EXPERIMENTAL STUDY

Blood Pressure-Independent Effect of Olmesartan on Albuminuria in Mice Overexpressing Renin

Its Beneficial Role in the ACE2/Ang (1-7)/Mas Axis and NADPH Oxidase Expression

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Summary

Enhanced renin-angiotensin activity contributes to hypertension, albuminuria, and glomerular hypertrophy. The angiotensin (Ang)-converting enzyme (ACE) 2/Ang (1-7)/Mas axis pathway acts against Ang II type 1 receptor (AT1R) signaling. We investigated whether olmesartan (Olm), an AT1R blocker, inhibits albuminuria independently of blood pressure and elucidated the potential mechanisms.

Three- to 4-month-old male mice overexpressing renin in the liver (Ren-TG) were given olmesartan (5 mg/kg/day) or hydralazine (Hyd) (3.5 mg/kg/day) orally for 2 months. Ren-TG mice had higher systolic blood pressure (SBP) than wild-type (WT) mice (158.2 ± 6.3 versus 112.8 ± 8.8 mmHg, n = 3-4, $P < 0.01$). Ren-TG mice treated with Olm or Hyd for 2 months had lower SBP than untreated Ren-TG mice. Urinary albumin excretion (UAE) was significantly increased in Ren-TG mice compared with WT mice (78.2 ± 31.2 versus 28.6 ± 13.8 μg/day, n = 5-6, $P < 0.01$). Olm treatment for 2 months reduced UAE, whereas Hyd treatment did not. Olm treatment reversed decreased gene and protein expressions of ACE2 and Mas receptor (Mas 1) in the kidney of Ren-TG mice and inhibited enhanced NADPH oxidase (Nox) 4 expression, whereas Hyd treatment had no influence. Furthermore, increased reactive oxygen species (ROS) in the kidney of Ren-TG mice were decreased by Olm treatment but not by Hyd treatment.

Olm treatment inhibits albuminuria and glomerular hypertrophy independently of blood pressure not only through its original AT1R blockade but also partly through the enhancement of the ACE2/Ang (1-7)/Mas axis and suppression of ROS generation.

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Key words: Renin-angiotensin system

Hypertension is one of the most important risk factors for chronic kidney diseases such as hypertensive nephrosclerosis. Enhanced activity of the renin-angiotensin system (RAS) causes hypertension and leads to organ damage, such as cardiac hypertrophy and renal dysfunction.[1,2] Animal studies using mice with a genetically clamped renin transgene in the liver (Ren-TG mice) demonstrated that elevated angiotensin (Ang) II causes high blood pressure, resulting in cardiac hypertrophy and renal damage.[3,4] It remains to be elucidated whether either high blood pressure or enhanced Ang II activity substantially contributes to RAS-induced renal dysfunction.

Angiotensin-converting enzyme (ACE)-related carboxypeptidase (ACE2), which is an enzyme similar to ACE, catalyzes the cleavage of Ang I to Ang (1-9) and Ang (1-7).[5-7] Ang (1-7) counterregulates Ang II actions through the activation of the Mas G protein-coupled receptor[8,9] and attenuates reactive oxygen species (ROS) partly through decrease in NADPH oxidase (Nox) expression,[10,11] which plays a key role in hypertension-induced ROS generation in the kidney.[12] Thus, the ACE2/Ang (1-7)/Mas axis pathway plays a protective role against RAS-induced renal damage.

Olmesartan (Olm) is an Ang II type 1 receptor (AT1R) antagonist that can inhibit albuminuria in chronic kidney disease and diabetic nephropathy.[13-15] Glomerular expression of ACE2 is reduced in diabetic mice with albuminuria,[16] and reduced ACE2 gene and protein expression has been confirmed in renal biopsies of patients with type 2 diabetes and kidney disease.[17] However, there are few reports of the expression of ACE2 when Olm is administered against hypertensive renal damage. In the present study, we investigated the inhibitory effects of Olm on

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urinary albumin excretion (UAE) in comparison with hydralazine (Hyd), a direct vasodilator, using Ren-TG mice, highlighting the role of the ACE2/Ang (1-7)/Mas receptor pathway and ROS generation.

Methods

Animals and reagents: Ren-TG mice (originally described as Ren-TG MK mice) were kindly provided by Drs. Oliver Smithies and Nobuyo Maeda (University of North Carolina at Chapel Hill, Chapel Hill, NC, USA). Briefly, a modified mouse renin transgene driven by a liver-specific albumin promoter/enhancer was inserted into the genome as a single copy at the liver-specific *ApoA1/ApoC3* locus. The resulting transgene expresses renin ectopically at constant high levels in the liver and leads to elevated plasma levels of active renin and Ang II.[3,4] Three- to 4-month-old male heterozygous Ren-TG mice (with one copy of the renin transgene), backcrossed to C57BL6/N mice for more than six generations, were used in the present study. The Ren-TG mice were administered olmesartan medoxomil (Olm; 5 mg/kg/day), hydralazine hydrochloride (Hyd; 3.5 mg/kg/day), or normal vehicle (Ren) for 2 months, as described previously.[1] The drugs were dissolved in the drinking water and given orally. Wild-type (WT) littermates were used as a control. The four groups (WT, Ren, Olm, and Hyd) were compared in the following experiments.

Olm was kindly provided by Daiichi-Sankyo (Tokyo, Japan). Hyd was purchased from Sigma-Aldrich (St. Louis, MO, USA). UAE was measured using ALBUWELL M TEST Kit (Exocell, Philadelphia, PA, USA). All procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (NIH) and were approved by the Institutional Animal Care and Use Committee of Hirosaki University Graduate School of Medicine.

Blood pressure measurement: Telemetry transmitters (PhysioTel®, HD-X11; Data Sciences International, St. Paul, MN, USA) were surgically implanted in an intramuscular pocket in the lower abdomen under isoflurane anesthesia. Catheter was inserted into the left internal carotid artery and the catheter tip was placed in the arch of aorta for the measurement of arterial blood pressure. Mice were singly housed following telemetry implantation and maintained on a 12-hour light-dark cycle (light on from 8:00 to 20:00). Seven days after the implantation, the transmitters were activated and the data collection was started. Systolic blood pressure (SBP) and pulse rate (PR) were monitored every 5 minutes for 10 seconds and stored. The average values of SBP and PR in the light period were calculated.[18]

Histological examination of the renal cortex: Kidney samples were fixed in 10% formalin. Paraffin-embedded tissues were cut into 5-μm sections and stained with periodic acid-Schiff (PAS) reagent, hematoxylin and eosin, and Masson's trichrome. The stained sections were examined with a Keyence all-in-one fluorescence microscope BZ-X710 (Keyence, Osaka, Japan). Glomerular hypertrophy was quantitatively assessed by the direct measurement of glomerular long diameter with PAS staining, and 10-12 randomly selected glomeruli of six mice were analyzed by the image analysis programs NIH Image J and BZ-X Analyzer (Keyence).

Analysis of mRNA expression by quantitative RT-PCR: Total RNA was extracted from the kidney cortex of the mice using RNeasy Protect Mini Kit (QIAGEN, Valencia, CA, USA). RNA was transcribed into first-strand cDNA using Omniscript RT kit (QIAGEN) according to the manufacturer's protocol. Quantitative reverse transcription polymerase chain reaction (RT-PCR) was performed using CFX connect (Applied Biosystems, Foster City, CA, USA) with TaqMan Universal PCR Master Mix (Applied Biosystems). The specific primers and probes (Applied Biosystems) were acquired to detect ACE2 (assay ID: Mm 001159003_m1), Mas1 (assay ID: Mm00434823_s1), Nox4 (assay ID: Mm00479246_m1), and glyceraldehyde-3-phosphatasedehydrogenase (GAPDH) (assay ID: Mm 99999915_g1 and Hs02758991_g1). The results were normalized by the mRNA expression levels of GAPDH.

Analysis of protein expression by western blot: The kidney was homogenized and centrifuged in 3% SDS buffer and 7.5% β-mercaptoethanol, and the supernatant was collected for western blot analysis. Protein concentrations were determined using Protein Quantification Assay (MACHEREY-NAGEL GmbH & Co. KG, Duren, Germany). The protein was separated by sodium dodecyl sulfide-polyacrylamide gel electrophoresis and electrophoretically transferred to a polyvinylidene fluoride membrane (Bio-Rad Laboratories, Berkeley, CA, USA). After blocking with 5% milk for 1 hour, the membranes were incubated with primary antibody for ACE2 (ab108252; abcam, Cambridge, UK) and Nox4 (ab133303; abcam) diluted to 1:2000 at 4°C overnight, respectively. Horseradish peroxidase and alkaline phosphate conjugated anti-rabbit antibody (ab6721; abcam) diluted 1:100000 were used for ACE2 and Nox4 as a secondary antibody. The primary antibody for beta-actin (13E5) Rabbit mAb (#5125; Cell Signaling Technology, Denvers, MA, USA) was diluted to 1:10000 at 4°C overnight. The protein bands were detected using Amersham ECL Prime Western Blotting Detection Reagents (GE Healthcare, Little Chalfont, UK). Densitometric analysis was performed with ChemiDoc™ XRS+ with Image Lab™ Software (Bio-Lad Laboratories), and a relative value of the target protein to beta-actin was calculated in each sample.

Evaluation of ROS generation in the kidney: To evaluate ROS generation in the kidney, we measured protein carbonyl levels, an indicator of oxidative stress, using OxiSelect™ Protein Carbonyl ELISA Kit (Cell Biolabs, Inc. San Diego, CA, USA) according to the manufacturer's instruction.[19]

Statistical analysis: All data are presented as means ± standard deviation (SD). Statistical analyses were performed using a commercially available software program (JMP Pro 13.1.1; SAS, Cary, NC, USA). Multiple comparisons were performed by analysis of variance followed by Tukey honest significant difference. A P-value < 0.05 was considered statistically significance.

Table. Physiological Parameters and Kidney Weight in Mice after Each Treatment for 2 Months

	WT	Ren	Olm	Hyd
	(n = 3)	(n = 4)	(n = 3)	(n = 3)
SBP (mmHg)	112.8 ± 8.8	158.2 ± 6.3†	123.5 ± 5.7	126.1 ± 6.5
PR (bpm)	594.1 ± 18.8	607.5 ± 24.3	620.3 ± 37.6	622.7 ± 28.4
	(n = 6)	(n = 6)	(n = 6)	(n = 6)
BW (g)	33.4 ± 3.0	33.7 ± 7.3	32.3 ± 3.0	32.3 ± 2.6
Kid/BW (mg/g)	5.4 ± 0.3	5.1 ± 0.5	5.6 ± 0.9	5.3 ± 0.7

Data are given as means ± SD. †P < 0.01 versus WT, Olm and Hyd groups. WT indicates wild type; Olm, olmesartan; Hyd, hydralazine; SBP, systolic blood pressure, PR, pulse rate; BW, body weight; and Kid, kidney.

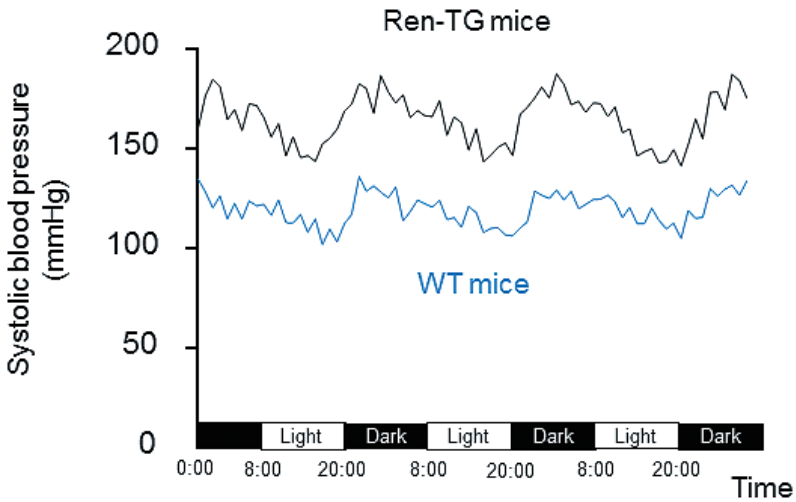

Figure 1. Representative recordings of systolic blood pressure by telemetry. Systolic blood pressures in WT mice (blue line) and Ren-TG mice (black line) recorded by telemetry system are shown.

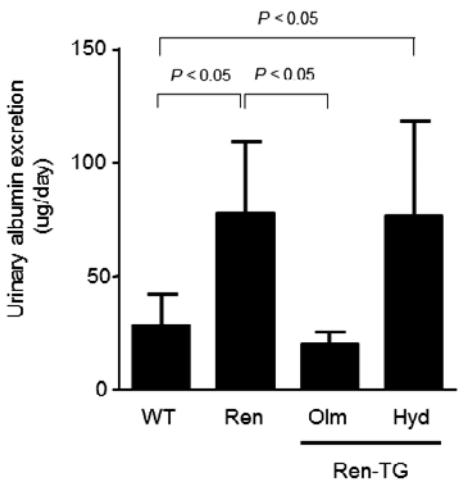

Figure 2. Assessment of urinary albumin excretion. Comparison of urinary albumin excretion (UAE) among the wild-type (WT), Ren-TG, olmesartan (Olm), and hydralazine (Hyd) groups. n = 5-6.

Results

Effects of Olm and Hyd treatment on SBP: After 2 months of treatment, the Ren group had higher SBP than the WT group (158.2 ± 6.3 versus 112.8 ± 8.8 mmHg, n = 3-4, P < 0.01) (Table). Representative recordings of SBP by telemetry system in both groups are shown in Figure 1. SBP in the Olm and Hyd groups after 2 months of treatment was significantly lower than that in the Ren group (123.5 ± 5.7 and 126.1 ± 6.5 mmHg, n = 3, P < 0.01 versus Ren group). There were no differences among the four groups in PR, body weight, and the ratio of kidney weight to body weight (Table).

Effects of Olm and Hyd treatment on UAE: UAE was significantly increased in the Ren group compared with the WT group (78.2 ± 31.2 versus 28.6 ± 13.8 μg/day, n = 5-6, P < 0.05) at the age of 6 months (Figure 2). Olm treatment for 2 months significantly reduced UAE to 20.3 ± 5.2 μg/day (n = 6, P < 0.05 versus Ren group). However, Hyd treatment for 2 months did not reduce UAE (77.0 ± 41.8 μg/day, n = 6).

Effects of Olm and Hyd treatment on glomerular hypertrophy: Histological assessment showed glomerular hypertrophy in the Ren group (Figure 3A). Olm treatment for 2 months attenuated glomerular hypertrophy, whereas Hyd treatment did not. Quantitative analysis showed that the long diameter of the glomeruli was significantly greater in the Ren group than in the WT group (1.44 ±

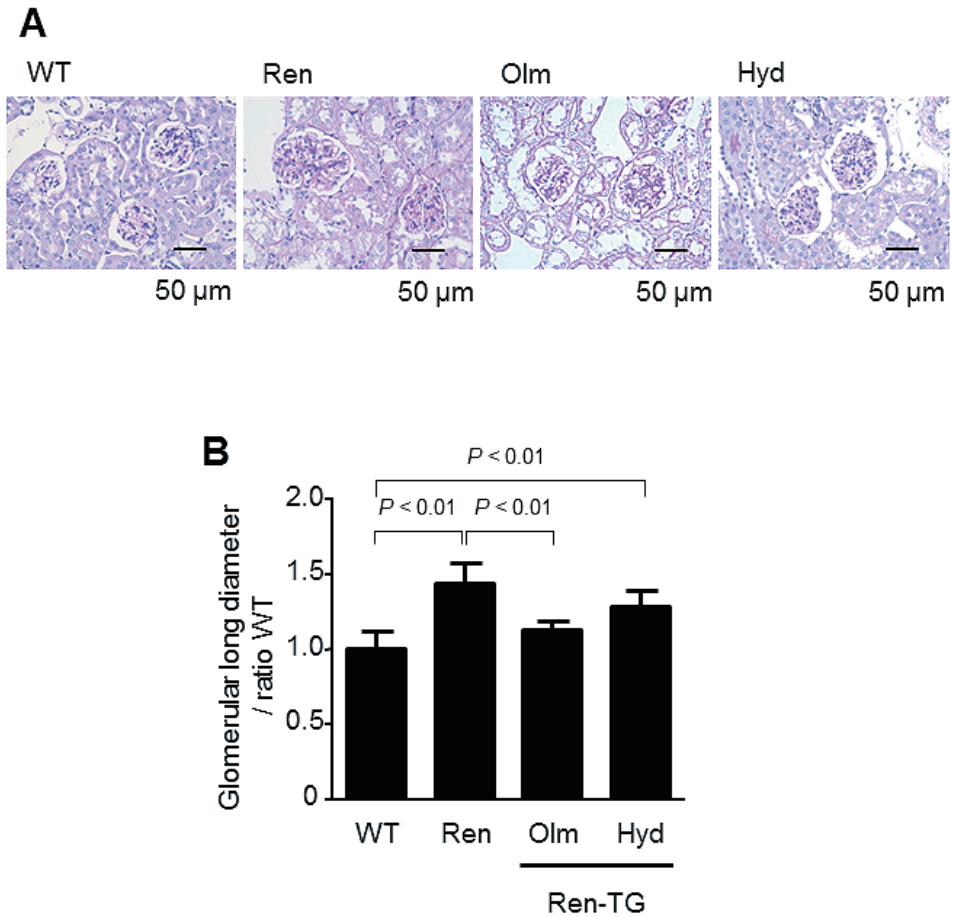

Figure 3. Assessment of glomerular hypertrophy. **A:** Representative images (400 ×) of kidneys stained with periodic acid-Schiff (PAS) in the wild-type (WT), Ren-TG, olmesartan (Olm), and hydralazine (Hyd) groups. **B:** Comparison of glomerular long diameter among the four groups. Data are shown as ratios to WT values (control). n = 5-6.

0.14 versus 1.00 ± 0.11, n = 5-6, P < 0.01) (Figure 3B). Olm treatment attenuated the increase in glomerular long diameter to a similar degree as in the WT group (1.13 ± 0.05, n = 6, P < 0.01 versus Ren group), whereas Hyd treatment had no effect (1.28 ± 0.11, n = 6).

Effects of Olm and Hyd treatment on gene and protein expressions of the ACE2/Mas axis: The gene expression of ACE2 (Figure 4A) and Mas 1 (Figure 4B) in the Ren group was significantly decreased compared with that in the WT group (0.77 ± 0.10 and 0.67 ± 0.07-fold, respectively, n = 5, P < 0.05 versus WT group). The decrease in gene expression of ACE2 observed in the Ren group was completely recovered with Olm treatment to the same degree as that in the WT group (1.01 ± 0.11-fold, n = 5, P < 0.01, versus Ren group); however, Hyd treatment did not improve this gene expression (0.81 ± 0.10-fold, n = 4) (Figure 4A). The decrease in gene expression of Mas 1 was also completely recovered with Olm treatment to the same degree as in that in the WT group (0.92 ± 0.10-fold, n = 5) but not with Hyd treatment (0.51 ± 0.10-fold, n = 5) (Figure 4B).

The protein expression of ACE2 in the Ren group was significantly decreased compared with that in the WT group (0.76 ± 0.07, n = 4, P < 0.05) (Figure 5A and 5B). The decrease in protein expression of ACE2 observed in the Ren group was completely recovered with Olm treatment (1.16 ± 0.12-fold, n = 4, P < 0.01, versus Ren group) but not with Hyd treatment (0.64 ± 0.13-fold, n = 4, P < 0.01 versus Ren group).

Effects of Olm and Hyd treatment on gene and protein expressions of NADPH oxidases: The activation of Nox plays a major role in Ang II-mediated ROS generation. The gene expressions of Nox1 and Nox2 did not differ among the four groups (data not shown). The gene expression of Nox4 was significantly higher in the Ren group than in the WT group (1.43 ± 0.14-fold, n = 5, P < 0.01) (Figure 4C). Olm treatment significantly decreased Nox4 expression (0.75 ± 0.13-fold, n = 5, P < 0.01, versus Ren group), whereas Hyd treatment did not (1.29 ± 0.20-fold, n = 5). The protein expression of Nox4 was significantly higher in the Ren group than in the WT group (1.82 ± 0.49-fold, n = 4, P < 0.05) (Figure 5C and D). Olm treatment significantly decreased Nox4 protein expression (0.86 ± 0.14-fold, n = 4, P < 0.01, versus Ren group), whereas Hyd treatment did not (1.32 ± 0.37-fold, n = 4).

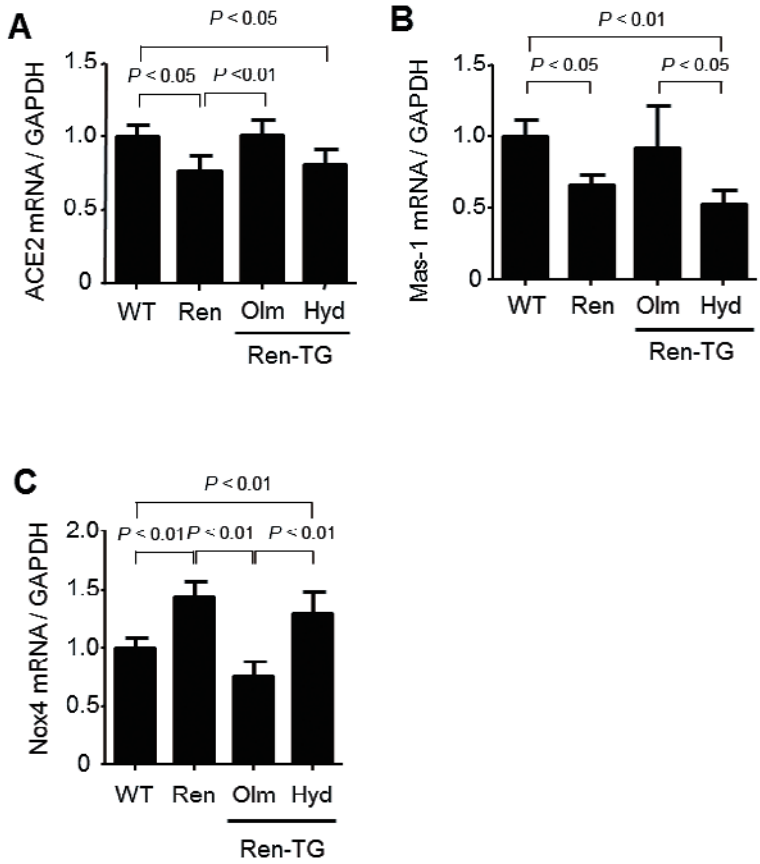

Figure 4. Assessment of gene expression in the kidney. Comparison of gene expression of angiotensin-converting enzyme 2 (ACE2) (**A**), Mas 1 (**B**), and NAPDH oxidase (Nox) 4 (**C**) in the kidney among the wild-type (WT), Ren-TG, olmesartan (Olm), and hydralazine (Hyd) groups. Data are normalized to glyceraldehyde-3-phosphatasedehydrogenase (GAPDH) and shown as ratios to WT values (control). n = 4-5.

Effects of Olm and Hyd treatment on ROS generation: Protein carbonyl levels in the kidney were increased in the Ren group compared with the WT group (0.34 ± 0.08 versus 0.15 ± 0.06 nmol/mg, n = 4, $P < 0.01$) (Figure 5E). Olm treatment inhibited increased protein carbonyl levels (0.13 ± 0.03 nmol/mg, n = 4, $P < 0.01$ versus Ren group), whereas Hyd treatment did not (0.36 ± 0.02 nmol/mg, n = 4).

Correlations between albuminuria and ACE2/Mas expression: The gene expressions of ACE2 and Mas 1 were both negatively correlated with UAE (r^2 = 0.621, $P <$ 0.01, and r^2 = 0.401, $P < 0.05$, respectively, both n = 14) (Figure 6A and B).

Discussion

Major findings: In the present study, we showed that treatment with Olm or Hyd for 2 months lowered elevated SBP to a similar degree, but only Olm treatment inhibited albuminuria and glomerular hypertrophy in Ren-TG mice. Olm treatment reversed the decreased gene and protein expressions of ACE2 observed in Ren-TG mice and inhibited the enhanced gene and protein expressions of Nox4, whereas Hyd treatment had no influence on them. Furthermore, ROS generation was increased in Ren-TG mice and was suppressed by Olm treatment but not by Hyd treatment. These findings indicate that Olm treatment inhibits albuminuria and glomerular hypertrophy independently of SBP not only through its original AT1R blockade but also through the enhancement of the ACE2/Ang (1-7)/Mas axis pathway and suppression of ROS generation.

Effects of Olm on albuminuria: Ren-TG mice are driven by a liver-specific albumin promoter/enhancer that is inserted into the genome as a single copy at the liver-specific *ApoA1/ApoC3* locus. The resulting transgene expresses renin ectopically at a constant high level in the liver and leads to elevated plasma levels of active renin and Ang II. As expected, Ren-TG mice have high blood pressure, albuminuria, and kidney disorders.[2-4] Importantly, their glomeruli are exposed to two stimuli: increased blood pressure and increased levels of plasma Ang II. To understand the different roles of these two stimuli in albuminuria, we administered two antihypertensives to Ren-TG mice: Olm (a depressor by AT1R blockade) and Hyd (a direct vasodilator). The results clearly showed that only Olm treatment inhibited UAE, although both antihypertensives similarly lowered elevated blood pressure in Ren-TG mice. A recent report showed that despite equiva-

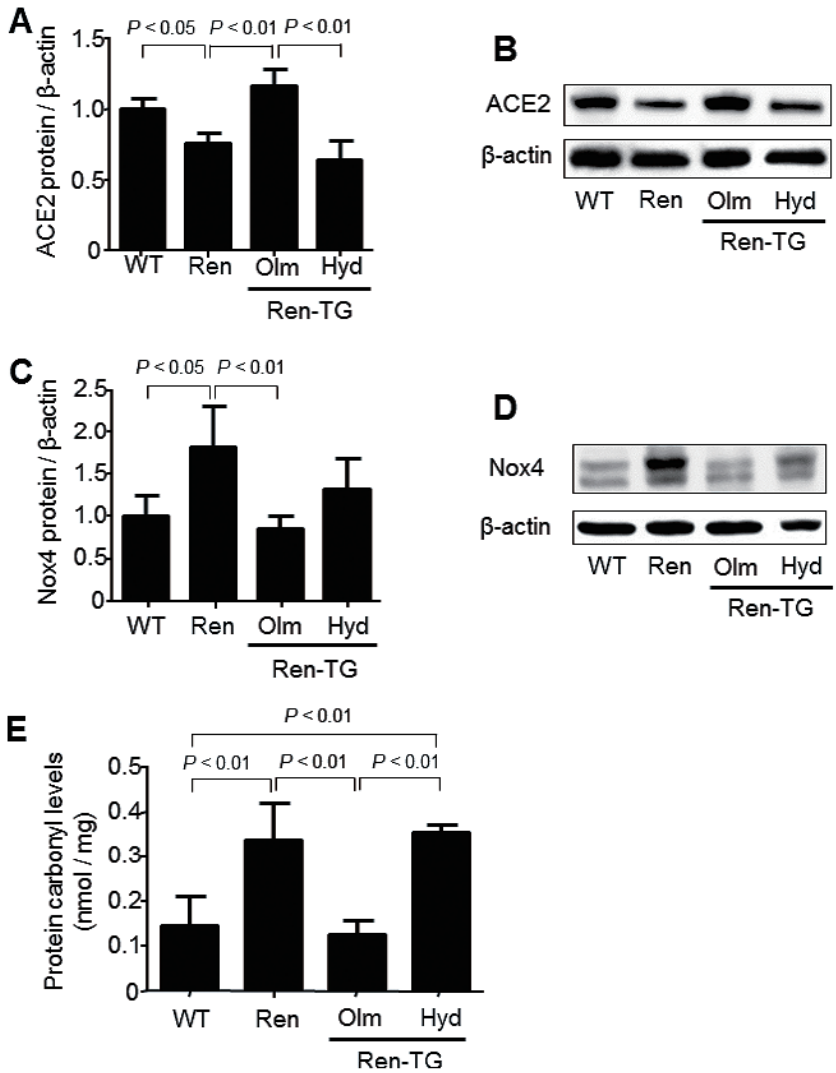

Figure 5. Assessment of protein expression and ROS generation in the kidney. Comparison of protein expression of angiotensin-converting enzyme 2 (ACE2) (**A** and **B**), and NAPDH oxidase (Nox) 4 (**C** and **D**) in the kidney among the wild-type (WT), Ren-TG, olmesartan (Olm), and hydralazine (Hyd) groups. Data are normalized to beta-actin and shown as ratios to WT values (control). $n = 4$. Comparison of protein carbonyl levels (**E**) in the kidney among the wild-type (WT), Ren-TG, olmesartan (Olm), and hydralazine (Hyd) groups. $n = 4$.

lent antihypertensive effects of nifedipine-CR (a calcium channel blocker), temocapril (an ACE inhibitor), and candesartan (an angiotensin receptor blocker), only candesartan suppressed albuminuria.[20] These findings indicate that enhanced AT1R signaling by increased RAS activity, not high blood pressure, is considerably important in the progression of albuminuria.

Potential mechanism involved in the inhibitory effects of Olm on albuminuria: Koka, *et al*. showed that Ang II acts to downregulate ACE2 in hypertensive kidney through the activation of the ERK 1/2 and p38 MAP kinase pathway.[21] Soler, *et al*. also showed that hypertensive renal disease markedly reduces ACE2 expression in the kidney cortex evaluated by ACE2 immunostaining.[22] Furthermore, Oudit, *et al*. showed that deletion of ACE2 leads to the development of Ang II-dependent renal damage,[23] suggesting that ACE2 acts as a renoprotective target in hypertensive renal disease. However, there are few reports about whether the expression of ACE2 in the kidney cortex increases when Olm is administered in hypertensive renal disease. The present study showed that the gene and protein expressions of the ACE2 and Mas 1 were decreased in Ren-TG mice and that Olm treatment, but not Hyd treatment, improved their expressions. Thus, Olm treatment may have renoprotective effects not only through AT1R blockade but also partly through the activation of the ACE2/Ang (1-7)/Mas axis pathway.

It is of considerable importance to elucidate the mechanism by which AT1R blockade causes elevation of ACE2 gene expression. Patel, *et al*. showed that activated Ang II-AT1R signaling generates ROS, which in turn results in the activation of p38 mitogen-activated protein

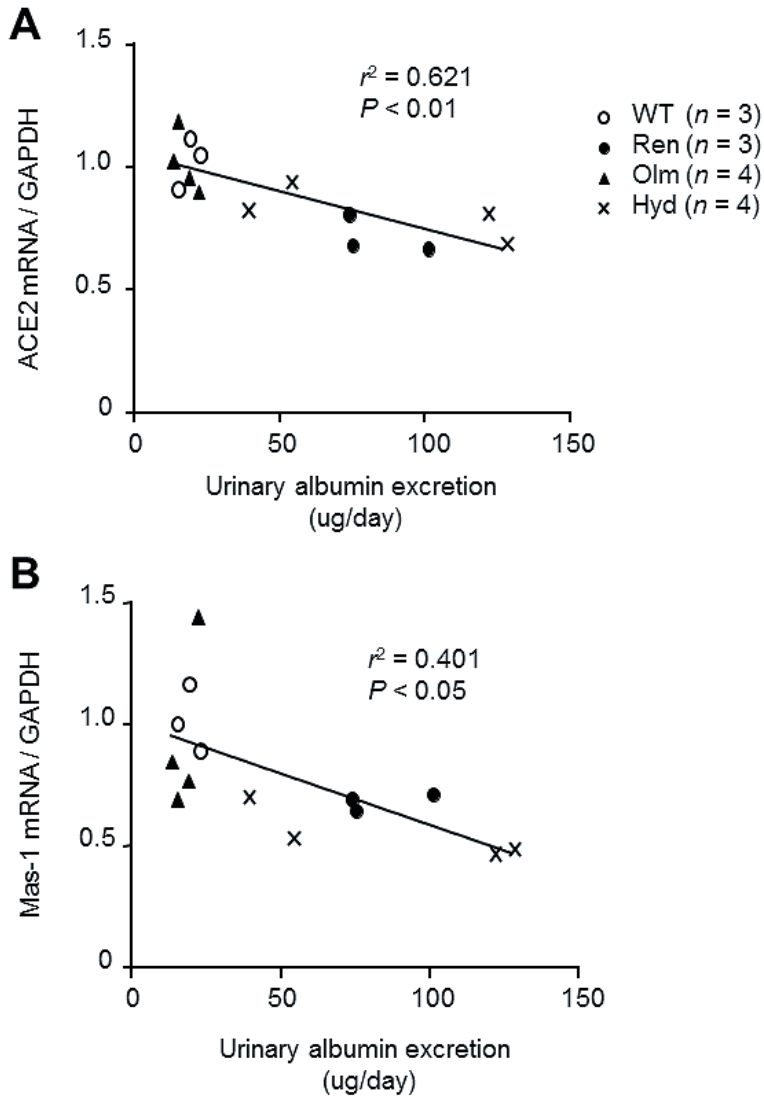

Figure 6. Correlations between albuminuria and ACE2/Mas-1 expression. Correlations between albuminuria and the gene expressions of ACE2 (**A**), and Mas-1 (**B**) in the kidney of the wild-type (WT), Ren-TG, olmesartan (Olm), and hydralazine (Hyd) groups. n = 3-4.

kinase (MAPK) signaling.[24] Moon, *et al*. also showed that Ang (1-7) attenuated Ang II-mediated NAPDH oxidase activation and ROS production in diabetic glomeruli and mesangial cells and that Ang II-induced NF-κB and MAPK signaling activation was also attenuated by Ang (1-7) in mesangial cells.[25] Furthermore, Wysocki, *et al*. showed that NADPH oxidase activity was increased in the kidney cortex of ACE2 knockout (ACE2KO) mice and that administration of an angiotensin receptor blocker (losartan) resulted in the normalization of NAPDH oxidase activity in the ACE2KO.[26] These reports indicate that the Ang II-AT1R signaling pathway regulates ACE2 expression partly through ROS-mediated p38 MAPK signaling. Because ROS are key regulators of ACE2 gene expression, we measured Nox4 expression in the kidney. We found that Nox4 expression was increased in Ren-TG mice and was suppressed by Olm treatment. We further evaluated ROS generation by protein carbonyl levels and showed that ROS generation increased in Ren-TG mice and was suppressed by Olm treatment but not by Hyd treatment. These findings indicate that the beneficial effects of Olm on ACE2 expression are partly mediated by the inhibition of Ang II-AT1R-associated ROS generation.

Recently, glomerular hypertrophy was identified as an integral feature of hypertensive nephropathy and appeared to precede rather than compensate for glomerulosclerosis.[27] Hyperfiltration is mediated by increased glomerular capillary pressure in hypertension and leads to progressive decrease in the glomerular filtration rate. In the present study, Ren-TG mice showed glomerular hypertrophy that was improved by Olm treatment but not by Hyd treatment. These findings indicate that Olm treatment reduced RAS-induced albuminuria not only by reducing hyperfiltration through lowering of capillary pressure but

also by partial anti-inflammatory effects through enhanced ACE2.[28,29]

Limitations: There are some limitations to the present study. First, we did not evaluate the detailed mechanisms that ACE2/Mas pathway is downregulated in Ren-TG mice and that Olm upregulates expressions of ACE2 and Mas 1. Further intensive studies are required to unravel this important issue. Second, we have not considered whether other ARBs have similar effects and have not considered the effects of other types of antihypertensive, such as calcium channel blockers and diuretics.[30]

Conclusions

The present study showed that Olm treatment inhibits albuminuria with hypertensive renal damage not only through its original AT1R blockade but also partly through the inhibition of ROS generation and the enhancement of the ACE2/Ang (1-7)/Mas axis pathway. The beneficial effects of Olm treatment on RAS-induced albuminuria have important clinical implications for renoprotective effects in humans.

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Disclosures

Conflicts of interest: Dr. Shimada received research funding from Daiichi-Sankyo. Dr. Okumura received Speakers' Bureau/Honorarium from Daiichi-Sankyo. Dr. Tomita received research funding from Boehringer Ingelheim, Bayer, Daiichi-Sankyo, and Pfizer and Speakers' Bureau/Honorarium from Boehringer Ingelheim, Bayer, and Daiichi-Sankyo. The other authors have no disclosures.

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