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A Novel Diabetes Mellitus Mouse Model, MAFA-Deficient and Beta Cell-Specific MAFK-Overexpressing Hybrid Transgenic Mice, Developed Severe Diabetic Nephropathy and Improved with TCV-116 (Candesartan Cilexetil) Treatment

Akiko FUJITA¹⁾, Keigyou YOH¹⁾, Homare SHIMOHATA²⁾, Naoki MORITO¹⁾, Masami OJIMA³⁾, Midori OKAMURA³⁾, Satoru TAKAHASHI³⁾, and Kunihiro YAMAGATA¹⁾

¹⁾Department of Nephrology, Division of Clinical Medicine and ³⁾Anatomy and Embryology, Division of Biomedical Science, Faculty of Medicine, University of Tsukuba, 1–1–1 Tennodai, Tsukuba, Ibaraki 305-8575 and ²⁾Department of Nephrology, Tokyo Medical University Ibaraki Medical Center, 3–20–1 Chuo, Ami Inashiki, Ibaraki 300-0395, Japan

Abstract: Many models of diabetic nephropathy have been reported. However, it is rare that the characteristic findings of severe human diabetic nephropathy, such as diffuse, nodular, and exudative lesions, are all detected in one model mouse. Previously, we reported that MAFA-deficient and beta cell-specific MAFK-overexpressing hybrid transgenic (*Mafa*^{-/-}*Mafk*⁺) mice develop diabetes mellitus and, after uninephrectomy, demonstrate these characteristic lesions. In this study, we administered TCV-116 (candesartan cilexetil) to *Mafa*^{-/-}*Mafk*⁺ mice after uninephrectomy and examined whether TCV-116 ameliorated the diabetic nephropathy. We also evaluated the utility of these mice as a model for developing treatments for diabetic nephropathy. We performed uninephrectomy of the *Mafa*^{-/-}*Mafk*⁺ mice at 8 weeks old. We then divided these mice into two groups as follows: 1) an untreated group and 2) a group treated with TCV-116 at 5 µg/g/day from 10 to 20 weeks. TCV-116 treatment did not affect serum glucose levels. However, in the treated group, urinary protein excretion, mesangial matrix expansion, enlargement of the kidney, and glomerular surface area were all improved relative to untreated mice. Oxidative stress is known to be increased in diabetic nephropathy and to be suppressed by TCV-116. The urinary level of 8-OHdG, an oxidative stress marker, at 20 weeks was lower in the TCV-116-treated group than in the untreated group. From these results, we concluded that the *Mafa*^{-/-}*Mafk*⁺ mouse is a useful model to analyze diabetic nephropathy and a useful tool for the development of new drugs to treat diabetic nephropathy.

Key words: anti angiotensin II receptor blockers, diabetic nephropathy model mouse, renin angiotensin system

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Address corresponding: K. Yamagata, Department of Nephrology, Division of Clinical Medicine, Faculty of Medicine, University of Tsukuba, 1–1–1 Tennodai, Tsukuba, Ibaraki 305-8575, Japan

Introduction

Diabetic nephropathy is a major complication of diabetes mellitus and is also the leading cause of end-stage renal disease [7]. Mouse models of diabetes mellitus are essential experimental tools for investigating the mechanisms by which diabetic nephropathy develops. A variety of mouse models of diabetic nephropathy have been reported, such as streptozotocin-induced mice [15], Akita mice [16], NOD mice [32], *ob/ob* mice [31], and *db/db* mice [26]. However, the major deficiency in animal models of diabetic nephropathy is the absence of severe human-like diabetic histopathological findings [4]. Previously, we have reported that MAFA [v-maf musculoaponeurotic fibrosarcoma oncogene family, protein A (avian)]-deficient and beta cell-specific MAFK [v-maf musculoaponeurotic fibrosarcoma oncogene family, protein K (avian)]-overexpressing hybrid transgenic (*Mafa*^{-/-}*Mafk*⁺) mice [27] develop diabetes mellitus at a young age, and after uninephrectomy, these mice demonstrate three characteristics of human diabetic nephropathy: namely, diffuse, nodular, and exudative lesions. *Mafa*^{-/-}*Mafk*⁺ mice are expected to be a useful tool for analyzing the development and treatment of diabetic nephropathy.

MAFA is a member of the large Maf family of transcription factors [2]. The *Mafa* gene is expressed in pancreatic beta cells, and its level of expression and the DNA binding activity of the resulting MAFA protein depend on the serum glucose concentration [10, 11]. We generated MAFA-deficient mice and determined that MAFA is a key regulator of glucose-stimulated insulin secretion, insulin transcription, and the maintenance of pancreatic islet structure *in vivo* [35]. MAFB and c-MAF are the two other members of the large Maf family, and they are also expressed in pancreatic beta cells [10, 12, 17, 21]. These transcription factors might activate the expression of the insulin gene. MAFK is a small Maf transcription factor and acts as a dominant negative protein to suppress the effect of the large Maf proteins [9]. In *Mafa*^{-/-}*Mafk*⁺ mice, the MAFA protein is absent, and the transcriptional activity of the other large Maf proteins is repressed in beta cells, which leads to the development of overt severe diabetes mellitus [27, 28].

The major therapeutic approaches that have been in-

vestigated for diabetic nephropathy include intensive glycemic control, restriction of dietary protein, and antihypertensive treatment involving inhibition of the renin angiotensin system (RAS) [23]. Many pathophysiological, genetic, and inflammatory issues in diabetic nephropathy have been reported to be related to RAS. There have been many reports that the inhibition of RAS with inhibitors of angiotensin converting enzyme or blockers of the angiotensin II receptor suppresses the progress of albuminuria and/or proteinuria in both experimental and clinical diabetic nephropathy [3, 7, 24].

In this study, we administered the angiotensin II receptor blocker TCV-116 (candesartan cilexetil) to *Mafa*^{-/-}*Mafk*⁺ mice after uninephrectomy and examined whether TCV-116 ameliorated diabetic nephropathy in these mice. We also evaluated the utility of these mice as a model for developing new drugs to treat diabetic nephropathy.

Materials and Methods

Animals

We generated *Mafa*^{-/-}*Mafk*⁺ mice as described previously [27]. All mice used had the ICR genetic background and were 8-week-old males. We performed uninephrectomy of the *Mafa*^{-/-}*Mafk*⁺ mice at 8 weeks old (W) by ablating the left renal artery and ureter. *Mafa*^{+/+} mice were used as wild-type (WT) mice (n=6). WT mice that had not undergone uninephrectomy were used as controls. Mice were fed a normal diet that comprised a commercial laboratory chow (MF, Oriental Yeast Co., Ltd., Tokyo, Japan) and were maintained under specific pathogen-free conditions in the Laboratory Animal Resource Center of the University of Tsukuba. All experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals at the University of Tsukuba, and the study was approved by the Institutional Review Board of the university.

Drug

The angiotensin II type 1 receptor antagonist, TCV-116, was synthesized by Takeda Chemical Industries, Ltd. (Osaka, Japan). After uninephrectomy, the *Mafa*^{-/-}*Mafk*⁺ mice were divided into two groups as follows: the AK group (n=6), which received no treatment, and the

TAK group (n=6), which was treated with TCV-116. The TAK group received TCV-116 (5 μ g/kg/day) given in drinking water from 10 to 20 weeks of age. Owing to the fact that the *Mafa*^{-/-}*Mafk*⁺ mice developed hyperglycemia, these mice showed polyuria and drank substantially more than 10 ml of water/day. The TCV-116 (5 μ g/kg/day) was administered in 5 ml of drinking water every day. After the mice had emptied the drink bottle, we refilled it with fresh water that did not contain TCV-116. By this approach, we could be sure that the mice received the exact dose of TCV-116. Vehicle, which comprised ethanol (0.1%, v/v), polyethylene glycol (0.1%, v/v), and sodium bicarbonate (5 mmol/l), was added to the TCV-116 to increase its solubility in water. The WT mice (n=6) did not receive TCV-116.

Analysis of urinary protein excretion, urinary excretion of creatinine, and renal serological assays

The urine of each mouse was collected over a 24-h period during housing in an individual metabolic cage. The amount of proteinuria was assessed by measuring the turbidity of the urine upon the addition of 3% sulphosalicylic acid. The urinary excretion of creatinine was measured using a LabAssayTM Creatinine kit (Wako, Osaka, Japan). Blood samples were collected from the tail vein of the mice. The concentrations of serum creatinine and fed glucose were measured using a DRI-CHEM 3500 automated analyzer for routine laboratory tests (Fujifilm Corporation, Tokyo, Japan).

Measurement of systolic blood pressure

Systolic blood pressure (SBP) was measured with a noninvasive tail cuff and pulse transducer system (BP-98A; Softron, Tokyo, Japan).

Histopathological analysis

Kidney tissues were fixed with 10% formalin and embedded in paraffin. Sections were stained with periodic acid-Schiff (PAS) and Masson's trichrome (MT). For histological analysis, the glomerular surface area of 30 glomeruli in each kidney section was measured using a BIOREVO BZ-9000 fluorescence microscope (Keyence, Osaka, Japan), and the mean glomerular surface area was determined.

Measurement of urinary levels of 8-hydroxy-2-deoxyguanosine

The urinary excretion of 8-hydroxy-2-deoxyguanosine (8-OHdG) was measured by competitive enzyme-linked immunosorbent assay using a commercially available kit, New 8-OHdG Check (Japan Institute for the Control of Aging, Shizuoka, Japan).

Statistical analysis

All data are expressed as means \pm SEM. Multiple data comparisons were performed by using one-way analysis of variance (ANOVA) with post hoc analysis (StatView 5.0, SAS Institute Inc., Cary, NC, USA). *P* values <0.05 were considered statistically significant.

Results

Effects on serum glucose, body weight, and blood pressure

The mean serum glucose levels of the AK and TAK mice were significantly higher than those of the WT mice throughout the observation period [1151.6 \pm 47.2 mg/dl (10 W) and 1320.0 \pm 113.4 mg/dl (20 W) for AK mice; 1124.5 \pm 65.0 mg/dl (10 W) and 1369.8 \pm 35.4 mg/dl (20 W) for TAK mice; 173.0 \pm 8.2 mg/dl (10 W) and 159.8 \pm 38.3 mg/dl (20 W) for WT mice; Fig. 1A]. There was no significant difference in mean body weight between the AK and TAK mice. However, the mean body weights of the AK and TAK mice were significantly lower than those of the WT mice [24.4 \pm 1.2 g (10 W) and 27.4 \pm 1.5 g (20 W) for AK mice; 27.1 \pm 1.0 g (10 W) and 29.6 \pm 1.7 g (20 W) for TAK mice; 35.4 \pm 1.0 g (10 W) and 41.2 \pm 1.9 g (20 W) for WT mice; Fig. 1B]. Systolic blood pressure in the TAK mice tended to decrease after TCV-116 treatment but was not significantly lower than that of the AK mice at 20W (119.5 \pm 3.8 mmHg (10 W) and 115.4 \pm 9.4 mmHg (20 W) for AK mice; 118.6 \pm 4.5 mmHg (10 W) and 101.4 \pm 6.3 mmHg (20 W) for TAK mice; 106.7 \pm 5.3 mmHg (10 W) and 119.8 \pm 5.8 mmHg (20 W) for WT mice; Fig. 1C).

Effects on urinary protein and serum creatinine

In the TAK mice, urinary protein excretion decreased from 2.51 \pm 0.60 mg/day at 10 W to 1.48 \pm 0.24 mg/day at 20 W. In contrast, urinary protein excretion in the AK

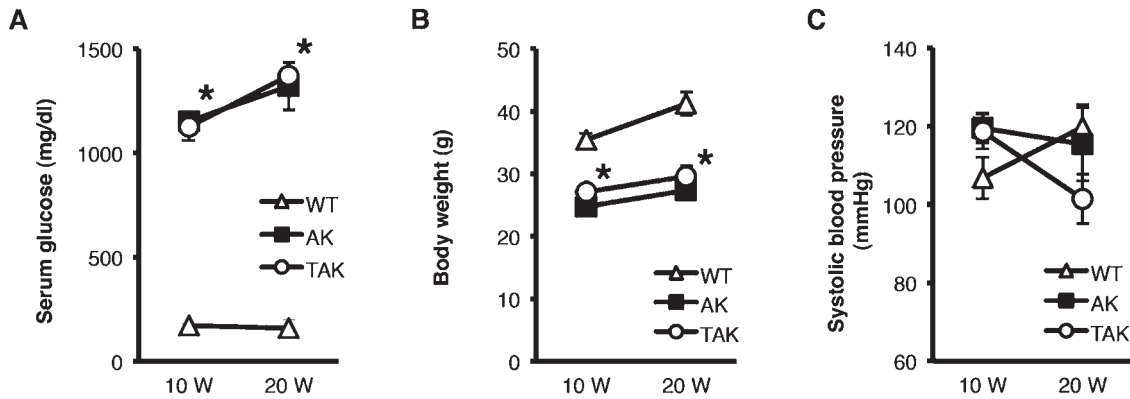


Fig. 1. Serum glucose (A), body weight (B), and systolic blood pressure (C) in wild-type (WT), *Mafa*^{-/-}*Mafk*⁺ (AK), and TCV-116-treated *Mafa*^{-/-}*Mafk*⁺ (TAK) mice. Open triangles, closed squares, and open circles indicate WT mice, AK mice, and TAK mice, respectively. Each bar represents the mean \pm SEM (* P <0.01, AK mice and TAK mice vs. WT mice). W, weeks old.

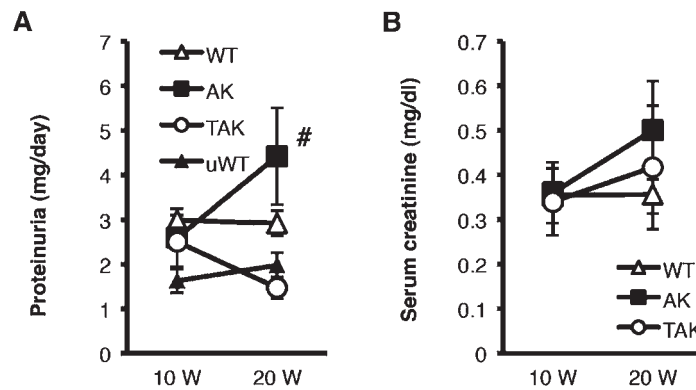


Fig. 2. Urinary protein (A) and serum creatinine (B) in wild-type (WT), *Mafa*^{-/-}*Mafk*⁺ (AK), and TCV-116-treated *Mafa*^{-/-}*Mafk*⁺ (TAK) mice. Open triangles, closed squares, and open circles indicate WT mice, AK mice, and TAK mice, respectively. Closed triangles indicate WT mice that had undergone uninephrectomy (uWT). Each bar represents the mean \pm SEM (# P <0.01, AK mice vs. TAK mice and uWT mice). W, weeks old.

group increased from 2.60 ± 0.66 mg/day (10 W) to 4.42 ± 1.09 mg/day (20 W). Urinary protein excretion in the TAK mice at 20 weeks of age was significantly lower than that in the AK mice (Fig. 2A). The level of proteinuria in the TAK mice was also lower than that in the WT mice [2.99 ± 0.13 mg/day (10 W), 2.92 ± 0.28 mg/day (20 W)], but the difference was not significant. We also examined the level of proteinuria in WT mice that had undergone uninephrectomy [$n=4$, 1.63 ± 0.27 mg/day (10 W), 1.98 ± 0.28 mg/day (20 W)]. There were no significant differences in the levels of proteinuria

between WT mice that had undergone uninephrectomy and TAK mice. In the analysis of renal function, the serum creatinine level in the TAK mice increased less than in the AK mice, but it was not significantly lower in the TAK mice than in the AK mice at 20 W [0.36 ± 0.07 mg/dl (10 W) and 0.50 ± 0.11 mg/dl (20 W) for AK mice; 0.34 ± 0.08 mg/dl (10 W) and 0.42 ± 0.14 mg/dl (20 W) for TAK mice; 0.35 ± 0.01 mg/dl (10 W) and 0.36 ± 0.04 mg/dl (20 W) for WT mice; Fig. 2B].

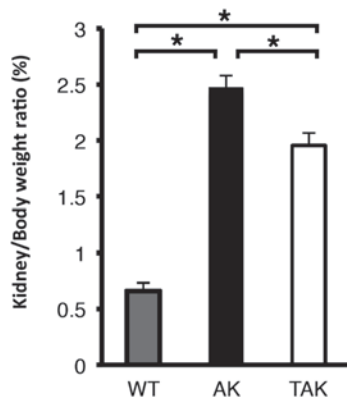


Fig. 3. The ratio of kidney weight to body weight in wild-type, *Mafa*^{-/-}*Mafk*⁺, and TCV-116-treated *Mafa*^{-/-}*Mafk*⁺ mice at 20 weeks old (**P*<0.01).

Effect on renal size

The mean single kidney weight to body weight ratio of the WT mice was $0.67 \pm 0.07\%$. The kidney size of the AK mice was enlarged at 20 W (a ratio of $2.47 \pm 0.11\%$). In contrast, the kidney weight to body weight ratio of the TAK mice at 20 W was $1.96 \pm 0.11\%$, and it was significantly lower than that of the AK mice (Fig. 3).

Histopathology

Next, we performed histological analysis of kidneys from 20-week-old AK, TAK, and WT mice. Glomerular hypertrophy, mesangial matrix expansion, and nodular lesions were observed in PAS-stained kidney sections from AK mice (Fig. 4C and 4D). However, mesangial matrix expansion was less obvious in the TAK mice (Fig. 4E and 4F). Furthermore, no nodular lesions were observed in the TAK mice. The mean glomerular surface area of the TAK mice was significantly smaller than that of the AK mice ($5071.5 \pm 176.8 \mu\text{m}^2$ for AK mice, $3855.8 \pm 20.8 \mu\text{m}^2$ for TAK mice, $3064.2 \pm 141.5 \mu\text{m}^2$ for WT mice; Fig. 5). The MT-stained sections showed that the amount of fibrosis was less in the TAK mice than in the AK mice (Fig. 4G and 4H).

Effect of Urinary 8-OHdG

We also evaluated oxidative stress by measuring the urinary excretion of 8-OHdG, which is accepted as a sensitive marker for oxidative DNA damage *in vivo*. In

the AK mice, urinary excretion of 8-OHdG increased from 380.3 ± 104.1 ng/day at 10 W to 523.9 ± 72.7 ng/day at 20 W. In contrast, the urinary levels of 8-OHdG in the TAK group decreased from 378.6 ± 41.4 ng/day at 10 W to 189.0 ± 25.3 ng/day at 20 W (Fig. 6).

Discussion

A variety of mouse models provide valuable insight into the development of diabetic nephropathy [4]. They are also expected to serve as a useful platform for testing novel therapeutic strategies. In general, diabetic nephropathy in humans is a late complication of diabetes, occurring in susceptible patients after 10–20 years of diabetes [30]. Consequently, mouse models of diabetes need to be maintained for extended periods after the onset of hyperglycemia, usually 3–8 months, in order to detect diabetic nephropathy [30]. The major limitations in many mouse models are a lack of observed hypertension and the absence of advanced pathological lesions [4, 30]. Previously, we have reported that *Mafa*^{-/-}*Mafk*⁺ mice that have not undergone uninephrectomy develop severe overt diabetes mellitus by 5 weeks old and show higher levels of proteinuria and serum creatinine at 10 weeks old than WT mice [27]. Furthermore, AK mice, which have undergone uninephrectomy, demonstrate advanced pathological lesions of diabetic nephropathy within 20 weeks [27]. In this study, we tried to ascertain whether AK mice might also be a useful model for testing drug of treatments for diabetic nephropathy. In the present study, the progression of nephropathy in AK mice was significantly suppressed by treatment with TCV-116. The results suggested that AK mice are not only a good model of diabetic nephropathy but also might be a useful tool for analysis of treatments for diabetic nephropathy.

Both AK mice and TAK mice developed diabetes mellitus, and there were no significant differences between the two groups with respect to serum glucose levels and body weight during the observation period (Fig. 1A and 1B). Some studies have reported that TCV-116 might improve glucose levels [1, 25]. However, there were no significant differences in serum glucose levels between the AK and TAK mice. In this study, we could not detect any beneficial effects of TCV-116 treatment on glucose

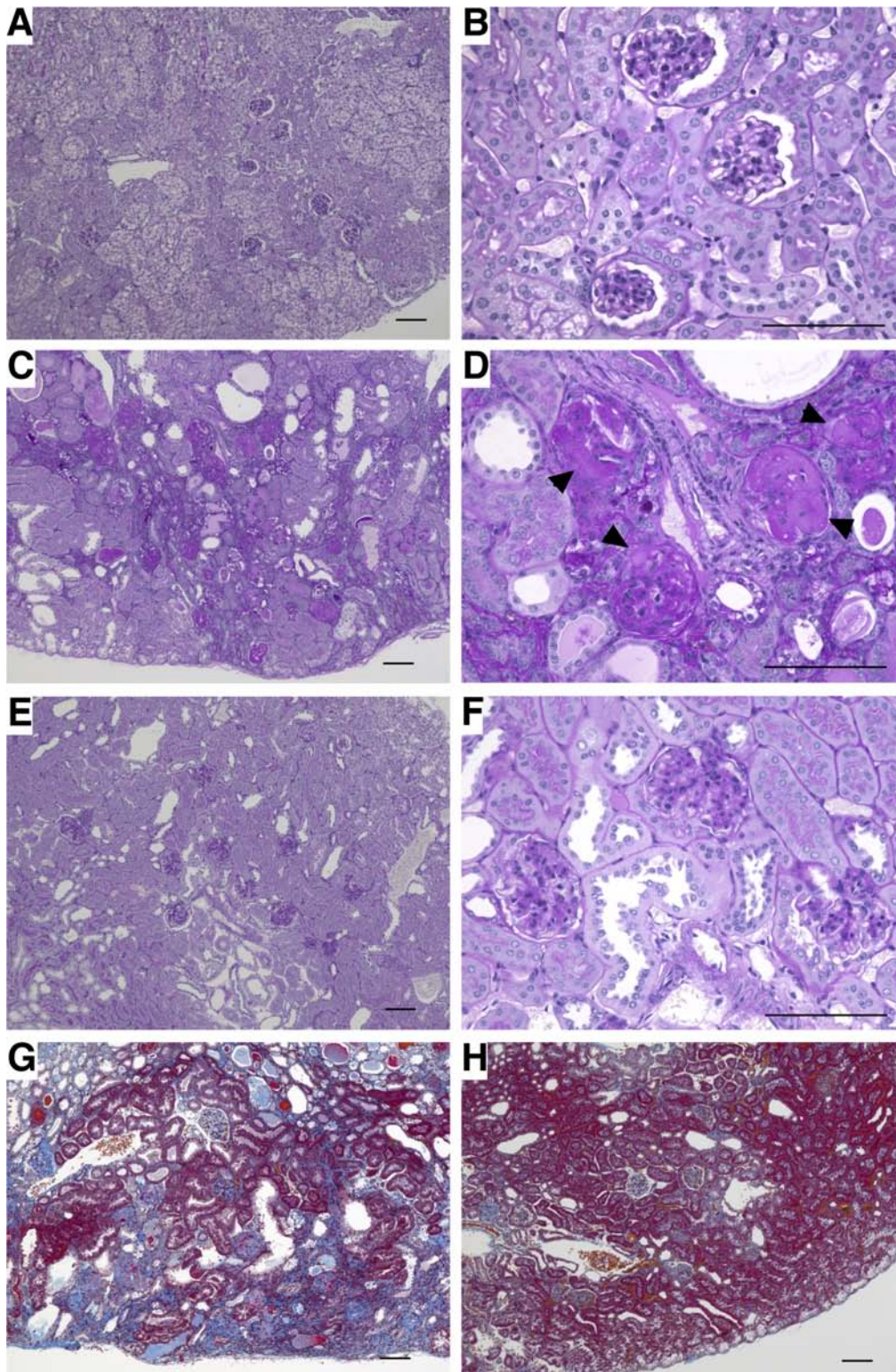


Fig. 4. Histological appearance of kidney sections at 20 weeks old. Wild-type (WT) mice (A, B), *Mafa*^{-/-}*Maflk*^{+/+} (AK) mice (C, D, G), and TCV-116-treated *Mafa*^{-/-}*Maflk*^{+/+} TAK mice (E, F, H). Periodic acid-Schiff (PAS) (A, C, E, ×100; B, D, F, ×400) and Mas-son's trichrome (MT) (G, H, ×100) staining. Glomerular hypertrophy, mesangial matrix expansion, and nodular lesions were observed in PAS-stained kidney sections from AK mice (C and D). The arrowheads show nodular lesions. In the MT-stained sections, the blue area, which shows fibrosis, was smaller in the TAK mice (H) than in the AK mice (G). Scale bar, 100 μ m.

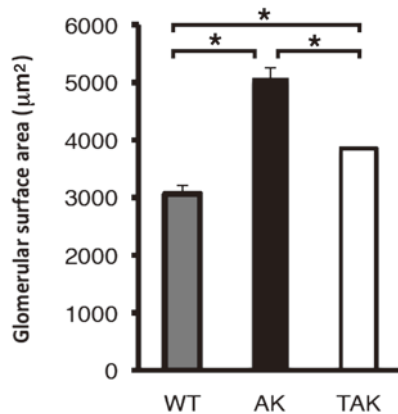


Fig. 5. Histological analysis of glomerular surface area in wild-type (WT), *Mafa*^{-/-}*Mafk*⁺ (AK), and TCV-116-treated *Mafa*^{-/-}*Mafk*⁺ (TAK) mice. The mean glomerular surface area of the AK group was significantly larger than those of the other groups. After TCV-116 treatment, the glomerular surface area was significantly decreased in the TAK group. Data are means \pm SEM. * P <0.01.

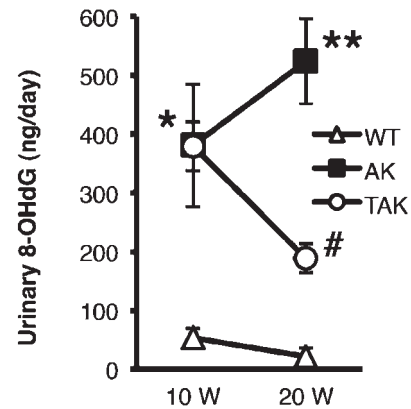


Fig. 6. Urinary 8-OHdG levels in wild-type (WT), *Mafa*^{-/-}*Mafk*⁺ (AK), and TCV-116-treated *Mafa*^{-/-}*Mafk*⁺ (TAK) mice. Open triangles, closed squares, and open circles indicate WT mice, AK mice, and TAK mice, respectively. The urinary 8-OHdG level in the TAK mice was significantly decreased relative to that in the AK mice at 20 weeks old. Data are means \pm SEM. * P <0.01, AK mice and TAK mice vs. WT mice; ** P <0.01, AK mice vs. TAK mice and WT mice; # P <0.05, TAK mice vs. WT mice. W, weeks old.

levels in TAK mice. The mean blood pressure of the TAK mice, which were treated with TCV-116, was lower than that of the AK mice at 20 W, but the difference was not significant. In contrast, the level of proteinuria in the TAK mice was significantly lower than that in the AK mice at 20 W. In the present study, treatment with TCV-116 (5 μ g/kg/day) was not sufficient to reduce blood pressure but did decrease proteinuria due to diabetic nephropathy effectively. The level of protein in the urine of the WT mice did not change from 10 to 20 weeks of age, and the proteinuria of the WT mice at 20 W was higher than that of the TAK mice. We also examined the levels of proteinuria in WT mice that had undergone uninephrectomy and found that the level of proteinuria was 1.63 ± 0.27 mg/day at 10 W and 1.98 ± 0.28 mg/day at 20 W. These results indicated that, in the TAK mice, the level of proteinuria was improved nearly to that of WT mice that had undergone uninephrectomy. The histological analysis showed that the AK mice developed severe diabetic nephropathy, with nodular lesions, mesangial matrix expansion, and fibrosis. In contrast, the TAK mice did not show severe nephropathy (Fig. 4). It is also known that kidney size may be enlarged in diabetic nephropathy [18]. In the present study,

the kidney size of the AK mice was enlarged (Fig. 3). Thus, the improvement of diabetic nephropathy in the TAK mice was also confirmed by the results of the histological and kidney size analyses.

Treatments that inhibit RAS suppress the progress of diabetic nephropathy in both animal models and humans [3, 7, 24]. The RAS system regulates renal vasomotor activity, maintains optimal salt and water homeostasis, and modifies cellular proliferation in the nephron [3]. On the basis of the homeostatic effects of the RAS system on the kidney, treatments that inhibit RAS have been reported to protect against glomerular capillary hypertension, proteinuria, and renal fibrosis [20, 29, 34]. In the current study, treatment of AK mice with the angiotensin II receptor blocker TCV-116 was effective in ameliorating proteinuria (Fig. 2), renal fibrosis (Fig. 4), and kidney enlargement (Fig. 3).

Diabetes introduces multiple sources of oxidative stress, and the stress factors appear early in the disease and worsen as the disease progresses [19, 33]. Increased oxidative stress in diabetes mellitus has been implicated in the pathogenesis of diabetic nephropathy [5, 8, 14].

It is also known that angiotensin II in the kidney generates reactive oxygen species [3, 7]. TCV-116 has been reported to have antioxidant activity [6, 22], and 8-OHdG is known as a sensitive marker for oxidative DNA damage *in vivo*. In the current study, the urinary levels of 8-OHdG in the AK and TAK mice were higher than those in the WT mice at 10 W. Hyperglycemia induces oxidative stress by various mechanisms [13, 33]. The higher urinary levels of 8-OHdG in the AK and TAK mice at 10 W might be induced by hyperglycemia. The AK and TAK mice displayed hyperglycemia at 20 W. However, the urinary level of 8-OHdG in the TAK mice was significantly lower than that of the AK mice at 20 W. This result suggested that TCV-116 treatment was effective in decreasing oxidative stress in TAK mice. It is also very interesting to consider the relationships of oxidative stress and RAS with MAFA and MAFK. However, it is unknown whether MAFA and/or MAFK increases oxidative stress or its effects on RAS. Further studies to define these issues may clarify the mechanisms that are responsible for the development of diabetic nephropathy.

In this study, we showed that, after uninephrectomy, *Mafa*^{-/-}*Mafk*⁺ mice developed severe diabetic nephropathy, which improved upon treatment with TCV-116. From these results, we concluded that the mouse model of diabetic nephropathy, namely *Mafa*^{-/-}*Mafk*⁺ mice subjected to uninephrectomy, is a useful model for analysis of diabetic nephropathy and a useful tool for development of new drugs that target this disease.

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