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Strict angiotensin blockade prevents the augmentation of intrarenal angiotensin II and podocyte abnormalities in type 2 diabetic rats with microalbuminuria

Akira Nishiyama^{a,b}, Toshitaka Nakagawa^c, Hiroyuki Kobori^d, Yukiko Nagai^c, Noriyuki Okada^e, Yoshio Konishi^e, Takashi Morikawa^e, Michiaki Okumura^e, Isseiki Meda^e, Hideyasu Kiyomoto^{b,f}, Naohisa Hosomi^f, Takefumi Mori^g, Sadayoshi Ito^g, and Masahito Imanishi^e

*a*Department of Pharmacology, Kagawa University Medical School, Kagawa, Japan

*b*Hypertension and Kidney Disease Research Center, Kagawa University Medical School, Kagawa, Japan

*c*Life Sciences Research Center, Kagawa University Medical School, Kagawa, Japan

*d*Department of Physiology, Hypertension and Renal Center of Excellence, Tulane University Health Sciences Center, New Orleans, Louisiana, USA

*e*Department of Internal Medicine, Osaka City General Hospital, Osaka

*f*Department of CardioRenal and Cerebrovascular Medicine, Kagawa University Medical School, Kagawa

*g*Department of Medicine, Tohoku University Graduate School of Medicine, Sendai, Japan

Abstract

Objectives—Beneficial effects of angiotensin II type 1 receptor blockers have been indicated for patients with diabetic nephropathy. We investigated the effects of an angiotensin II type 1 receptor blocker, telmisartan, on intrarenal angiotensin II levels and the progression of albuminuria or glomerular injury in type 2 diabetic Otsuka Long–Evans Tokushima Fatty rats with microalbuminuria.

Methods and Results—Otsuka Long–Evans Tokushima Fatty rats were randomly treated with telmisartan (10 mg/kg/day, orally), hydralazine (25 mg/kg/day in drinking water) or vehicle from the initiation of albuminuria (13 weeks old). At this age, Otsuka Long–Evans Tokushima Fatty rats showed low but detectable albuminuria (1.0 ± 0.1 mg/day) and higher systolic blood pressure, postprandial blood glucose and kidney angiotensin II levels than age-matched nondiabetic Long–Evans Tokushima Otsuka rats. At 35 weeks of age, vehicle-treated Otsuka Long–Evans Tokushima Fatty rats did not show apparent glomerular injury or tubulointerstitial fibrosis but did exhibit severe albuminuria (72.6 ± 5.9 mg/day) and accumulation of cytoplasmic granules containing albumin in podocytes. Otsuka Long–Evans Tokushima Fatty rats also showed higher systolic blood pressure, postprandial blood glucose, collagen gene expression, desmin staining (a marker of podocyte injury) and angiotensin II levels than Long–Evans Tokushima Otsuka rats. Treatment with telmisartan did not affect postprandial blood glucose but decreased systolic blood pressure, collagen gene expression, desmin staining and angiotensin II levels. Telmisartan also prevented the development of albuminuria (0.6 ± 0.1 mg/day at 35 weeks old) and accumulation of cytoplasmic granules. Hydralazine treatment

Correspondence to Akira Nishiyama, MD, PhD, Department of Pharmacology, Kagawa University Medical School, 1750-1 Ikenobe, Miki-cho, Kita-gun, Kagawa 761-0793, Japan, Tel: +81 87 898 5111 (ext. 2502); fax: +81 87 891 2126; e-mail: akira@kms.ac.jp.

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resulted in a similar reduction in systolic blood pressure and partially attenuated the albuminuria (35.4 ± 1.8 mg/day at 35 weeks old) but did not affect the other parameters.

Conclusion—The present results suggest the contribution of augmented intrarenal angiotensin II levels to the initiation and progression of albuminuria as well as podocyte abnormalities in type 2 diabetic rats. Angiotensin II blockade may inhibit the transition from microalbuminuria to overt nephropathy through prevention of intrarenal angiotensin II augmentation, independently of changes in blood pressure and glucose levels.

Keywords

albuminuria; angiotensin II; Otsuka Long–Evans Tokushima Fatty rat; telmisartan; type 2 diabetes

Introduction

Diabetic nephropathy is a major complication in diabetes mellitus and a leading cause of end-stage renal failure, which causes disabilities and a high mortality rate in patients with this disease [1]. The progression of proteinuria increases the risk for renal [1] and cardiovascular [2] diseases in type 2 diabetes. In the first stages of diabetic nephropathy, low concentrations of albumin are excreted in the urine (microalbuminuria) [1,3]. Recent epidemiological studies have revealed that microalbuminuria is also a risk factor for cardiovascular morbidity and mortality [4,5]. Therefore, prevention of the development of microalbuminuria from an early stage is a key treatment goal for type 2 diabetic nephropathy and associated cardiovascular events.

Although the mechanisms underlying the development of microalbuminuria in diabetes are extremely complex, a potential role of the renin–angiotensin system has been suggested [6–13]. In type 2 diabetic hypertensive patients with microalbuminuria, lowering blood pressure by angiotensin II (AngII) blockade with angiotensin II type 1 (AT_1) receptor blockers (ARBs) or angiotensin-converting enzyme inhibitors (ACEIs) inhibits the transition to overt nephropathy [6–9]. Large-scale clinical trials have also shown that treatment with ARBs or ACEIs is more effective for reducing microalbuminuria than other traditional antihypertensive therapies [6,8–10]. Further studies have demonstrated that ARBs inhibit the transition from microalbuminuria to overt nephropathy in normotensive type 2 diabetic patients [11,12]. Collectively, these data obtained by clinical studies [6,9–12] suggest that the preventive effects of ARBs and ACEIs on the progression of microalbuminuria in type 2 diabetes are independent of their blood pressure-lowering effects. However, whether intrarenal AngII levels are actually augmented at the initiation or during the progression of microalbuminuria in type 2 diabetes or both remains unclear. Moreover, the effects of AngII blockade on intrarenal AngII levels and morphological changes in renal tissues have not been investigated during the transition from microalbuminuria to macroalbuminuria.

In the present study, we aimed to determine whether intrarenal AngII levels are elevated at the initiation or during the progression of microalbuminuria in type 2 diabetic Otsuka Long–Evans Tokushima Fatty (OLETF) rats or both, which exhibit pathological features of renal injury similar to those of human type 2 diabetes [13–15]. We also examined the effects of an ARB, telmisartan, on intrarenal AngII levels as well as the transition from microalbuminuria to macroalbuminuria and renal morphological changes.

Methods

Animals

All experimental procedures were performed according to the guidelines for the care and use of animals established by the Osaka City General Hospital and Kagawa University Medical School. A total of 56 male 7-week-old OLETF rats and 24 age-matched Long–Evans Tokushima Otsuka (LETO) rats (genetic control for OLETF rats) were supplied by Otsuka Pharmaceutical Co. Ltd. (Tokushima, Japan). After obtaining basal measurements, seven LETO and seven OLETF rats were sacrificed at 13 weeks of age. The remaining LETO rats ($n = 17$) were treated with vehicle (0.5% methyl cellulose; Nacalai Tesque, Inc., Kyoto, Japan), while OLETF rats were randomly divided into groups for treatment with telmisartan [10 mg/kg/day; orally (p.o.), $n = 17$], hydralazine (25 mg/kg/day in drinking water; $n = 16$) or vehicle ($n = 16$). The doses of telmisartan and hydralazine were determined on the basis of previous studies on rats [13,16]. At 22 weeks of age, seven rats in each group were sacrificed. The remaining animals were continued to receive their treatment and were sacrificed at 35 weeks of age.

Systolic blood pressure (SBP) was measured in conscious rats by tail-cuff plethysmography (BP-98A; Softron Co., Tokyo, Japan), and 24-h urine samples were collected using metabolic cages. After decapitation, the right renal artery was clamped, and the right kidney was removed. Half of the right kidney was homogenized in cold methanol and processed for measurements of the AngII content [13,17,18], while the other half of the kidney was snap-frozen in liquid nitrogen and stored at -80°C . Then, retrograde perfusion with isotonic saline was performed after cross-clamping the abdominal aorta above the renal arteries and cutting the inferior caval vein near the renal vein. Half of the left kidney was removed and fixed in 10% buffered paraformaldehyde, while the other half of the kidney was perfused with 2.5% glutaraldehyde in 0.1 mol/l phosphate buffer at a pressure of 95–130 mmHg (depending on individual blood pressure).

Real-time reverse transcription-PCR

The mRNA levels of glyceraldehydes-3-phosphate dehydrogenase (GAPDH) and type I and type IV collagens in renal cortical tissues were analyzed by real-time reverse transcription-PCR using a LightCycler FastStart DNA Master SYBR Green I kit (Roche Applied Science, Indianapolis, Indiana, USA), as previously described [13]. All data are expressed as the relative differences between OLETF and LETO rats after normalization to the corresponding GAPDH expression.

Histological examination

Kidneys were fixed with 10% formalin (pH 7.4), embedded in paraffin, sectioned into 3- μm slices and stained with periodic acid-Schiff (PAS; Mass Histology Service, Worcester, Massachusetts, USA) and Masson's trichrome (Mass Histology Service) reagents. The extents of glomerular sclerosis and tubulointerstitial fibrosis were quantitatively evaluated by an automatic image analysis system using PAS-stained and Masson's trichrome-stained sections, as described previously [18]. The severity of visceral epithelial cell (podocyte) injury was evaluated by immunohistochemistry for desmin, as described previously [19]. The ratio of the affected lesions to each glomerulus or microscopic field was calculated using the Image-Pro Plus software (Media Cybernetics, Silver Spring, Maryland, USA). For each glomerulus or tubulointerstitium, the area of an affected lesion in which the intensity was beyond a threshold level calculated according to the background signal was measured automatically by the software and then divided by the total area. A total of 20 glomeruli or consecutive microscopic fields were examined for each rat, and the average percentages of affected lesions were obtained for each rat.

Kidneys were also fixed with 2.5% glutaraldehyde in 0.1 mol/l phosphate buffer, washed with same buffer, dehydrated with a graded series of ethanol, embedded in EPON 812 resin (TAAB Laboratories Equipment Ltd., Berkshire, UK), sectioned into 0.5- μ m slices and stained with toluidine blue (Nacalai Tesque, Inc.) to evaluate the rate of appearance of cytoplasmic granules in the glomeruli. A total of 20 glomeruli were examined for each rat, and the number of granules-positive glomeruli were counted. The average percentage of granules-positive glomeruli was obtained for each rat. The above histological analyses were performed by an outsourcing company or a robotic system with an image analysis software in a blinded manner to avoid any biases. Kidney samples were also evaluated by electron microscopy, as previously described [20]. The ultrastructure of the podocytes was analyzed using a JEM-1200EX microscopy (JEOL Ltd., Tokyo, Japan), while immunocytochemical detection of albumin with an antirat albumin antibody (Bethyl Laboratories, Inc., Montgomery, Texas, USA) and colloidal gold particles (EY laboratories, Inc., San Mateo, California, USA) was performed as described previously [20].

Other analytical procedures

Albumin in the urine was determined using an enzyme-linked immunosorbent assay kit (Shibayagi Co. Ltd., Gunma, Japan). Preliminary experiments showed that the urinary albumin concentrations were similar between untreated LETO and OLETF rats at 7 and 9 weeks of age. However, at 13 weeks of age, OLETF rats exhibited detectable urinary albumin concentrations that were significantly higher than those of LETO rats. Therefore, we defined the appearance of low, but abnormal, levels of albumin in the urine as microalbuminuria in OLETF rats at 13 weeks of age [calculated urinary excretion rate of albumin ($U_{\text{albumin}}V$) is nearly 1.0 mg/day]. Although it obviously remains unclear whether this definition of microalbuminuria in OLETF rats is similar to that in other animal models and humans, recent studies indicate that $U_{\text{albumin}}V$ is less than 1.0 mg/day in normal rats [21,22].

The AngII content in the kidneys was measured by a radioimmunoassay as described previously [13,17,18]. Postprandial blood glucose (PPBG) levels were measured with a glucose analyzer (Sanwa-Kagaku, Co. Ltd., Nagoya, Japan). Renal cortical tissue collagen content was determined on the basis of hydroxyproline concentration [13,23].

Statistical analysis

Values are presented as means $\pm$ SE. Statistical comparisons of differences were performed using one-way or two-way analysis of variance combined with the Newman-Keuls post-hoc test. $P<0.05$ was considered statistically significant.

Results

Postprandial blood glucose and systolic blood pressure

The temporal profiles of PPBG and SBP are shown in Fig. 1(a, b). Vehicle-treated OLETF rats showed higher PPBG levels than vehicle-treated LETO rats from 13 to 35 weeks of age (Fig. 1a). OLETF rats treated with telmisartan or hydralazine showed similar PPBG levels to vehicle-treated OLETF rats. During the observation period, SBP remained unaltered in vehicle-treated LETO rats, whereas vehicle-treated OLETF rats progressively developed hypertension. Treatment of OLETF rats with telmisartan or hydralazine resulted in similar reductions in SBP (Fig. 1b). From 13 to 35 weeks of age, the body weight of vehicle-treated OLETF rats was higher than that of vehicle-treated LETO rats. None of the treatments affected the body weight of OLETF rats (data not shown).

Urinary excretion rate of albumin

The temporal profiles of urinary excretion rate of albumin ($U_{\text{albumin}}V$) are shown in Fig. 1(c, d) (logarithmic scale). At 7 and 9 weeks of age, the urinary albumin concentrations were nearly undetectable in both untreated LETO and OLETF rats, and the calculated $U_{\text{albumin}}V$ values did not differ significantly between these animals. At 13 weeks of age, OLETF rats exhibited detectable urinary albumin concentrations. The average $U_{\text{albumin}}V$ of vehicle-treated OLETF was 1.0 ± 0.1 mg/day and significantly higher than the values for LETO rats (0.08 ± 0.02 mg/day). Therefore, in the present experiments, we defined microalbuminuria in the rats as a $U_{\text{albumin}}V$ value more than 1.0 mg/day. After 13 weeks of age, $U_{\text{albumin}}V$ of vehicle-treated OLETF rats progressively increased with age (72.6 ± 5.9 mg/day at 35 week of age). Treatment with telmisartan prevented the progression of albuminuria in OLETF rats. Furthermore, the $U_{\text{albumin}}V$ value was significantly decreased after treatment with telmisartan (0.64 ± 0.08 mg/day at 35 weeks of age) and did not exceed the level observed at 13 weeks of age. Treatment with hydralazine also attenuated the progression of $U_{\text{albumin}}V$ in OLETF rats (35.4 ± 1.8 mg/day at 35 weeks of age). However, the effects of hydralazine on $U_{\text{albumin}}V$ were much weaker than those of telmisartan, as shown in Fig. 1(c, d).

Histological findings

The histological findings after staining with PAS and Masson's trichrome at 35 weeks of age are shown in Fig. 2(a, b). In 7, 13 and 22-week-old LETO and OLETF rats, no obvious glomerular sclerosis or tubulointerstitial fibrosis was observed (data not shown). Similarly, there were no significant findings except for minor alterations due to aging in the glomeruli and tubulointerstitial space of LETO and OLETF rats, even at 35 weeks of age. Quantitative analyses confirmed that neither the PAS-positive area in glomeruli nor the Masson's trichrome-positive area in the tubulointerstitium was differed significantly among the groups at this age (Fig. 2a, b).

The glomerular histological findings for toluidine blue staining, podocyte ultrastructure, albumin immunolabeling and desmin staining are shown in Figs 3 and 4, respectively. At 13 weeks of age, neither LETO nor OLETF rats showed any obvious cytoplasmic granules in podocytes or other glomerular changes. Furthermore, there was no significant difference between the desmin-positive areas in these animals (data not shown). However, at 22 weeks of age, significant accumulation of cytoplasmic granules in podocytes was observed in vehicle-treated OLETF rats but not in vehicle-treated LETO rats (Fig. 3a). Furthermore, the desmin-positive area was significantly higher in OLETF rats than in LETO rats (Fig. 3b). In OLETF rats, treatment with telmisartan significantly decreased the rate of appearance of cytoplasmic granules and the desmin-positive area in glomeruli to values that did not differ significantly from those of vehicle-treated LETO rats. On the contrary, these morphological changes did not differ between vehicle-treated and hydralazine-treated OLETF rats (Fig. 3a, b). Similar results were also observed at 35 weeks of age (Fig. 4a–c). Immunocytochemical detection of albumin by electron microscopy revealed that the albumin immunosignals were highly concentrated inside the cytoplasmic granules in podocytes of 35-week-old OLETF rats, although no apparent foot process loss was observed (Fig. 4c, d).

Angiotensin II contents in the kidney

Figure 5a shows the AngII contents in the kidney at 13, 22 and 35 weeks of age. At 13 weeks of age, the AngII contents in the kidney was higher in OLETF rats (180 ± 11 fmol/g) than in LETO rats (101 ± 7 fmol/g). Augmentation of the kidney AngII levels in OLETF rats was also observed at 22 and 35 weeks of age (199 ± 19 and 224 ± 20 fmol/g, respectively), compared with age-matched LETO rats (119 ± 8 and 109 ± 7 fmol/g, respectively). Treatment with telmisartan prevented the augmentation of kidney AngII levels in OLETF rats (94 ± 3 and 120 ± 4 fmol/g at 22 and 35 weeks of age, respectively). On the contrary, the kidney AngII levels did not differ

between vehicle-treated and hydralazine-treated OLETF rats (179 ± 18 and 228 ± 22 fmol/g at 22 and 35 weeks of age, respectively).

Collagen mRNA levels and contents in renal cortical tissues

Figure 5(b, c) shows the mRNA levels of type I collagen and type IV collagen in renal cortical tissues at 13, 22 and 35 weeks of age. At 13 weeks of age, the levels of type I collagen mRNA in LETO and OLETF rats were similar. However, the level of type IV collagen mRNA was significantly higher in OLETF rats than in LETO rats at this age. At 22 and 35 weeks of age, the levels of types I and IV collagen mRNA in vehicle-treated OLETF rats were significantly higher than those in vehicle-treated LETO rats. In OLETF rats, treatment with telmisartan decreased the expression of types I and IV collagen mRNA to the levels that did not differ from those in LETO rats. On the contrary, treatment with hydralazine did not alter the levels of types I and IV collagen mRNA in OLETF rats (Fig. 5b, c). The collagen contents in the renal cortical tissues did not differ among the animals at each age (data not shown).

Discussion

Diabetic nephropathy develops slowly over years and decades and represents a major complication in diabetes mellitus as well as a leading cause of end-stage renal failure [1]. Microalbuminuria, an early sign of renal insufficiency, generally worsens to overt macroalbuminuria in type 2 diabetes. On the basis of clinical evidence that treatment with ARBs or ACEIs inhibits the progression of microalbuminuria to overt nephropathy in type 2 diabetic patients [6–12], most national guideline groups have recommended the use of ARBs or ACEIs in preference to other antihypertensive agents for hypertensive diabetic patients with microalbuminuria [24–27]. However, it remains undetermined whether the intrarenal renin–angiotensin system is actually activated at the initiation of microalbuminuria in type 2 diabetes. The present study demonstrates that intrarenal AngII levels are augmented at the initiation of microalbuminuria and during the progression of albuminuria in type 2 diabetic OLETF rats, prior to the manifestation of overt nephropathy. We also found that treatment with an ARB prevented the augmentation of intrarenal AngII levels as well as the transition from microalbuminuria to macroalbuminuria. These data suggest that augmented intrarenal AngII levels play a critical role in the initiation of microalbuminuria and transition to overt nephropathy in type 2 diabetes.

Early morphological changes in the glomeruli and tubulointerstitium are clinically silent during the progression of albuminuria [1]. Several studies showed that OLETF rats exhibit apparent glomerular and interstitial injuries after 50 weeks of age [13–15]. In the present study, OLETF rats exhibited detectable albuminuria from 13 weeks of age and then progressed to overt albuminuria in an age-dependent manner. Although vehicle-treated OLETF rats showed a markedly increased U_{albumin} at 35 weeks of age, histological analyses of PAS-stained and Masson's trichrome-stained sections failed to show any significant glomerular and tubulointerstitial injuries. However, more careful assessment of the morphology revealed the accumulation of cytoplasmic granules in podocytes and enhanced desmin staining (a marker of podocyte injury) [19]. Furthermore, renal cortical collagen gene expression was already enhanced in OLETF rats from 13 or 22 weeks of age, although the collagen contents in the renal cortical tissues remained unchanged during the observation period. Thus, these data suggest that podocyte abnormalities and enhanced collagen gene expression exist before glomerular and tubulointerstitial injuries become apparent in OLETF rats.

In the present study, treatment with an ARB, telmisartan, and nonspecific vasodilator, hydralazine, showed similar antihypertensive effects in OLETF rats. However, telmisartan almost completely prevented the progression of albuminuria, whereas hydralazine partially attenuated it. We also found that telmisartan prevented the podocyte abnormalities but

hydralazine did not. These data are consistent with the concept that the antialbuminuric effects of AngII blockade are associated with its protective effects on podocyte injury [28,29]. In this regard, Suzuki *et al.* [30] have recently demonstrated that AngII blockade with an ARB ameliorates proteinuria by preventing reductions in the functional molecules, such as nephrin, in the slit diaphragm located between the adjacent foot processes of podocytes. On the contrary, moderate effects of hydralazine on albuminuria might be simply associated with reduction in blood pressure reduction, as suggested by recent clinical studies [31,32]. As recent studies also indicate possible changes in proximal tubular reabsorption of albumin during the development of diabetic nephropathy [33,34], further studies are needed to examine the effects of ARBs on proximal tubular reabsorption of albumin in OLETF rats.

Within the kidney, AngII is distributed in the interstitial fluid, tubular fluid and the intracellular compartments [15,35]. The interstitial and intratubular compartments contribute to the disproportionately high total AngII levels [35–37]. However, the intrarenal distribution of the augmented AngII in OLETF rats was not investigated in the present study. In addition, the mechanisms by which intrarenal AngII levels are augmented at the initiation of albuminuria and during the progression of albuminuria remain to be determined. Consistent with previous observations in hypertensive rats [18,37,38], the present study showed that elevated kidney AngII levels were significantly decreased by AngII AT₁ receptor blockade with an ARB in OLETF rats, suggesting that some of the renoprotective effects of telmisartan are accompanied by reductions in intrarenal AngII levels. Recent studies have demonstrated that ARBs decrease intrarenal AngII levels through prevention of AT₁ receptor-mediated uptake of AngII and intrarenal production of AngII by overexpression of angiotensinogen in AngII-infused hypertensive rats [18,35,39–41]. Importantly, a growing body of evidence also indicates that the long-term beneficial effects of ARBs in treating renal injury can be explained in part by their effects on blocking AngII formation and AngII uptake, thereby inhibiting the actions of intracellular AngII [40,41]. However, it is not clear from the present study whether uptake of AngII occurs similarly in OLETF rats as shown in AngII-infused rats. Furthermore, we cannot address the issues related to the precise mechanisms responsible for the telmisartan-induced reduction in intrarenal AngII levels in OLETF rats.

Early studies reported a significant accumulation of protein absorption droplets in podocytes of rats treated with an intraperitoneal injection of albumin [42]. Interestingly, the cytoplasmic granules observed in podocytes of OLETF rats were similar to those observed in the albumin-injected rats [42]. Furthermore, we found that the effects of telmisartan on the rate of appearance of cytoplasmic granules were similar to those on U_{albumin}/V among the groups. Previous studies showed that accumulation of cytoplasmic granules was associated with the progression of proteinuria in streptozocin-induced diabetic rats [43], diabetic spontaneously hypertensive rats/N-cp [43] and rats overexpressing the human AT₁ receptor in podocytes [29]. In the present study, immunocytochemical detection of albumin by electron microscopy revealed the albumin immunosignals were highly concentrated inside the cytoplasmic granules in podocytes of 35-week-old OLETF rats. Collectively, these data suggest that the cytoplasmic granules in the podocytes of OLETF rats are accumulations of protein absorption droplets.

In summary, the present study indicates that both the initiation and progression of microalbuminuria are associated with augmented intrarenal AngII levels and podocyte abnormalities, before morphological injuries to the renal tissues become apparent in type 2 diabetic rats. Furthermore, strict AngII blockade from an early stage can suppress the augmentation of intrarenal AngII levels as well as the development of microalbuminuria, independently of its effects on blood pressure and glucose metabolism. These data support the concept that strict blockade of AngII from an early stage could be an effective strategy for preventing the development of type 2 diabetic nephropathy later in life. However, more careful assessments of the podocytes histopathology, glomerular slit membrane components and

intrarenal renin–angiotensin system are required. In addition, further analyses for the intratrenal AngII activity on type 2 diabetic patients with microalbuminuria are necessary in the future. We therefore propose further clinical studies using a recently developed method for measuring urinary human angiotensinogen [44].

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References

1. Makino H, Nakamura Y, Wada J. Remission and regression of diabetic nephropathy. *Hypertens Res* 2003;26:515–519. [PubMed: 12924617]
2. de Zeeuw D, Remuzzi G, Parving HH, Keane WF, Zhang Z, Shahinfar S, et al. Albuminuria, a therapeutic target for cardiovascular protection in type 2 diabetic patients with nephropathy. *Circulation* 2004;110:921–927. [PubMed: 15302780]
3. Mogensen CE. Microalbuminuria predicts clinical proteinuria and early mortality in maturity-onset diabetes. *N Engl J Med* 1984;310:356–360. [PubMed: 6690964]
4. Hillege HL, Fidler V, Diercks GF, van Gilst WH, de Zeeuw D, van Veldhuisen DJ, et al. Prevention of Renal and Vascular End Stage Disease (PREVEND) Study Group. Urinary albumin excretion predicts cardiovascular and noncardiovascular mortality in general population. *Circulation* 2002;106:1777–1782. [PubMed: 12356629]
5. Dinneen SF, Gerstein HC. The association of microalbuminuria and mortality in noninsulin-dependent diabetes mellitus. A systematic overview of the literature. *Arch Intern Med* 1997;157:1413–1418. [PubMed: 9224218]
6. Ruggenti P, Fassi A, Ilieva AP, Bruno S, Iliev IP, Brusegan V, et al. Bergamo Nephrologic Diabetes Complications Trial (BENEDICT) Investigators: preventing microalbuminuria in type 2 diabetes. *N Engl J Med* 2004;351:1941–1951. [PubMed: 15516697]
7. Viberti G, Mogensen CE, Groop LC, Pauls JF, European Microalbuminuria Captopril Study Group. Effect of captopril on progression to clinical proteinuria in patients with insulin-dependent diabetes mellitus and microalbuminuria. *JAMA* 1994;271:275–279. [PubMed: 8295285]
8. Burnier M, Zanchi A. Blockade of the renin-angiotensin-aldosterone system: a key therapeutic strategy to reduce renal and cardiovascular events in patients with diabetes. *J Hypertens* 2006;24:11–25. [PubMed: 16331093]
9. Viberti G, Wheeldon NM, MicroAlbuminuria Reduction with VALsartan (MARVAL) Study Investigators. Microalbuminuria reduction with valsartan in patients with type 2 diabetes mellitus: a blood pressure-independent effect. *Circulation* 2002;106:672–678. [PubMed: 12163426]
10. Sasso FC, Carbonara O, Persico M, Iafusco D, Salvatore T, D'Ambrosio R, et al. Irbesartan reduces the albumin excretion rate in microalbuminuric type 2 diabetic patients independently of hypertension: a randomized double-blind placebo-controlled crossover study. *Diabetes Care* 2002;25:1909–1913. [PubMed: 12401731]
11. Zandbergen AA, Baggen MG, Lamberts SW, Bootsma AH, de Zeeuw D, Ouwendijk RJ. Effect of losartan on microalbuminuria in normotensive patients with type 2 diabetes mellitus. A randomized clinical trial. *Ann Intern Med* 2003;139:90–96. [PubMed: 12859158]
12. Makino H, Haneda M, Babazono T, Moriya T, Ito S, Iwamoto Y, et al. INNOVATION Study Group. Prevention of transition from incipient to overt nephropathy with telmisartan in patients with type 2 diabetes. *Diabetes Care* 2007;30:1577–1578. [PubMed: 17389334]

13. Nagai Y, Yao L, Kobori H, Miyata K, Ozawa Y, Miyatake A, et al. Temporary angiotensin II blockade at the prediabetic stage attenuates the development of renal injury in type 2 diabetic rats. *J Am Soc Nephrol* 2005;16:703–711. [PubMed: 15647337]
14. Yagi K, Kim S, Wanibuchi H, Yamashita T, Yamamura Y, Iwao H. Characteristics of diabetes, blood pressure, and cardiac and renal complications in Otsuka Long-Evans Tokushima Fatty rats. *Hypertension* 1997;29:728–735. [PubMed: 9052888]
15. Kawano K, Hirashima T, Mori S, Saitoh Y, Kurosumi M, Natori T. Spontaneous long-term hyperglycemic rat with diabetic complications. Otsuka Long-Evans Tokushima Fatty (OLETF) strain. *Diabetes* 1992;41:1422–1428. [PubMed: 1397718]
16. Wienen W, Richard S, Champeroux P, Audeval-Gerard C. Comparative antihypertensive and renoprotective effects of telmisartan and lisinopril after long-term treatment in hypertensive diabetic rats. *J Renin Angiotensin Aldosterone Syst* 2001;2:31–36. [PubMed: 11881063]
17. Nishiyama A, Seth DM, Navar LG. Renal interstitial fluid concentrations of angiotensins I and II in anesthetized rats. *Hypertension* 2002;39:129–134. [PubMed: 11799091]
18. Kobori H, Ozawa Y, Suzuki Y, Nishiyama A. Enhanced intrarenal angiotensinogen contributes to early renal injury in spontaneously hypertensive rats. *J Am Soc Nephrol* 2005;16:2073–2080. [PubMed: 15888567]
19. Nagase M, Yoshida S, Shibata S, Nagase T, Gotoda T, Ando K, Fujita T. Enhanced aldosterone signaling in the early nephropathy of rats with metabolic syndrome: possible contribution of fat-derived factors. *J Am Soc Nephrol* 2006;17:3438–3446. [PubMed: 17082236]
20. Ueno M, Dobrogowska DH, Vorbrott AW. Immunocytochemical evaluation of the blood-brain barrier to endogenous albumin in the olfactory bulb and pons of senescence-accelerated mice (SAM). *Histochem Cell Biol* 1996;105:203–212. [PubMed: 8681038]
21. Kim JJ, Li JJ, Jung DS, Kwak SJ, Ryu DR, Yoo TH, et al. Differential expression of nephrin according to glomerular size in early diabetic kidney disease. *J Am Soc Nephrol* 2007;18:2303–2310. [PubMed: 1759973]
22. Sullivan JC, Semprun-Prieto L, Boesen EI, Pollock DM, Pollock JS. Sex and sex hormones influence the development of albuminuria and renal macrophage infiltration in spontaneously hypertensive rats. *Am J Physiol Regul Integr Comp Physiol* 2007;293:R1573–R1579. [PubMed: 17699561]
23. Nishiyama A, Yao L, Nagai Y, Miyata K, Yoshizumi M, Kagami S, et al. Possible contributions of reactive oxygen species and mitogen-activated protein kinase to renal injury in aldosterone/salt-induced hypertensive rats. *Hypertension* 2004;43:841–848. [PubMed: 14769808]
24. Buse JB, Ginsberg HN, Bakris GL, Clark NG, Costa F, Eckel R, et al. American Heart Association. American Diabetes Association: primary prevention of cardiovascular diseases in people with diabetes mellitus: a scientific statement from the American Heart Association and the American Diabetes Association. *Circulation* 2007;115:114–126. [PubMed: 17192512]
25. Mancia G, De Backer G, Dominiczak A, Cifkova R, Fagard R, Germano G, et al. Management of Arterial Hypertension of the European Society of Hypertension; European Society of Cardiology. 2007 guidelines for the management of arterial hypertension: the Task Force for the Management of Arterial Hypertension of the European Society of Hypertension (ESH) and of the European Society of Cardiology (ESC). *J Hypertens* 2007;25:1105–1187. [PubMed: 17563527]
26. Japanese Society of Hypertension. Japanese Society of Hypertension guidelines for the management of hypertension (JSH 2004). *Hypertens Res* 2006;29(Suppl):S1–S105. [PubMed: 17366911]
27. Chobanian AV, Bakris GL, Black HR, Cushman WC, Green LA, Izzo JL Jr, et al. National Heart, Lung, and Blood Institute Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure. National High Blood Pressure Education Program Coordinating Committee: the seventh report of the Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure: the JNC 7 report. *JAMA* 2003;289:2560–2572. [PubMed: 12748199]
28. Yoo TH, Li JJ, Kim JJ, Jung DS, Kwak SJ, Ryu DR, et al. Activation of the renin-angiotensin system within podocytes in diabetes. *Kidney Int* 2007;71:1019–1027. [PubMed: 17361112]
29. Hoffmann S, Podlich D, Hahnel B, Kriz W, Gretz N. Angiotensin II type 1 receptor overexpression in podocytes induces glomerulosclerosis in transgenic rats. *J Am Soc Nephrol* 2004;15:1475–1487. [PubMed: 15153558]

30. Suzuki K, Han GD, Miyauchi N, Hashimoto T, Nakatsue T, Fujioka Y, et al. Angiotensin II type 1 and type 2 receptors play opposite roles in regulating the barrier function of kidney glomerular capillary wall. *Am J Pathol* 2007;170:1841–1853. [PubMed: 17525253]
31. Casas JP, Chua W, Loukogeorgakis S, Vallance P, Smeeth L, Hingorani AD, et al. Effect of inhibitors of the renin-angiotensin system and other antihypertensive drugs on renal outcomes: systematic review and meta-analysis. *Lancet* 2005;366:2026–2033. [PubMed: 16338452]
32. Ogawa S, Takeuchi K, Mori T, Nako K, Tsubo Y, Ito S. Effects of monotherapy of temocapril or candesartan with dose increments or combination therapy with both drugs on the suppression of diabetic nephropathy. *Hypertens Res* 2007;30:325–334. [PubMed: 17541211]
33. Pollock CA, Poronnik P. Albumin transport and processing by the proximal tubule: physiology and pathophysiology. *Curr Opin Nephrol Hypertens* 2007;16:359–364. [PubMed: 17565279]
34. Tojo A, Onozato ML, Kurihara H, Sakai T, Goto A, Fujita T. Angiotensin II blockade restores albumin reabsorption in the proximal tubules of diabetic rats. *Hypertens Res* 2003;26:413–419. [PubMed: 12887133]
35. Navar LG, Nishiyama A. Why are angiotensin concentrations so high in the kidney? *Curr Opin Nephrol Hypertens* 2004;13:107–115. [PubMed: 15090867]
36. Nishiyama A, Seth DM, Navar LG. Renal interstitial fluid angiotensin I and angiotensin II concentrations during local angiotensin-converting enzyme inhibition. *J Am Soc Nephrol* 2002;13:2207–2212. [PubMed: 12191964]
37. Nishiyama A, Seth DM, Navar LG. Angiotensin II type 1 receptor-mediated augmentation of renal interstitial fluid angiotensin II in angiotensin II-induced hypertension. *J Hypertens* 2003;21:1897–1903. [PubMed: 14508196]
38. Nishiyama A, Yoshizumi M, Rahman M, Kobori H, Seth DM, Miyatake A, et al. Effects of AT1 receptor blockade on renal injury and mitogen-activated protein activity in Dahl salt-sensitive rats. *Kidney Int* 2004;65:972–981. [PubMed: 14871417]
39. Kobori H, Prieto-Carrasquero MC, Ozawa Y, Navar LG. AT1 receptor mediated augmentation of intrarenal angiotensinogen in angiotensin II-dependent hypertension. *Hypertension* 2004;43:1126–1132. [PubMed: 15037565]
40. Zhuo JL, Imig JD, Hammond TG, Orengo S, Benes E, Navar LG. Ang II accumulation in rat renal endosomes during Ang II-induced hypertension: role of AT(1) receptor. *Hypertension* 2002;39:116–121. [PubMed: 11799089]
41. Zhuo JL. Intracrine renin and angiotensin II: a novel role in cardiovascular and renal cellular regulation. *J Hypertens* 2006;24:1017–1020. [PubMed: 16685198]
42. Andrews PM. A scanning and transmission electron microscopic comparison of puromycin aminonucleoside-induced nephrosis to hyperalbuminemia-induced proteinuria with emphasis on kidney podocyte pedicel loss. *Lab Invest* 1977;36:183–197. [PubMed: 839733]
43. Gross ML, Ritz E, Schoof A, Adamczak M, Koch A, Tulp O, et al. Comparison of renal morphology in the streptozotocin and the SHR/N-cp models of diabetes. *Lab Invest* 2004;84:452–464. [PubMed: 14767489]
44. Katsurada A, Hagiwara Y, Miyashita K, Satou R, Miyata K, Ohashi N, et al. Novel sandwich ELISA for human angiotensinogen. *Am J Physiol Renal Physiol* 2007;293:F938–F945. [PubMed: 17634399]

Abbreviations

ACEI

angiotensin converting enzyme inhibitor

AngII

angiotensin II

ARB

AT₁ receptor blocker

GAPDH

glyceraldehydes-3-phosphate dehydrogenase

LETO	Long-Evans-Tokushima-Otsuka
OLETF	Otsuka-Long-Evans-Tokushima-Fatty
PAS	periodic acid-Schiff
PPBG	postprandial blood glucose
U_{albmin}V	urinary excretion rate of albumin

Fig. 1.

Profiles of (a) PPBG, (b) SBP, and (c) urinary excretion rate of albumin ($U_{albumin}V$) (d; logarithmic scale). Vehicle-treated OLETF rats develop hypertension, diabetes and albuminuria. In these animals, treatment with telmisartan or hydralazine does not affect the PPBG but decreases the SBP to similar levels. At 13 weeks of age, vehicle-treated OLETF rats exhibit detectable urinary albumin concentrations. The progression of albuminuria is prevented by treatment with telmisartan but not with hydralazine. LETO, control Long-Evans Tokushima Otsuka; OLETF, Otsuka Long-Evans Tokushima Fatty; PPBG, postprandial blood glucose; SBP, systolic blood pressure.

Fig. 2. Photomicrographs of glomeruli or tubulointerstitium stained with (a) PAS and (b) Masson's trichrome reagents (original magnification, $\times 200$, respectively). Neither the PAS-positive area in glomeruli nor Masson's trichrome-positive area in the tubulointerstitium differs significantly among the groups at 35 weeks of age. LETO, Long-Evans Tokushima Otsuka; OLETF, Otsuka Long-Evans Tokushima Fatty; PAS, periodic acid-Schiff.

Fig. 3. Glomerular histological findings for (a; original magnification, $\times 200$) toluidine blue staining and (b; original magnification, $\times 200$) desmin immunohistochemistry at 22 weeks of age. (a) Significant accumulation of cytoplasmic granules in podocytes is observed in vehicle-treated OLETIF rats. (b) The desmin-positive area is also significantly higher in OLETIF rats than in LETO rats. In OLETIF rats, treatment with telmisartan significantly decreases the rate of appearance of cytoplasmic granules and the desmin-positive area in glomeruli to the levels that does not differ significantly from those in vehicle-treated LETO rats. On the contrary, these values were similar between vehicle-treated and hydralazine-treated OLETIF rats (a, b).

* $P < 0.05$ vs. LETO rats, $^{\dagger}P < 0.05$; OLETF + vehicle vs. OLETF + telmisartan or hydralazine.
LETO, Long–Evans Tokushima Otsuka; OLETF, Otsuka Long–Evans Tokushima Fatty.

Fig. 4. (a; original magnification, ×200) Glomerular histological findings for toluidine blue staining, (b; original magnification, ×200) desmin immunohistochemistry, (c; original magnification, ×3000) podocyte ultrastructure and (d; original magnification, ×3000) albumin immunohistochemistry at 35 weeks of age. Significant accumulation of cytoplasmic granules and higher desmin-positive area in glomeruli are observed in vehicle-treated OLETF rats (c, d; arrows), although no apparent foot process loss is seen (c, d). Immunocytochemical detection of albumin by electron microscopy reveals that albumin immunosignals are highly concentrated inside the cytoplasmic granules in podocytes of 35-week-old OLETF rats (d). In OLETF rats, treatment with telmisartan significantly decreases the rate of appearance of

cytoplasmic granules and the desmin-positive area in glomeruli to the levels that does not differ significantly from those in vehicle-treated LETO rats. On the contrary, these values were similar between vehicle-treated and hydralazine-treated OLETF rats (a-c). * $P < 0.05$ vs. LETO rats, [†] $P < 0.05$; OLETF + vehicle vs. OLETF + telmisartan or hydralazine. LETO, Long-Evans Tokushima Otsuka; OLETF, Otsuka Long-Evans Tokushima Fatty.

Fig. 5.

Kidney (a) AngII contents, renal cortical mRNA levels of (b) type I collagen and (c) type IV collagen. Vehicle-treated OLETF rats show augmented kidney AngII levels and mRNA levels of collagen types I and IV from 13 or 22 weeks of age. In OLETF rats, treatment with telmisartan reduces these levels, whereas treatment with hydralazine does not. Data for mRNA levels are expressed as the relative differences between OLETF and LETO rats after normalization to GAPDH expression. * $P < 0.05$ vs. LETO rats, † $P < 0.05$; OLETF + vehicle vs. OLETF + telmisartan or hydralazine. AngII, angiotensin II; GAPDH, glyceraldehydes-3-phosphate dehydrogenase; LETO, Long-Evans Tokushima Otsuka; OLETF, Otsuka Long-Evans Tokushima Fatty.