, body weights were measured at 0, 7, 28 and 56 days after STZ-administration.

*Histological analysis : periodic acid Schiff (PAS) staining and immunohistochemistry*

The STZ-injected animals were sacrificed by cervical dislocation at 28 and 56 days after administration. Right kidneys were fixed with 4% paraformaldehyde in 0.1M phosphate buffer (pH 7.3) and embedded in paraffin by a routine procedure. Four-μm-thick sections were stained with PAS-hematoxylin for detection of basement membrane in glomerulus and brush border in proximal tubules. The neighboring sections were immunostained with polyclonal rabbit anti-renin[17] or polyclonal rabbit anti-angiotensin II (Peninsula Lab., Belmont, USA) antiserum.

Gene-specific primer pairs in RAS

| Genes (Accession No.) | | 1 st PCR (Primer position) | 2 nd PCR (Primer position) |
|---|---|---|---|
| *Agt* (NM007428) | Forward | 5'-GATGAACTTGCCACTGGAGG-3' (797-816) | 5'-CGATGAGAGGTTTCTCTCAG-3' (871-890) |
| | Reverse | 5'-TTGAGTTCGAGGAGGATGC-3' (1291-1309) | 5'-CGGGTTCTCTATCCAAGTCA-3' (1108-1127) |
| *Renin* (J00621) | Forward | 5'-GTGGGTGGAATCACTGTGAC-3' (524-543) | 5'-TTATCTCGGCTCCTACGAGC-3' (911-931) |
| | Reverse | 5'-GTGGCAGAGGGCCTTAGC-3' (1242-1259) | 5'-TCCAACGCGATTGTTATGC-3' (1210-1228) |
| *Ace* (J03940) | Forward | 5'-TCCACTGGCAAGGTCTGCTT-3' (500-519) | 5'-CTTCCTCACGAAGCTATGCC-3' (576-595) |
| | Reverse | 5'-ACTGGTGACATCGAGGTTGG-3' (957-976) | 5'-GCACTACCATGTCGTAGATG-3' (922-941) |
| *Agtr 1* (S37491) | Forward | 5'-GCATCATCTTTGTGGTGGG-3' (127-145) | 5'-TTGTTGACTTTGCCTCTGTG-3' (252-271) |
| | Reverse | 5'-TATGCAGATGGTGATGGGCA-3' (871-890) | 5'-GAAGAAAAGCACAATCGCC-3' (749-767) |
| *Agtr 2* (U04828) | Forward | 5'-ACCTGCATGAGTGTCGATAG-3' (537-556) | 5'-CTGTCTCAAAGAAGGAATCC-3' (582-601) |
| | Reverse | 5'-GTTGGAAGCGGTTTCCAACA-3' (1091-1110) | 5'-ACAGCTGTTGGTGAATCC-3' (1050-1067) |

Briefly, deparaffinized sections were incubated with 0.1% $H_2O_2$ in methanol and then 10% normal goat serum 30min each for elimination of non-specific reactions. Sections were reacted with anti-renin or anti-angiotensin II diluted at 1：3000 each at 4 ℃ overnight, and then incubated using a Histofine kit (Nichirei, Tokyo, Japan). Immunoreaction was visualized with 3, 3'-diaminobezidine-$H_2O_2$ solution.

*Estimation of diabetic nephropathy*

To estimate diabetes, monitoring of the blood glucose concentration and β-cell localization of the pancreas were performed. Briefly, glucose levels obtained from tail blood were measured by Refrotron Plus (Roche, Tokyo, Japan) at 1 day before and at 7, 21, 49 days after the injection of STZ according to the manufacturer's protocol. Since the maximum detectable glucose level by Refrotron Plus was 600mg/dl, measured values over that concentration were recorded as 600mg/dl. In the pancreas, the effects of STZ were estimated by insulin immunohistochemistry, for which deparaffinized sections were immunostained with polyclonal guinea pig anti-porcine insulin antiserum (Zymed, San Francisco, USA).

*LMD and RT-nested PCR*

Left kidneys of STZ-injected (28 and 56 days) and sham-control (56 days) animals were immediately embedded in OCT compound (Sakura FineTech., Tokyo, Japan), and 4-μm cryostat sections were cut and mounted on glass slides coated with RNase-free membrane film (Meiwa Shoji, Osaka, Japan). They were fixed with absolute methanol for 1 min and stained with 1 % toluidine blue for 10 sec. LMD was performed using an LS-Pro 300 apparatus (Meiwa Shoji) to pick up 4 areas, including 1) the arterial area of the cortex con-

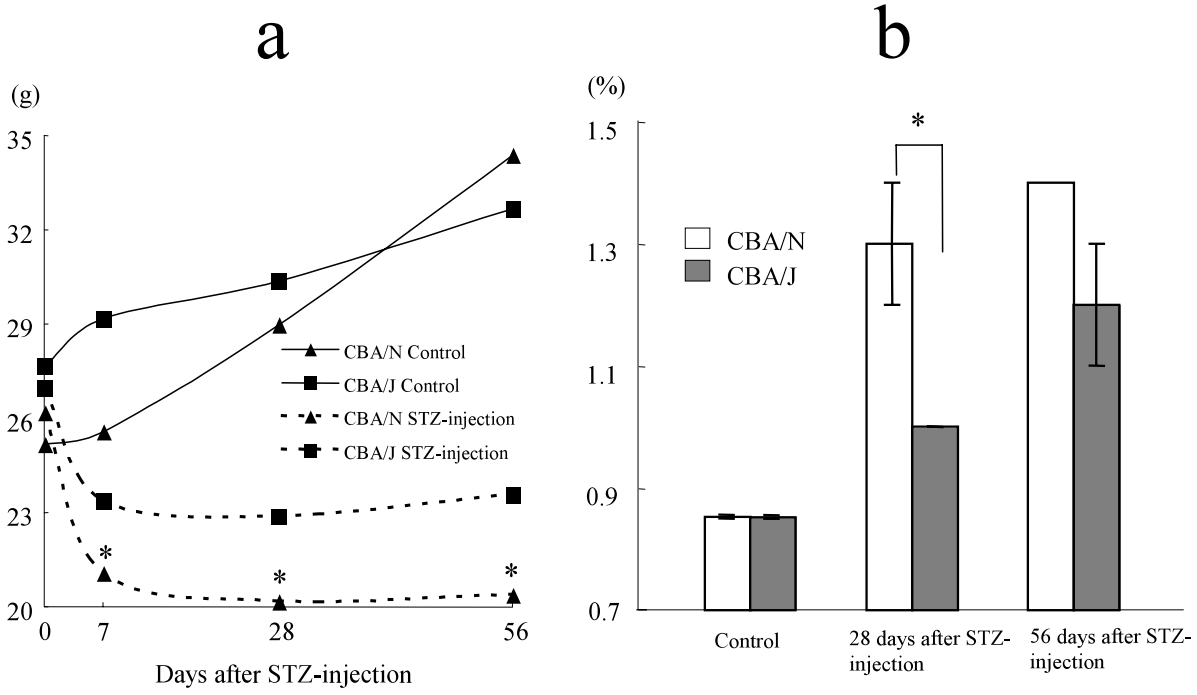


Figure 1. Changes of body weight (a) and kidney weight ratio (b) after STZ administration. (a) The decrease in body weight is more marked in CBA/N than that in CBA/J. (b) The increase of kidney /body weight ratio (%) is also higher and earlier in CBA/N than in CBA/J. The data are presented as the mean ± SD. Significant differences are indicated with asterisks : *P<0.01.*

taining the arcuate and interlobular arteries, 2) the glomerulus, 3) the tubular area of the cortex containing proximal and distal tubules and cortical collecting ducts, and 4) the tubular area of the inner layer of the outer medulla containing proximal straight tubules, the nephron loop and mellulary collecting ducts. Total RNAs treated with DNaseI (Quiagen, Tokyo, Japan) were isolated from LMD samples by using an RNeasy Micro kit (Quiagen, Tokyo, Japan), according to manufacturer's protocol.

All total RNA solutions isolated by LMD was reverse-transcribed with ReverTraAce (Toyobo, Osaka, Japan) and amplified with Biolase DNA polymerase (BioLine, Luckenwalde, Germany) under the following PCR conditions: 5 min at 95℃, 35 cycles of 40sec at 95℃, 30sec at 60℃, and 1 min at 72℃ followed by 5 min at 72℃. To detect high quality production from the smallest aliquots, nested PCR was performed using RAS-related specific primer pairs (Table 1).

*Statistical estimation*

All values are presented as means ± standard errors. Statistical analysis was performed by two-way ANOVA with a post hoc test and then by Bonferroni's multiple range test. *P* values of less than 0.01 were considered statistically significant.

## Results

*Changes of body weight, kidney weight, insulin immunoreactivity and blood glucose level after STZ administration*

In these experimental periods, the body weights of both CBA/N and CBA/J mice were sharply decreased at 7 days after injection, though the decrease ratio in CBA/N was more marked than in CBA/J (Fig. 1). The difference of body weight between the two strains in-

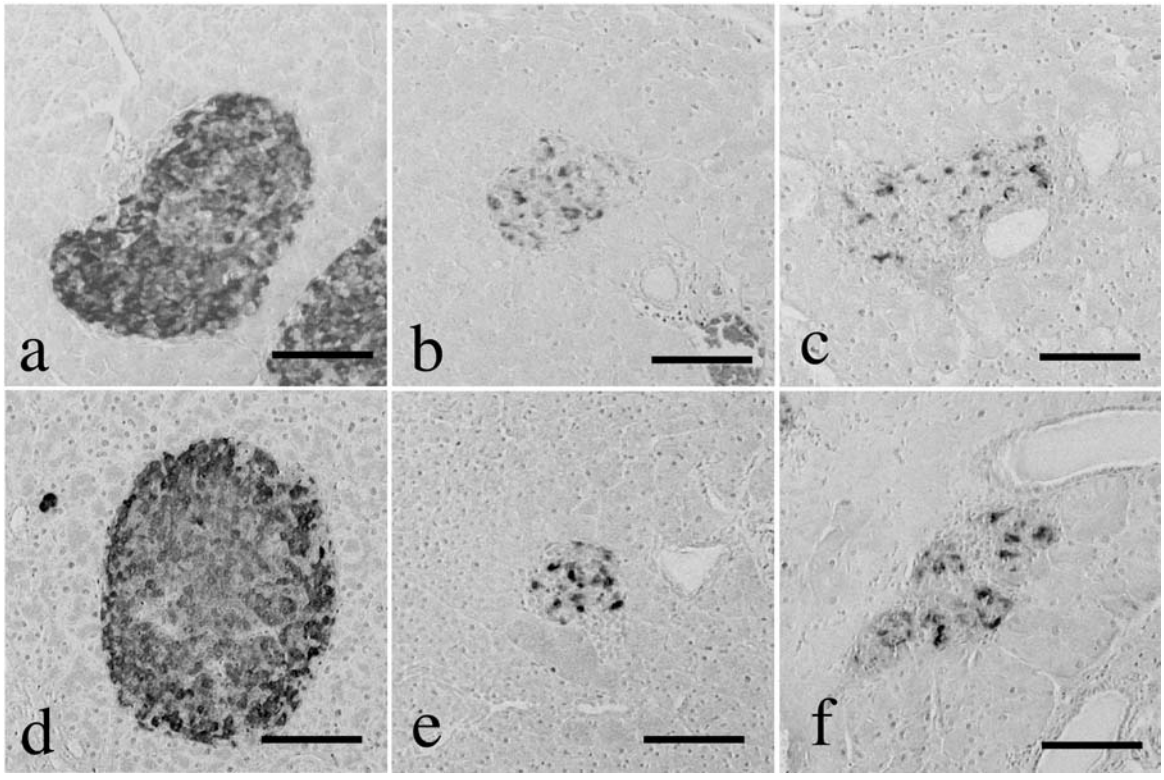


Figure 2. Immunohistochemistry for insulin in pancreatic islets after STZ-administration. Similar disappearing nature of insulin-bearing cells is observed in both strains. a, b, c: CBA/N. d, e, f: CBA/J. a, d: sham-control, b, e: 28 days after STZ-injection, c, f: 56 days after STZ-injection. Bar, 100μm.

jected was maintained throughout the experimental period. In sham-control mice, the body weights were gradually increased by physiological growth, and there was no significant difference between the two strains. The kidney/body weight ratio in CBA/N was increased at 28 days after STZ-injection, earlier than in CBA/J, but thereafter it showed similar high values at 56 days in both strains. The extent of β-cell damage was further estimated by insulin immunostaining, resulting in a similar reduction of insulin-bearing cells in both strains (Fig. 2). As shown in Figure 3, there was a gradual increase of the blood glucose concentration in both mouse strains from 7 to 49 days after STZ-injection, and their values were significantly higher than those in sham-treated controls ($P<0.01$). No significant difference of blood glucose levels was observed in any period between the two strains. These results indicated that type 1 diabetes was clearly induced in both mouse strains. Additionally, they suggested that CBA/N mice had a greater response after STZ-administration than CBA/J mice, although there was no significant difference in the blood glucose level or pancreatic β-cell damage.

*Histological alterations including immunohistochemistry for renin and angiotensin II in the diabetic kidney*

In DN, it was observed histopathologically that, compared to the control kidney, the expanded distal tubules and collecting ducts showed thin-layered epithelial cells, and that PAS-positive granules, indicating lysosomal dense bodies, were sometimes deposited on the epithelial cells of distal tubules and col-

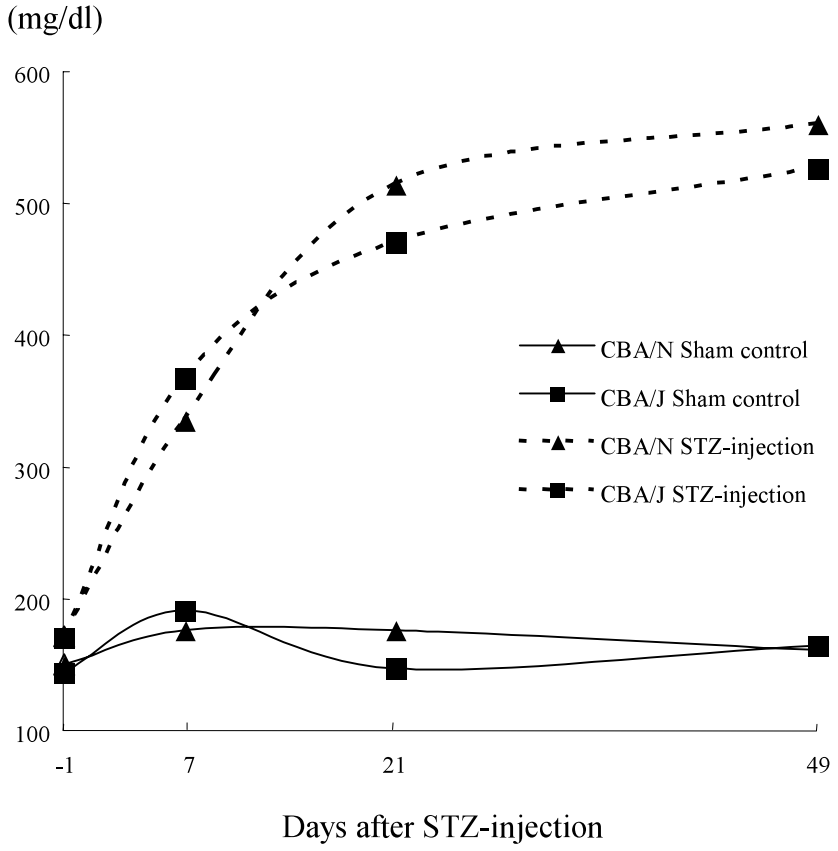


Figure 3. Changes of blood glucose concentration after STZ-administration. In both strains, there is a gradual increase of the blood glucose concentration, with no significant difference in any period between the two strains.

lecting ducts, especially in the case of 56 days after STZ-injection (Fig. 4). These pathological changes of the tubular region were earlier and more intense in CBA/N mice than in CBA/J mice. In glomeruli, slight mesangial hypercellularity was demonstrated in both strains.

In the normal condition, renin-immunoreactive cells were restricted to the vascular pole of glomerulus, i.e. were juxtaglomerular cells, as in a previous report[17], whereas there was no expression of angiotensin II-immunoreactivity throughout the kidney. In DN, distinct immunoreactivities for renin and angiotensin Ⅱ were newly distributed on the flattened epithelial cells in the dilated distal tubules in the cortex as well as the collecting ducts in the cortex and medulla (Fig. 5). These reactions were especially marked in the apical region of cytoplasm compared to the basal region. It was additionally noted that these immunohistochemical changes from normal into diabetic kidneys were demonstrated more intensely in CBA/N mice than in CBA/J mice. Renin-immunoreactive cells in both strains and angiotensin II-immunoreactive cells in CBA/N mice tended to increase at 28 days compared to those at 56 days, but the latter cells in CBA/J maintained a similar distribution between 28 and 56 days after injection. These results suggested that angiotensin II, a major functional mediator in the RAS, as well as renin, the trigger enzyme for RAS activation, were demonstrated more intensely on

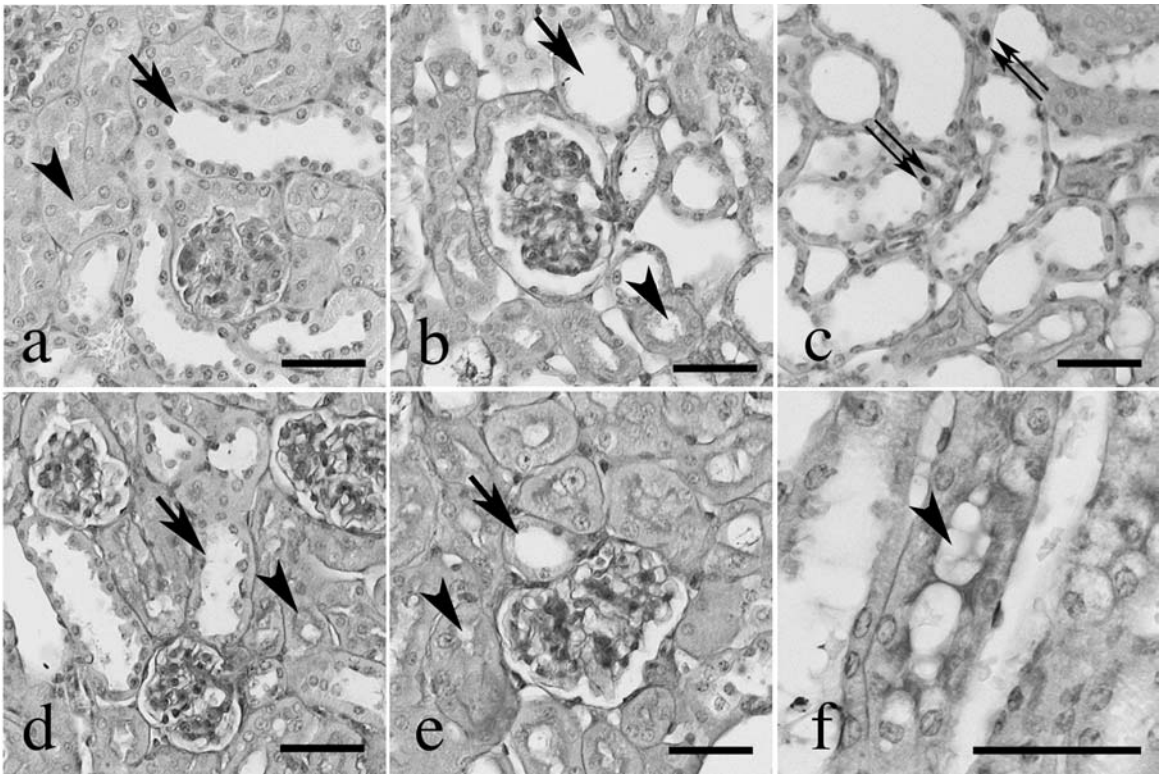


Figure 4. Histopathological alterations in the diabetic kidney. The expansion of distal tubules/collecting ducts (arrows) and the diminishment of the PAS-positive brush border in proximal tubules (arrowheads) are observed as well as inclusion of PAS-positive granules (double arrows). These changes are earlier and more intense in CBA/N mice than in CBA/J mice. a, b, c, f: CBA/N, d, e: CBA/J. a, d: 28 days after STZ-injection, b, c, e, f: 56 days after STZ-injection. Bar, 50μm.

distal tubules and collecting ducts in CBA/N mice than in CBA/J mice.

*Changes of RAS-related gene expression*

Microdissectional analysis of the control kidney showed that expression of renin mRNA predominantly occurred in the renal cortical artery, glomerulus and in part in medullary tubules (Table 2). *Ace* mRNA was expressed on the cortical artery and in cortical tubule areas, whereas *Agtr 1* mRNA was expressed through wide areas without glomeruli. There was no or very little expression of *Agt* and *Agtr 2* mRNAs. No difference was observed in RAS expression between non-diabetic CBA/N and CBA/J mice.

In the present study, LDM-nested PCR performed was not really quantitative because of the very small aliquots used for detection, so the dynamics of expression in each mRNA were estimated by the numbers of positive samples. In DN, renin mRNA on medullary tubules was induced in CBA/J mice but reduced in CBA/N mice, whereas no changes of renin expression were observed in any areas of the artery, glomerulus or cortical tubules. *Agt* mRNA was newly induced in the cortical and medullary tubule areas, where its expression was higher and earlier in CBA/J mice than in CBA/N mice. At 56 days after STZ-injection, *Agt* mRNA was additionally expressed on the cortical artery as well as in the above areas; however, no expression was detected on the glomerulus in either strain in

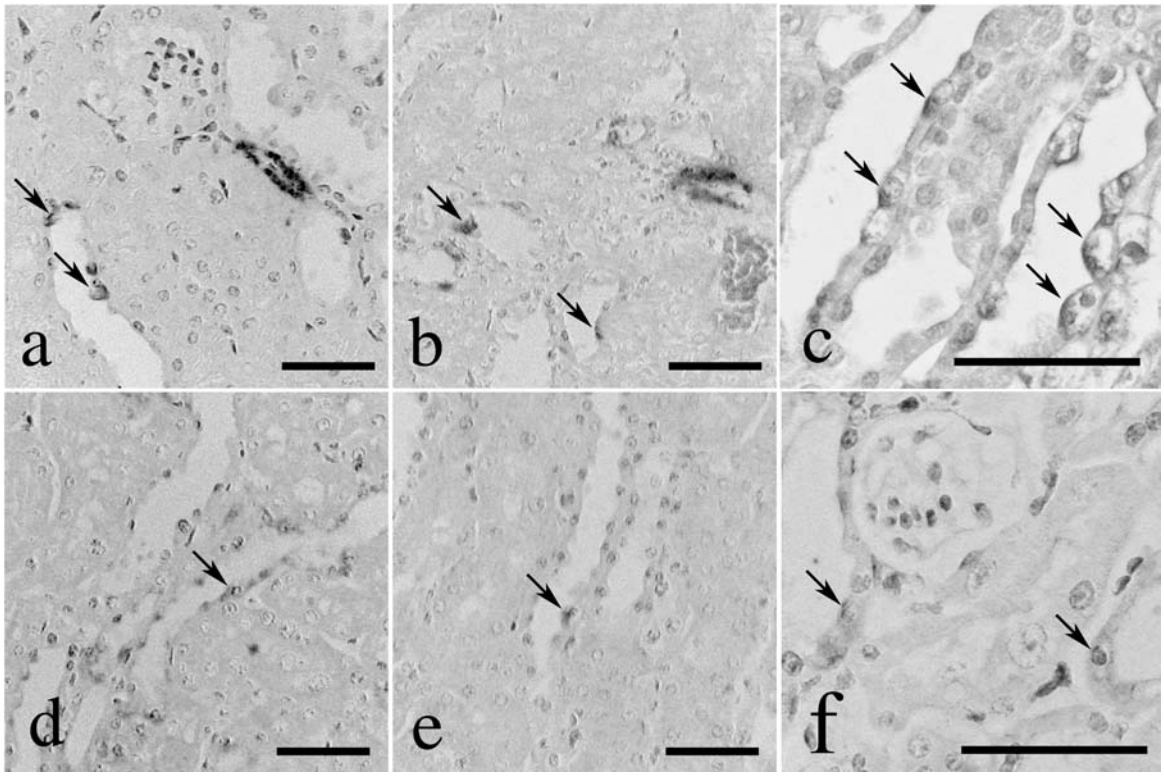


Figure 5. Immunohistochemistry for renin (a, b, c) and angiotensin II (d, e, f) after STZ-administration. Both reactivities are detected on the epithelial cells of distal tubules and collecting ducts (arrows). a, c, d, f: CBA/N, b, e: CBA/J. a, b: 28 days after STZ-injection, c, d, e, f: 56 days after STZ-injection. Bar, 50μm.

any experimental period. *Ace* mRNA expression was increased on the glomerulus at 28 days in CBA/J, but not in CBA/N mice; on the other hand, it was decreased on cortical tubules at 56 days in CBA/N, but not in CBA/J mice. Expression of *Agtr1* mRNA in the cortical tubule area was decreased during the experimental period in both strains, whereas it was slightly increased on the glomerulus in CBA/J, but not in CBA/N. No expression of *Agtr 2* mRNA was detected in either strain in DN. These results suggest that RAS activation in DN is totally higher in CBA/J, *Ren1 Ren2* mice than in CBA/N, *Ren1* mice.

## Discussion

*Strain difference in STZ-induced diabetes*

It has been reported that there is a mouse strain difference in STZ-induced diabetes[32]. In C57BL/KsJ mice, constant hyperglycemia occurred at lower doses of STZ (140mg/kg); on the other hand, BALB/cJ, DBA/2J, AKR/J, CBA/J and C3H/HeJ mice showing more resistance to STZ-injection did not become consistently hyperglycemic until doses of 300mg/kg were given. Additionally, it has been reported that C57BL/6J mice are more sensitive to STZ than BALB/c mice as judged by the extent of pancreatic insulin depletion and β-cell death in vivo and *in vitro*[4]. In the present study, to compare the effects on inbred *Ren1* and *Ren1 Ren2* strains, we chose CBA/N and CBA/J mice as STZ-resistant models, so as to examine the possible effects due to the difference of the duplicated renin gene. The clinical estimation showed that the decrease of body

Incidence of expression in RAS-related genes with LMD-RT nested PCR

| | | CBA/N | | | | | CBA/J | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Sham control | n=3 | Area1 | Area2 | Area3 | Area4 | n=3 | Area1 | Area2 | Area3 | Area4 |
| *Renin* | | 1 | 2 | 0 | 2 | | 3 | 3 | 0 | 1 |
| *Agt* | | 0 | 0 | 0 | 0 | | 0 | 0 | 0 | 0 |
| *Ace* | | 3 | 1 | 3 | 1 | | 3 | 0 | 3 | 1 |
| *Agtr 1* | | 0 | 2 | 2 | 3 | | 0 | 2 | 2 | 3 |
| *Agtr 2* | | 0 | 0 | 1 | 1 | | 0 | 1 | 0 | 0 |
| 28 days after STZ | n= 5 | | | | | n= 5 | | | | |
| *Renin* | | 2 | 4 | 1 | 0 | | 4 | 5 | 0 | 3 |
| *Agt* | | 0 | 0 | 2 | 0 | | 0 | 0 | 5 | 4 |
| *Ace* | | 4 | 1 | 4 | 1 | | 5 | 3 | 5 | 1 |
| *Agtr 1* | | 0 | 2 | 2 | 1 | | 2 | 4 | 5 | 0 |
| *Agtr 2* | | 0 | 0 | 0 | 0 | | 0 | 0 | 0 | 0 |
| 56 days after STZ | n= 5 | | | | | n= 5 | | | | |
| *Renin* | | 3 | 2 | 0 | 0 | | 4 | 3 | 0 | 3 |
| *Agt* | | 2 | 0 | 2 | 2 | | 1 | 0 | 4 | 2 |
| *Ace* | | 4 | 1 | 0 | 1 | | 5 | 2 | 4 | 2 |
| *Agtr 1* | | 0 | 1 | 0 | 0 | | 1 | 2 | 2 | 1 |
| *Agtr 2* | | 0 | 0 | 0 | 0 | | 0 | 0 | 0 | 0 |

Numerical values show the numbers of positive samples in each LMD-RT nested PCR. Area 1 means the arterial area of the cortex containing the arcuate and interlobular arteries. Area 2 means the glomerular region. Area 3 means the tubular area of the cortex containing proximal and distal tubules and cortical collecting ducts. Area 4 means the tubular area of the inner layer of the outer medulla containing proximal straight tubules, the nephron loop and medullary collecting ducts.

weight by acute hypoinsulinemia and the increase of kidney weight ratio by hypertrophy was more intense in CBA/N than in CBA/J, suggesting the most representative strain difference between the two animals due to duplication of the renin gene, although they both are classified as STZ-resistant strains.

In humans, type 1 diabetes is a T-cell-mediated autoimmune disease, which is determined by a combination of genetic and environmental factors[11]. The genetic factors associated with islet antigens have been subjected to more intensive study and two genetic regions of major importance have been identified : the human leukocyte antigen locus and the insulin gene[15]. In the present study, both strains had the same haplotype, H-$2^k$, and showed similar β-cell damage after STZ-injection. These results reveal the importance for diagnosis and therapy of diabetes to find new genetic factors by analysis of the present mouse strains.

*Pathological changes in DN*

Histological changes in DN were already been reported in the early 1970s. DN is characterized by discrete structural alterations, including renal hypertrophy, thickening of basement membranes, and progressive glomerular accumulation of extracellular matrix

components[23,27]. The development of irreversible renal changes in diabetes mellitus occurrs in the glomerular and tubular compartments, finally showing glomerulosclerosis and tubulointerstitial fibrosis[26,35,39]. These changes are always preceded by early hypertrophy[10]. In rats, structural alterations of the macula densa and the proximal tubules have been demonstrated in the early phase of STZ-administration[31,41]. In ICR-derived mouse strains, which are known to be STZ-susceptible, glomerular changes are characterized by marked glomerular hypertrophy, an increase in the mesangial matrix and glomerular segmental sclerosis[38]. In NOD mice derived from the ICR strain, renal hypertrophy and slight glomerular injury were observed in early stages and structural alteration of the proximal straight tubules were followed at later stages during the acute phase of diabetes[21].

In the present study, renal lesions were newly detected at distal tubules and collecting ducts that showed flattened epithelial cells, as well as the previously reported alterations including slight hypertrophy of the glomerulus and a decrease of PAS staining in the brush borders of proximal straight tubules. These results suggest that renal alteration in STZ-resistant mice or in the CBA strain might be characterized by changes in distal tubules and collecting ducts, which have not been reported in any experimental models.

*Differences of RAS expression between CBA/N and CBA/J mice*

In the present study, CBA/J mice showed slow expression of renin and angiotensin II and then light renal damage, although RAS activation occurred relatively early during the experimental period. On the other hand, CBA/N had immediate and high expression of renin and angiotensin II and then marked renal damage, although RAS activation was relatively late compared to CBA/J. Many cytokines, including angiotensin II, respond to high glucose circumstances and induce TGF-β, which activates proliferation of mesangial extracellular matrix and tubular hypertrophy in renal injury[39]. This pathway from angiotensin II to renal injury might function similarly in both CBA/N and CAN/J, because the extent of renal damage corresponded with the expression of angiotensin II in both strains. It is unclear how *Ren2* associates with the pathway from RAS activation into angiotensin II production in DN. The renin precursor, prorenin, is produced in blood circulation as is active renin[30]. Additionally, a transgenic rat carrying mouse *Ren2* has a high prorenin concentration in systemic circulation[24]. Circulating active renin that binds to the renin receptor has a strong activity for production of angiotensin I, followed by production of angiotensin II by ubiquitous Ace, whereas prorenin shows relatively weaker activity for angiotensin I production than renin[25]. It might be speculated that prorenin has an antagonistic effect for production of angiotensin I when there is a high level of prorenin in the kidney as well as in blood circulation, resulting in prevention of renal damage in the early DN stage. Additionally, the chymase family, of which many subtypes exist in mice[13,20], might play some important roles, when it is upregulated in DN, directly converting Agt into angiotensin II in different ways in CBA/N and CBA/J.

In any case, the results demonstrating new expression of renin and angiotensin II in distal tubules suggest that intrarenal RAS functions via an autocrine/paracrine mechanism, playing an important role in the onset of various renal pathological changes.

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