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Early Treatment With Olmesartan Prevents Juxtamedullary Glomerular Podocyte Injury and the Onset of Microalbuminuria in Type 2 Diabetic Rats

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BACKGROUND

Studies were performed to determine if early treatment with an angiotensin II (Ang II) receptor blocker (ARB), olmesartan, prevents the onset of microalbuminuria by attenuating glomerular podocyte injury in Otsuka Long-Evans Tokushima Fatty (OLETF) rats with type 2 diabetes mellitus.

METHODS

OLETF rats were treated with either a vehicle, olmesartan (10 mg/kg/day) or a combination of nonspecific vasodilators (hydralazine 15 mg/kg/day, hydrochlorothiazide 6 mg/kg/day, and reserpine 0.3 mg/kg/day; HHR) from the age of 7–25 weeks.

RESULTS

OLETF rats were hypertensive and had microalbuminuria from 9 weeks of age. At 15 weeks, OLETF rats had higher Ang II levels in the kidney, larger glomerular desmin-staining areas (an index of podocyte injury), and lower gene expression of nephrin in juxtamedullary glomeruli, than nondiabetic Long-Evans Tokushima Otsuka (LETO) rats. At 25 weeks, OLETF rats showed overt albuminuria, and higher levels of Ang II in the kidney and larger glomerular desmin-staining areas in superficial and juxtamedullary glomeruli compared to LETO rats. Reductions in mRNA levels of nephrin were also observed in superficial and juxtamedullary glomeruli. Although olmesartan did not affect glucose metabolism, it decreased blood pressure and prevented the renal changes in OLETF rats. HHR treatment also reduced blood pressure, but did not affect the renal parameters.

CONCLUSIONS

This study demonstrated that podocyte injury occurs in juxtamedullary glomeruli prior to superficial glomeruli in type 2 diabetic rats with microalbuminuria. Early treatment with an ARB may prevent the onset of albuminuria through its protective effects on juxtamedullary glomerular podocytes.

Keywords: *angiotensin II receptor blockers (ARBs); blood pressure; hypertension; juxtamedullary glomeruli; microalbuminuria; olmesartan; type 2 diabetes mellitus*

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Diabetic nephropathy is a major complication in type 2 diabetes mellitus and a leading cause of end-stage renal failure.[1] The first sign of diabetic nephropathy is the appearance of albumin in the urine (microalbuminuria).[2] Although the mechanisms underlying the occurrence of microalbuminuria in type 2 diabetes are extremely complex, the potential role of the renin-angiotensin system has been suggested.[3–11] Large-scale clinical trials have shown that in hypertensive type 2 diabetic patients with microalbuminuria, lowering blood pressure by blockade of the renin-angiotensin system with angiotensin II (Ang II) type 1 (AT1) receptor blockers (ARBs) or angiotensin-converting enzyme inhibitors is more effective in reducing microalbuminuria than any other conventional antihypertensive therapies.[3–7] These findings suggest that the antialbuminuric effects of renin-angiotensin system inhibition are independent of their blood pressure-lowering effects.[12]

Recently, the Randomized Olmesartan and Diabetes Microalbuminuria Prevention (ROADMAP) study was conducted to examine if an ARB can prevent the onset of

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microalbuminuria.[13] It has been demonstrated that early treatment with the ARB, olmesartan, significantly reduced the occurrence rate of microalbuminuria in type 2 diabetes patients.[14] However, the precise mechanisms by which intensive Ang II blockade during a prediabetic state prevents the occurrence of microalbuminuria are not completely understood.

A growing body of evidence has indicated that one of the most important mechanisms in the development of albuminuria is injury to glomerular epithelial cells (podocytes).[15–17] Therefore, we hypothesized that the beneficial effect of an ARB on the onset of microalbuminuria is associated with its protective effect on podocyte injury. In particular, we aimed to characterize the mechanisms by which microalbuminuria develops in early type 2 diabetic nephropathy by focusing on the heterogeneity of glomerular podocyte abnormalities in type 2 diabetic Otsuka Long-Evans Tokushima Fatty (OLETF) rats, which exhibit pathological features of renal injury similar to those found in human type 2 diabetic patients with hypertension, obesity, and hyperinsulinemia.[10,11,18–20]

METHODS

Experimental procedures were conducted according to the guidelines for the care and use of animals established by Kagawa University (Kagawa, Japan).

Animals. Eighty-two male 5-week-old OLETF rats and 30 male age-matched Long-Evans Tokushima Otsuka (LETO) rats (genetic control for OLETF rats) were supplied by Otsuka Pharmaceutical (Tokushima, Japan).

After measuring physical and laboratory data at baseline, each five LETO and OLETF rats were killed at 5 and 7 weeks of age, respectively. The remaining OLETF rats were then randomly treated with one of the following foods: standard diet (n = 20); olmesartan (0.02% in chow, 10–15 mg/kg/day; Daiichi-Sankyo, Tokyo, Japan): (n = 24); HHR (hydralazine 0.03%, hydrochlorothiazide 0.012%, reserpine 0.006% in chow; Sigma Chemical, St. Louis, MO, for each): (n = 24). The remaining LETO rats (n = 20) were fed a standard diet. The doses of olmesartan and HHR were determined on the basis of previous studies on rats.[10,21] At 15 weeks of age, 2 OLETF rats and 10 LETO rats treated with standard diet and 12 OLETF rats treated with olmesartan and HHR were killed. The remaining rats continued to receive their treatment until 25 weeks of age (12 OLETF rats and 12 LETO rats with a standard diet, and 12 OLETF rats with olmesartan and 12 OLETF rats HHR). Systolic blood pressure (SBP) was measured in conscious rats by tail-cuff plethysmography (BP-98A; Softron, Tokyo, Japan). Detailed methods for sample preparation and histological analyses are available in the **Supplementary Methods** online.

Real-time reverse transcription-polymerase chain reaction (RT-PCR). The regions of superficial glomeruli and juxtamedullary glomeruli in renal cortex tissues were separated and dissected by a laser capture microdissection system. RT-PCR for nephrin, podocin, and 18s was done after mRNA isolation from laser capture microdissection samples. The mRNA expression levels of endothelial nitric oxide synthase (*eNOS*), vascular endothelial growth factor (*VEGF*), and VEGF receptor 1 and 2 (*VEGFR1* and *VEGFR2*) in renal cortical tissues were measured by RT-PCR. Data for mRNA levels were expressed as the relative differences between OLETF rats and LETO rats after normalization of 18s expression. All information relating to mRNA primers is available in the **Supplementary Methods** online.

Other analytical procedures. Detailed information regarding other analytical procedures is available in the **Supplementary Methods** online.

Statistical analyses. Values are means ± s.e.m. Statistical comparisons of differences were carried out using one-way or two-way ANOVA combined with the Newman–Keuls *post-hoc* test. $P < 0.05$ was considered significant.

RESULTS

SBP, body weight, kidney weight, visceral fat weight, and blood glucose

The serial profiles of SBP are shown in **Figure 1a**. At 5 and 7 weeks of age, each group of OLETF rats showed similar SBP. During the observation period, vehicle-treated OLETF rats progressively developed hypertension. OLETF rats treated with olmesartan or HHR resulted in similar reductions in SBP. Kidney weight and visceral fat weight per body weight ratios were higher in OLETF rats than in LETO rats. The serial profiles of body weight, postprandial blood glucose, and kidney weight and visceral fat weight per body weight are available in the **Supplementary Figure S1a** and **Supplementary Table S1** online.

Urinary excretion rate of albumin (UalbV) and urinary protein excretion

The profiles of UalbV are shown in **Figure 1b**. At 5 and 7 weeks of age, UalbV between untreated LETO and OLETF rats was similar, and calculated UalbV values did not significantly differ between these rats. At 9 weeks of age, vehicle-treated OLETF rats showed microalbuminuria (1.0 ± 0.2 mg/day), whereas LETO rats did not (0.2 ± 0.02 mg/day). After 9 weeks of age, UalbV of vehicle-treated OLETF rats progressively increased with age and resulted in massive proteinuria at 25 weeks of age. Treatment with olmesartan prevented the onset of microalbuminuria (>1.0 mg/day) in OLETF rats until 25 weeks of age (0.44 ± 0.1 mg/day at 25 weeks of age). Treatment with HHR also attenuated the progression of UalbV in OLETF rats. However, the effects of HHR on UalbV were much less than those of olmesartan (21.5 ± 2.0 mg/day at 25 weeks of age). The serial profiles of urinary protein excretion are available in the **Supplementary Figure S1b** online.

Plasma triglyceride, glycated albumin, insulin, adiponectin, and creatinine levels

OLETF rats showed higher plasma triglyceride, insulin, and glycated albumin levels than that of LETO rats at 25 weeks of

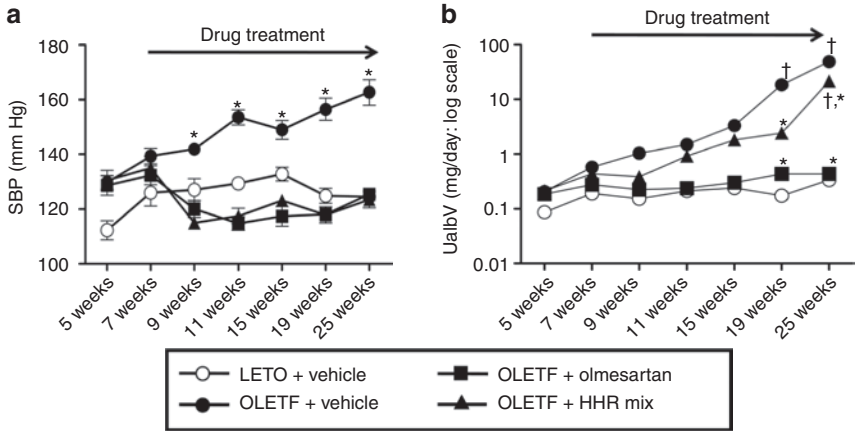

Figure 1 | Profiles of (**a**) SBP and (**b**) UalbV. The onset of microalbuminuria is prevented by treatment with olmesartan but not with HHR. *$P < 0.05$; OLETF vs. LETO, †$P < 0.05$; OLETF + vehicle vs. OLETF + olmesartan or HHR. HHR, hydralazine, hydrochlorothiazide, and reserpine; LETO, Long-Evans Tokushima Otsuka rats; OLETF, Otsuka Long-Evans Tokushima Fatty rats; SBP, systolic blood pressure; UalbV, urinary excretion rate of albumin.

age. Treatment with olmesartan decreased plasma triglyceride level, but did not decrease plasma insulin and glycated albumin levels. Plasma adiponectin levels were similar between olmesartan-treated rats and vehicle-treated rats in OLETF rats at 25 weeks of age. At 15 and 25 weeks of age, no significant difference in creatinine level was observed in OLETF rats given any antihypertensive treatment. Detailed data are available in the **Supplementary Table S1** online.

Histological findings

At 15 and 25 weeks of age, vehicle-treated LETO rats and OLETF rats showed no obvious glomerular sclerosis in PAS-staining areas. Glomerular size was not significantly different among the groups at 15 weeks of age. At 25 weeks of age, the size of superficial glomeruli in OLETF rats was larger than those in LETO rats. Juxtamedullary glomeruli tended to be larger than superficial glomeruli in OLETF rats, but these changes were not statistically significant. Detailed data for PAS-staining areas and glomerular sizes are available in the **Supplementary Figure S2** online.

The glomerular histological findings for the immunostaining of desmin in superficial and juxtamedullary glomeruli are shown in **Figure 2a,b**, respectively. At 5 and 7 weeks of age, there was no significant difference in the desmin-staining area between vehicle-treated LETO rats and OLETF rats (data not shown). However, at 15 weeks of age, the glomerular desmin-positive area was significantly larger in vehicle-treated OLETF rats than in LETO rats in only juxtamedullary glomeruli, but not in superficial glomeruli. Afterwards, the glomerular desmin-positive area became larger in vehicle-treated OLETF rats than in LETO rats in juxtamedullary and superficial glomeruli at 25 weeks of age. In OLETF rats, treatment with olmesartan prevented enlargement of the glomerular desmin-positive area in juxtamedullary and superficial glomeruli to values that did not differ significantly from those of LETO rats until 25 weeks of age. Conversely, the glomerular desmin-positive area was not different between vehicle- and HHR-treated OLETF rats except in juxtamedullary glomeruli at 15 weeks of age.

At 25 weeks of age, observation of podocyte ultrastructure by electron microscopy revealed marked podocyte foot process effacement in vehicle-treated OLETF rats, whereas olmesartan-treated OLETF rats showed a marked reduction in podocyte foot process effacement resembling that seen in vehicle-treated LETO rats. In contrast, HHR-treated OLETF rats at 25 weeks of age did not show as much reduction in podocyte foot process effacement as compared with the olmesartan-treated OLETF rats (**Figure 2c**).

Immunofluorescence microscopy of the slit diaphragm-associated molecules nephrin and podocin revealed loss of immunostaining continuity in vehicle- and HHR-treated OLETF rats at 25 weeks of age, which showed massive albuminuria. Moreover, unbound nephrin and podocin were observed in a merged image. On the other hand, immunofluorescence continuity and the binding between nephrin and podocin were maintained in OLETF rats treated with olmesartan compared with vehicle-treated OLETF rats at 25 weeks of age (**Figure 3a**).

Oil red O-staining showed lipid to accumulate predominantly in proximal tubular cells, but not in glomerulus of OLETF rats at 25 weeks of age. Treatment with olmesartan attenuated lipid accumulation in proximal tubular cells. Reduction in the CD34-staining area in glomerular capillary was not observed in OLETF rats at 25 weeks of age. Podocyte number, determined by mean WT-1^+ cells per glomerular cross section, was similar in OLETF rats and LETO rats at 25 weeks of age. Any treatment did not change podocyte number. Detailed data are available in the **Supplementary Figures S3** and **S4** online.

Gene expression of the slit diaphragm-associated molecules nephrin and podocin and endothelial markers

Figure 3b–d shows the mRNA levels of the slit diaphragm-associated molecules nephrin and podocin in superficial and juxtamedullary glomeruli. At 15 weeks of age, the mRNA levels of nephrin and podocin in the superficial glomeruli of LETO and OLETF rats were similar (**Figure 3b,c**). However, in juxtamedullary glomeruli, the mRNA levels of nephrin and podocin were significantly lower in OLETF rats than those

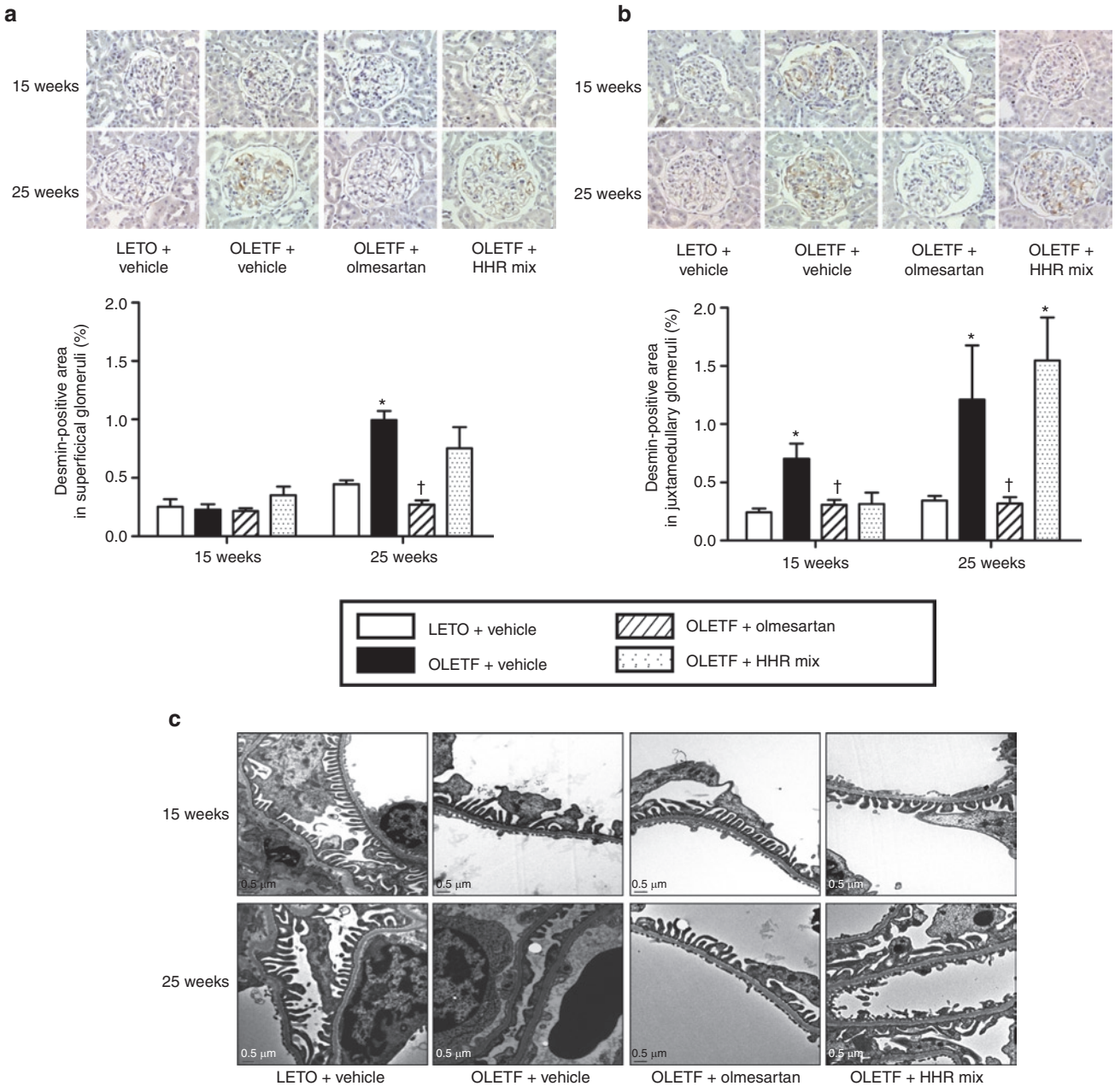

Figure 2 | Photomicrographs of glomeruli stained with desmin (original magnification ×200) and their quantitative analyses data in (**a**) superficial and (**b**) juxtamedullary glomeruli, as well as electron microscopy findings at (**c**) 15 and 25 weeks of age. The glomerular desmin-positive area is significantly larger in vehicle-treated OLETF rats than in LETO rats in juxtamedullary glomeruli, whereas that in superficial glomeruli is not altered between rats at 15 weeks of age. At 25 weeks of age, the desmin-positive area is larger in vehicle-treated OLETF rats than in LETO rats in juxtamedullary and superficial glomeruli. In OLETF rats, treatment with olmesartan prevents the enlargement of the glomerular desmin-positive area in juxtamedullary and superficial glomeruli to levels that are not different from those in LETO rats. Twenty-five-week-old rats demonstrated a marked effacement of podocyte foot processes in vehicle-treated OLETF rats that was largely abrogated in olmesartan-treated OLETF rats, which closely resembled that seen in vehicle-treated LETO rats. Original magnification ×3,000; bar = 0.5 μm. *$P < 0.05$; OLETF vs. LETO, †$P < 0.05$; OLETF + vehicle vs. OLETF + olmesartan or HHR. HHR, hydralazine, hydrochlorothiazide, and reserpine; LETO, Long-Evans Tokushima Otsuka rats; OLETF, Otsuka Long-Evans Tokushima Fatty rats.

in LETO rats at this age (**Figure 3d,e**). At 25 weeks of age, the mRNA levels of nephrin and podocin in vehicle-treated OLETF rats were lower than those in LETO rats in superficial and juxtamedullary glomeruli. In OLETF rats, treatment with olmesartan restored the levels of the gene expression of nephrin and podocin to the levels that did not differ from those in LETO rats. However, treatment with HHR did not alter the mRNA levels of nephrin and podocin in OLETF rats. At 15 and 25 weeks of age, mRNA levels of *eNOS*, *VEGF*, *VEGFR1*, and *VEGF2* in vehicle-treated OLETF rats were higher than those in LETO rats. In OLETF rats, treatment with olmesartan or HHR mix reduced expression of these genes to levels similar to those in LETO rats. Detailed data are available in the **Supplementary Figure S5** online.

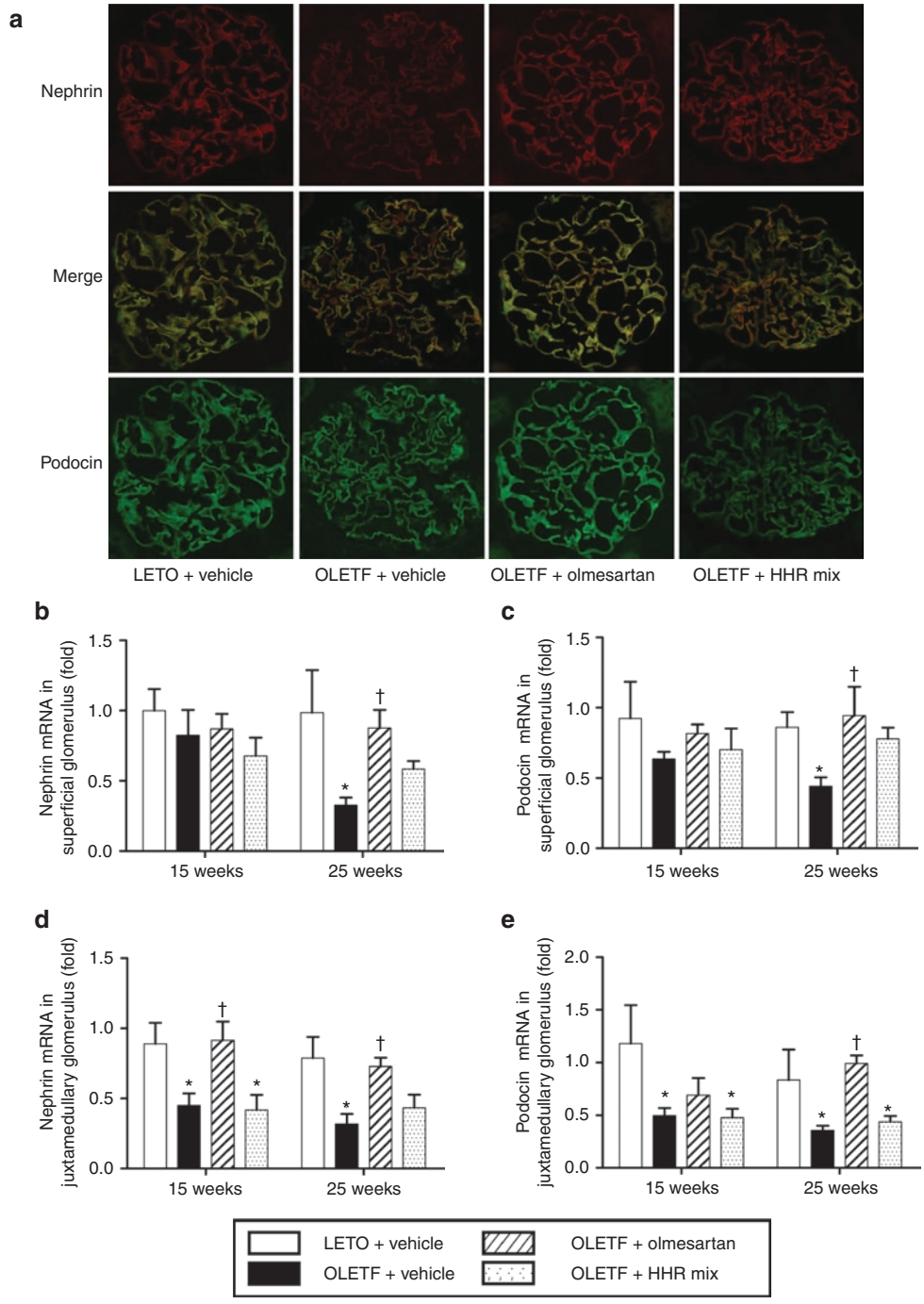

Figure 3 | (**a**) Immunofluorescence microscopy and (**b–e**) mRNA levels of nephrin and podocin. Immunofluorescence microscopy of nephrin and podocin reveals loss of immunostaining continuity and unbound nephrin and podocin in vehicle- and HHR-treated OLETF rats at 25 weeks of age. At 15 weeks of age, only in juxtamedullary glomeruli, the mRNA levels of nephrin and podocin are significantly lower in OLETF rats than those in LETO rats (**Figure 3d,e**). At 25 weeks of age, the mRNA levels of nephrin and podocin in vehicle-treated OLETF rats are lower than those in LETO rats in superficial and juxtamedullary glomeruli. In OLETF rats, treatment with olmesartan restores the gene expression of nephrin and podocin to levels that are not different from those in LETO rats. **P* < 0.05; OLETF vs. LETO, †*P* < 0.05; OLETF + vehicle vs. OLETF + olmesartan or HHR. HHR, hydralazine, hydrochlorothiazide, and reserpine; LETO, Long-Evans Tokushima Otsuka rats; OLETF, Otsuka Long-Evans Tokushima Fatty rats.

Ang II content in the kidney

Figure 4 shows Ang II content in the kidney. At 15 weeks of age, intrarenal Ang II contents were significantly higher in vehicle-treated OLETF rats than those in LETO rats (183 ± 33 vs. 107 ± 10 fmol/g). Ang II levels in the kidney were progressively augmented at 25 weeks of age in vehicle-treated OLETF rats (376 ± 27 fmol/g). Treatment with olmesartan prevented an increase in intrarenal Ang II levels in OLETF rats (26 ± 2 and 110 ± 17 fmol/g at 15 and 25 weeks of age, respectively). Ang II contents in the kidney did not differ

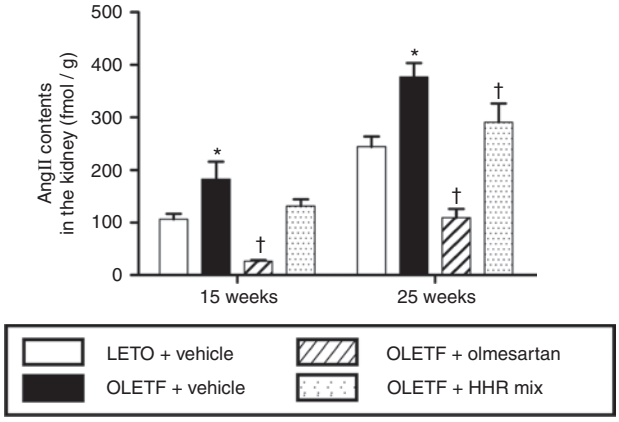

Figure 4 | Ang II contents in the kidney. Treatment with olmesartan prevents an increase in intrarenal Ang II levels in OLETF rats. *$P < 0.05$; OLETF vs. LETO, †$P < 0.05$; OLETF + vehicle vs. OLETF + olmesartan or HHR. Ang II, angiotensin II; HHR, hydralazine, hydrochlorothiazide, and reserpine; LETO, Long-Evans Tokushima Otsuka rats; OLETF, Otsuka Long-Evans Tokushima Fatty rats.

between vehicle- and HHR-treated OLETF rats (131 ± 13 and 291 ± 36 fmol/g at 15 and 25 weeks of age, respectively).

Glomerular DHE staining

DHE staining in glomeruli was significantly higher in vehicle-treated OLETF rats than in LETO rats at 15 and 25 weeks of age. Treatment with olmesartan prevented the increase in DHE staining in glomeruli until 25 weeks of age. Detailed data are available in the **Supplementary Table S2** online.

DISCUSSION

The present study demonstrated that podocyte injury occurs in juxtamedullary glomeruli prior to superficial glomeruli in type 2 diabetic OLETF rats with microalbuminuria. These data indicate, for the first time, that the initiation of microalbuminuria is accompanied by juxtamedullary glomerular podocyte injury in type 2 diabetes. We also showed that early treatment with olmesartan (an ARB) could prevent the occurrence of microalbuminuria in OLETF rats, as was recently demonstrated by the ROADMAP study in type 2 diabetic patients with normoalbuminuria.[14] Our data also indicate that these effects of olmesartan were associated with the prevention of juxtamedullary podocyte injury.

Early morphological changes in the glomeruli are usually subclinical and asymptomatic prior to the development of albuminuria.[1,22] Several studies have shown that OLETF rats exhibit apparent glomerular sclerosis and interstitial injuries with maintained plasma creatinine levels after 50 weeks of age.[10,18,19] In the present study, OLETF rats exhibited microalbuminuria (>1.0 mg/day) from 9 weeks of age and then progressed to overt proteinuria in an age-dependent manner. Although diabetic OLETF rats had a marked increase in UalbV at 25 weeks of age, histological analyses failed to show significant glomerular sclerosis. However, a more careful assessment of the morphology with desmin immunostaining revealed that podocyte injury exists only in juxtamedullary glomeruli with the presence of microalbuminuria at 15 weeks of age.

Contribution of podocyte functional abnormalities to the progression of proteinuria has been indicated by recent studies.[23–25] In the present study, we documented that juxtamedullary glomerular podocyte injury was observed at the initiation of microalbuminuria in type 2 diabetic rats. Kim *et al.*[26] found that the expression of nephrin, which is a functional molecule located between slit diaphragms or two adjacent foot processes of podocytes, plays a critical role in proteinuria associated with diabetes,[23,24,27] and that nephrin expression was significantly reduced in large glomeruli compared with small glomeruli in streptozotocin-induced diabetic rats. In the present study, nephrin and podocin were observed to exist separately in a merged image. We also showed that nephrin expression was markedly reduced in juxtamedullary glomeruli, whereas it was not altered in superficial glomeruli of type 2 diabetic rats with microalbuminuria. These data are consistent with the notion that the urinary albumin excretion considerably derives from juxtamedullary glomeruli during the early phase of albuminuria.

It has been reported that mechanical stretch induces morphological and cytoskeletal change in podocytes *in vitro*.[28] Recent reports also showed that mechanical stretch on cultured podocytes stimulated Ang II production, resulting in a reduction of nephrin expression, but not podocyte apoptosis.[29] These observations, combined with our findings, suggest that podocyte abnormalities, such as loss of nephrin and podocin, occur prior to podocyte detachment, or foot process effacement, in the juxtamedullary glomeruli of early diabetic nephropathy accompanied with hypertension. While glomerular size in juxtamedullary glomeruli tended to be larger than those in superficial glomeruli in OLETF rats at 15 weeks of age, these differences were not statistically significant. Because half of the left kidneys for paraffin embedding were perfused using saline, rather than formalin, it is possible that the glomerular size measurements lacked accuracy.

Interestingly, the present study also showed that treatment with an ARB prevented microalbuminuria via the protection of nephrin and podocin expression, whereas treatment with HHR did not. Thus, the protective effect of ARBs against podocyte injury cannot be explained simply by its blood pressure-lowering effects. Hoffmann[26,30] showed that transgenic rats overexpressing the AT1 receptor in podocytes developed proteinuria without changes in blood pressure. These data are consistent with a recent *in vitro* study,[31] where Ang II directly reduced nephrin expression in cultured podocytes. Nevertheless, we also observed that HHR treatment had moderate effects on juxtamedullary glomerular podocyte injury, as determined by desmin-staining, at the onset of microalbuminuria. These results suggest that a reduction in blood pressure can, in part, attenuate glomerular damage, as suggested by recent clinical studies.[32,33] Mori *et al.*[11] reported that juxtamedullary glomerular injury is mainly induced by high renal perfusion pressure, whereas superficial glomeruli are directly injured by Ang II in Ang II-infused hypertensive rats.

Previous studies have shown that glomerular capillary endothelial cell proliferation determined by CD34-staining and increases in renal *eNOS* and *VEGF* expression occurred

in diabetic rats.[34,35] In the present study, endothelial deterioration was not observed, but increased eNOS, VEGF, and VEGFR2 expression was observed in kidneys of 25-week-old OLETF rats. From these results, we speculate that eNOS and VEGF increase to maintain endothelial structure during the development of diabetic nephropathy. Furthermore, WT-1 analysis revealed that reduced podocyte number was not observed, although podocyte dysfunction, such as reductions in nephrin and podocin, was observed in OLETF rats. These data suggest that podocyte dysfunction, but not podocyte loss, plays an important role in the onset of microalbuminuria.

ARBs reduce intrarenal Ang II levels through prevention of AT1 receptor-mediated uptake of Ang II and/or intrarenal production of Ang II by overexpression of angiotensinogen.[36] In the present study, we observed that treatment with an ARB—olmesartan—strongly inhibits augmentation of intrarenal Ang II levels in OLETF rats, decreasing them to levels even lower than those in LETO rats. On the other hand, we previously showed that treatment with telmisartan, another ARB,[11] and temporary treatment with temocapril, an angiotensin-converting enzyme inhibitor,[10] could suppress development of both renal injury and augmentation of intrarenal Ang II in OLETF rats.

Previous studies have shown that olmesartan elicits beneficial effects against metabolic disorder.[37,38] In the present study, short-term treatment with olmesartan significantly decreased plasma triglyceride level, but did not change fat weight, glucose metabolism, and plasma insulin and adiponectin levels in 25-week-age OLETF rats. Thus, it is possible that the renoprotective effect of olmesartan is not mediated through its antimetabolic effect under the present experimental conditions. Lipid accumulation was observed in proximal tubular cells, but not glomeruli, of OLETF rats.

In conclusion, the present study demonstrated, for the first time, that the onset of microalbuminuria is associated with podocyte abnormalities in the juxtamedullary glomeruli prior to morphological glomerular injury in type 2 diabetic rats. Furthermore, our data also support the observations of the ROADMAP studies[14] and the concept that selective inhibition of Ang II in the early stages of type 2 diabetes could prevent diabetic nephropathy.

Supplementary material is linked to the online version of the paper at http://www.nature.com/ajh

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