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Influence of Olmesartan on Sirtuin 1 mRNA Expression in 5/6 Nephrectomized Spontaneously Hypertensive Rats

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ABSTRACT

Background Recent studies revealed that sirtuin 1 (SIRT1) has a relation to the mechanism of transforming growth factor-beta (TGF-beta) mediated apoptosis in glomerular mesangial cells and plays an important role in blood pressure regulation. It has been suggested that SIRT1 contributes to the renoprotective effect of angiotensin receptor blocker (ARB), but this has not yet become clearly recognized. In this study, we examined the relationship between SIRT1 and the therapeutic effect of olmesartan on renal injury in nephrectomized spontaneously hypertensive rats (SHRs).

Methods Male Wistar rats and 5/6 nephrectomized (5/6Nx) SHRs were assigned to 5 groups as follows: group A, Wistar rats; group B, Wistar rats administered high-dose olmesartan (15 mg/kg/day); group C, 5/6Nx SHRs; group D, 5/6Nx SHRs administered low-dose (3 mg/kg/day) olmesartan; and group E, 5/6Nx SHRs administered high-dose olmesartan. The drugs were administered for 12 weeks. Blood pressure and urinary protein excretion were measured every 4 weeks. Serum creatinine, glomerular sclerosis, SIRT1 mRNA level, TGF-beta mRNA level and *klotho* mRNA level were measured at the end of the examination.

Results Systolic blood pressure, urinary protein excretion, serum creatinine and glomerular sclerosis in Wistar rats were significantly lower than that of 5/6Nx SHRs. Among 5/6Nx SHRs, high doses of olmesartan significantly decreased urinary protein excretion, serum creatinine and glomerular sclerosis compared to the non-treated and low-dose olmesartan groups. Expression of SIRT1 and *klotho* mRNA were significantly down-regulated in 5/6Nx SHRs; however, olmesartan did not attribute to any change in gene expression. Expression of TGF-beta mRNA was significantly increased in 5/6Nx SHRs, and olmesartan did not affect the level of TGF-beta mRNA expression.

Conclusion Expression of SIRT1 is decreased in 5/6Nx SHRs compared to Wistar rats. Olmesartan suppressed glomerular sclerosis, but did not increase the expression of SIRT1, suggesting that the renoprotective effect of olmesartan is independent of the SIRT1 pathway.

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Abbreviations: 5/6Nx, 5/6 nephrectomized; ARB, angiotensin receptor blocker; IGS, index of glomerular sclerosis; PPAR, peroxisome proliferator-activated receptor; SHR, spontaneously hypertensive rat; Sir2, silent information regulator 2; SIRT1, sirtuin 1; TGF-beta, transforming growth factor-beta

Key words angiotensin receptor antagonists; chronic renal failure; sirtuin; spontaneously hypertensive rat

Recent studies revealed that caloric restriction increases the life span of rats and positively affects various diseases such as diabetes, atherosclerosis, neurodegenerative diseases, cancer and age-associated renal injury.[1] The relationship between such favorable effects and caloric restriction was found from the observation of budding yeast. Guarente and colleagues[2] discovered that Sir2 (silent information regulator 2), the first sirtuin family member, increases life span. In mammals, seven homologues of Sir2 [sirtuin 1 (SIRT1) to sirtuin 7] have been detected. SIRT1 is mainly expressed in organs such as the kidney, liver and brain, and adipose tissue. Expression of SIRT1 increases with caloric restriction, whereas it decreases with senescence. SIRT1 inhibits transforming growth factor-beta (TGF-beta)-induced apoptosis in glomerular mesangial cells.[3]

There are convincing data that the renin-angiotensin system is a major mediator of renal injuries. Blood pressure control using angiotensin receptor blocker (ARB) can reduce the progression of nephropathy.[4] Several studies report that SIRT1 plays an important role in blood pressure regulation.[5–8] It is considered that SIRT1 contributes to the renoprotective effect of renin-angiotensin system blockade.[1]

Angiotensin II participates in various renal lesions and ARB has a kidney protective effect. It is reported that telmisartan, an angiotensin II type 1 receptor blocker, increases the expression of SIRT1 mRNA.[9] SIRT1 also may contribute to kidney protection by ARB, but this has not yet been clearly proven.

In this study, we examined the correlation between SIRT1 and the therapeutic effect of olmesartan, another ARB on renal injury in nephrectomized spontaneously hypertensive rats (SHRs).

Table 1. Primer sequences

	Forward primer	Reverse primer
TGF-beta	5'-**CCTGCCCCTACATTTGGA**-3'	5'-**TGGTTGTAGAGGGCAAGGAC**-3'
Sirtuin 1	5'-**CAGTGAGAAAATGCTGGCCTA**-3'	5'-**TTGGTGGTACAAACAGGTATTGA**-3'
Klotho	5'-**CAAGAAGTTCATAATGGAAAGCTTAAA**-3'	5'-**ATGCGGTGTACCCAATGAC**-3'
Beta-actin	5'-**CTGGCTCCTAGCACCATGA**-3'	5'-**TAGAGCCACCAATCCACACA**-3'

TGF-beta, transforming growth factor-beta.

MATERIALS AND METHODS

Animals

Male SHRs of the Izumo strain were obtained from Japan SLC (Shizuoka, Japan). As controls, male Wistar rats were obtained from Japan SLC. All rats were maintained in a room at a controlled temperature of 24 ± 2 ˚C with a 12-h light-dark cycle. Animals were given standard pellet chow and tap water. All experimental procedures were carried out in accordance with the Animal Experimentation Guidelines of Tottori University.

Establishment of model

Male 6-week-old SHRs underwent 5/6-nephrectomy. A surgical excision of approximately 2/3 of the renal cortex of the left kidney was performed. One week later, the right kidney was removed. One week after the surgery baseline measurements of body weight, blood pressure, urine volume and urinary protein were performed. Blood pressure was measured in the conscious state using the tail-cuff method with a sphygmomanometer (Softron, Tokyo, Japan). Urine was collected from individual rats housed in metabolic cages for 24 h. Olmesartan as ARB was administered orally once a day using a gastric tube. Rats were divided into 5 experimental groups: group A, Wistar rats (n = 8); group B, Wistar rats administered high-dose olmesartan (15 mg/kg/day, n = 8); group C, SHRs (n = 8); group D, SHRs administered low-dose olmesartan (3 mg/kg/day, n = 8) and group E, SHRs administered high-dose olmesartan (15 mg/kg/day, n = 8). Dosages of olmesartan were selected based on previous reports.[10, 11] The drug was administered once a day for 12 weeks. Measurements of body weight, blood pressure, urine volume and urinary protein was performed every 4 weeks in each group.

After 12 weeks of drug administration, rats were sacrificed under pentobarbital anesthesia. Blood samples were obtained prior to death. Serum samples were frozen and stored at –80 ˚C, and serum creatinine was measured. Remnant kidneys were removed, and a part of each kidney sample was fixed in 10% buffered formalin and embedded in paraffin for histological analysis.

RNA extraction and reverse transcription PCR analysis

Tissue samples were homogenized and total RNA was extracted using an RNasey Mini Kit (Qiagen, Hilden, Germany). RNA concentration was determined by measuring absorbance at 260 nm and the RNA quality was verified by electrophoresis on an ethidium-bromide-stained 1% agarose gel. About 2 μg of total RNA was reverse transcribed in a final volume of 10 μL containing 4 μL of 5 × standard buffer, 2 μL of 0.1 M dTT, 1 μL of M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA), 1 μL of 0.5 μg/μL Random Primer (Promega, Madison, WI), 1 μL of 20 mM dNTPs and 1 μL of RNase Inhibitor (Wako Pure Chemical Industries, Osaka, Japan). Samples were incubated at 37 ˚C for 60 min, followed by 95 ˚C for 5 min and cooling to 4 ˚C for 5 min.

Real-time PCR

For quantitative real-time PCR, we used 10 μL of reverse transcribed samples: EXPRESS qPCR Super mix Premixed ROX (Invitrogen), 5 μL; forward primer (10 μM), 0.9 μL; reverse primer (10 μM), 0.9 μL; a TaqMan Probe (2 μM), 1.2 μL (Roche) and a cDNA sample, 2 μL. mRNA levels of TGF-beta, SIRT1 and *klotho* were assessed by real-time PCR assays using beta-actin as a housekeeping gene. The forward and reverse primer sequences used for this study are shown in Table 1. The thermal cycler conditions were as follows: hold at 95 ˚C for 30 s and 60 ˚C for 1 min.

Histological analysis

Three-micrometer sections of formalin-fixed, paraffin-embedded kidney samples were stained with periodic acid-Schiff and periodic acid-methenamine silver. To calculate focal glomerular sclerosis, 100 to 150 glomeruli from each stained specimen were examined. The degree of sclerosis in each glomerulus was subjectively graded on a scale of 0 to 4 as follows: Grade 0, no change; Grade 1, sclerotic area less than or equal to 1/4 of the glomerulus or the presence of distinct adhesion between the capillary tuft and Bowman's capsule; Grade 2, sclerosis of 1/4 to 1/2 of the total glomerular area; Grade 3, sclerosis of 1/2 to 3/4 of the total glomerular

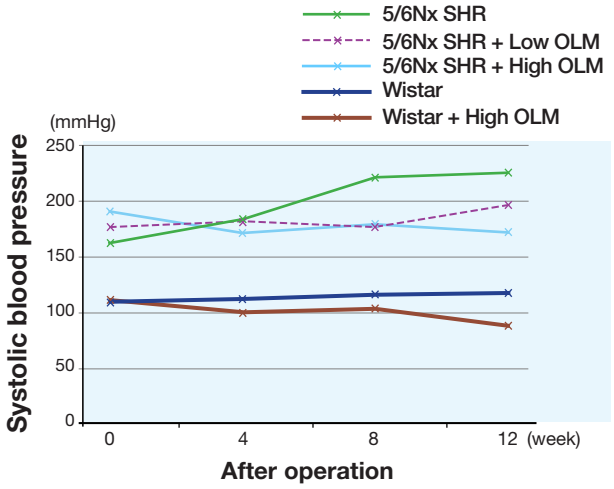


Fig. 1. Systolic blood pressure during the experimental period. The systolic blood pressure is higher in SHRs than in Wistar rats. At week 12, olmesartan significantly decreased the systolic blood pressure dose dependently ($P < 0.01$). Low OLM, olmesartan 3 mg/kg/day; High OLM, olmesartan 15 mg/kg/day; 5/6Nx SHR, 5/6 nephrectomized spontaneously hypertensive rat.

area; and Grade 4, sclerosis of more than 3/4 of the glomerulus. The index of glomerular sclerosis (IGS) was calculated by using the following formula as previously reported[10]:

$$IGS = \frac{(1 \times N1) + (2 \times N2) + (3 \times N3) + (4 \times N4)}{(N0 + N1 + N2 + N3 + N4)}$$

where N is the number of glomeruli at each grade of sclerosis.

Statistical analysis

Statistical significance of intergroup differences in quantitative data was assessed by Student's *t*-test (StatFlex version 6 for Windows, Artec, Osaka, Japan). $P < 0.05$ was considered significant.

RESULTS

Blood pressure

The systolic and diastolic blood pressure in the rats during the 12-week experimental period is shown in Fig. 1. The systolic blood pressure was higher in 5/6Nx SHRs than in Wistar rats during the 12-week experimental period. The systolic pressure increased throughout the experimental period in the nephrectomized group (group C). At week 12, olmesartan decreased systolic blood pressure dose dependently (groups D and E) ($P < 0.01$). However, there was no significant difference in systolic blood pressure between the high-dose olmesartan group (group E) and low-dose group (group D) at week 4 and week 8. There was a similar trend for diastolic pressure (data not shown).

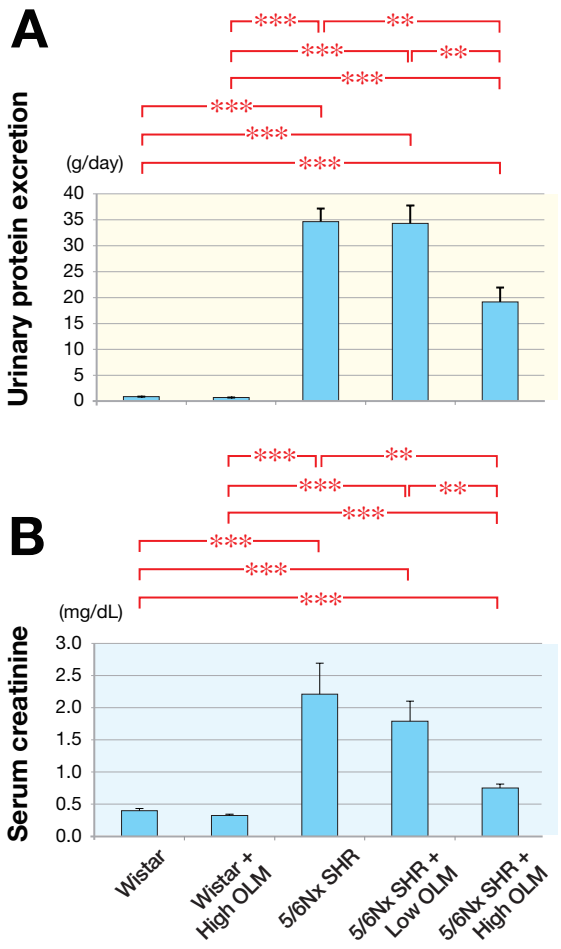


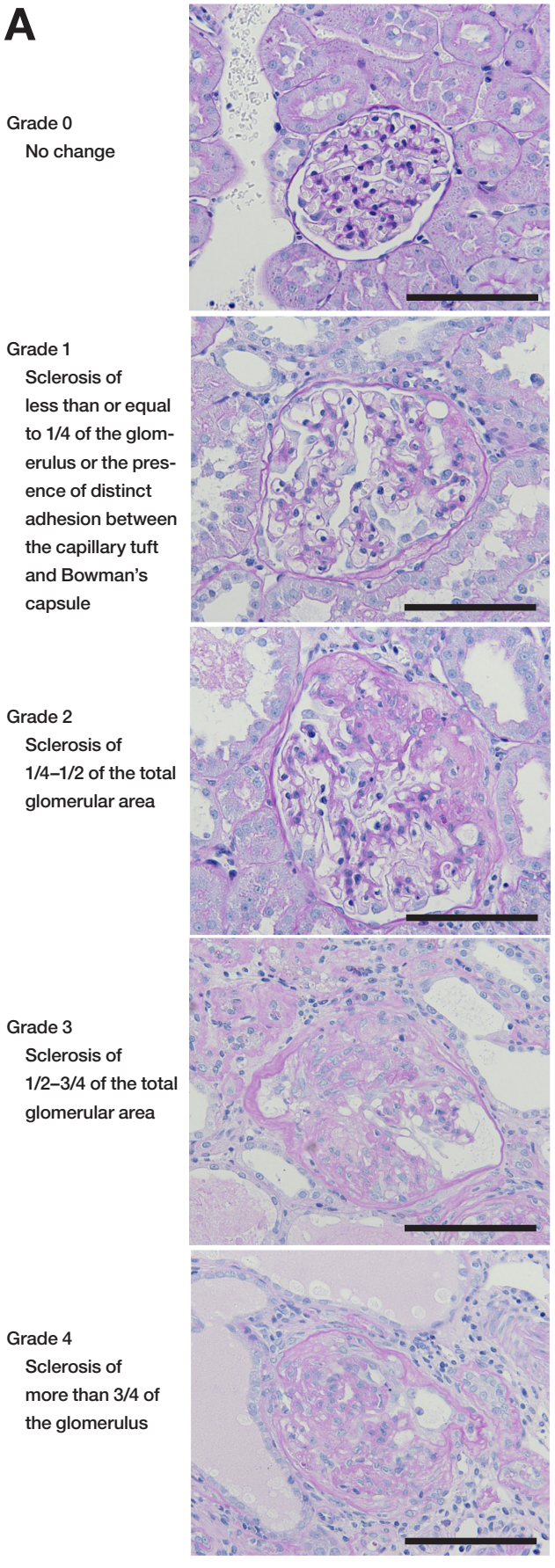
Fig. 2. Urinary protein excretion and serum creatinine.
A: Urinary protein excretions are lower in the Wistar rats than in the SHRs. Among SHRs, the high-dose olmesartan group show lower urinary protein excretion.
B: The serum creatinine levels are significantly higher in the SHRs than in the Wistar rats. Among SHRs, the high-dose olmesartan group show lower serum creatinine, while there is no difference between the low-dose group and non-treated group.
$^{**}P < 0.01$, $^{***}P < 0.001$. Abbreviations, refer to the legends of Fig. 1.

Proteinuria

Figure 2A shows total urinary protein excretion in each group at the end of the examination. Urinary protein levels were lower in the Wistar rats (groups A and B) than SHRs (groups C, D and E). Among SHRs, those treated with high-dose olmesartan (group E) showed lower urinary protein excretion.

Serum creatinine

Figure 2B shows serum creatinine in each group at week 12. The serum creatinine levels were significantly higher in SHRs than Wistar rats. Among SHRs, those treated with high-dose olmesartan (group E) showed lower se-



rum creatinine, while there was no difference between those treated with low-dose olmesartan (group D) and non-treated rats (group C).

Histological findings in the kidney

Figure 3 shows the IGS in each group. In the Wistar rats (groups A and B), only a few sclerotic changes of glomeruli and interstitial fibrosis were observed. The value of the index treated with high-dose olmesartan group (group E) was significantly lower than that of low-dose olmesartan group (group D) and non-treated group (group C), respectively.

mRNA quantification of TGF-beta, SIRT1 and *klotho*

The SIRT1 mRNA level in the nephrectomized rats was significantly decreased when compared with that in the Wistar rats. Administration of olmesartan did not change in the level of SIRT1 expression among SHRs (Fig. 4A). The level of TGF-beta mRNA expression in the non-treated SHRs (group C) was significantly higher than the Wistar rats (groups A and B). Olmesartan did not affect the level of TGF-beta mRNA expression among each group (Fig. 4B). The *klotho* mRNA level in the nephrectomized rats was significantly lower than that in the Wistar rats, and olmesartan did not affect the level of *klotho* mRNA expression (Fig. 4C).

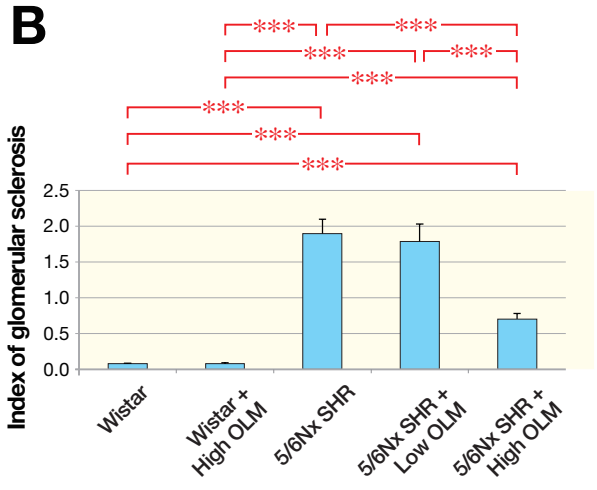


Fig. 3. Index of glomerular sclerosis (IGS).
A: Classification of glomerular sclerosis by periodic acid-Schiff stain. Bar = 200 μm.
B: The values of the index in the high-dose olmesartan groups are significantly lower than those in the low-dose groups and non-treated groups. ***$P < 0.001$. Abbreviations, refer to the legends of Fig. 1.

$$\text{IGS} = \frac{(1 \times N1) + (2 \times N2) + (3 \times N3) + (4 \times N4)}{(N0 + N1 + N2 + N3 + N4)}$$

N, the number of glomeruli at each grade of sclerosis.

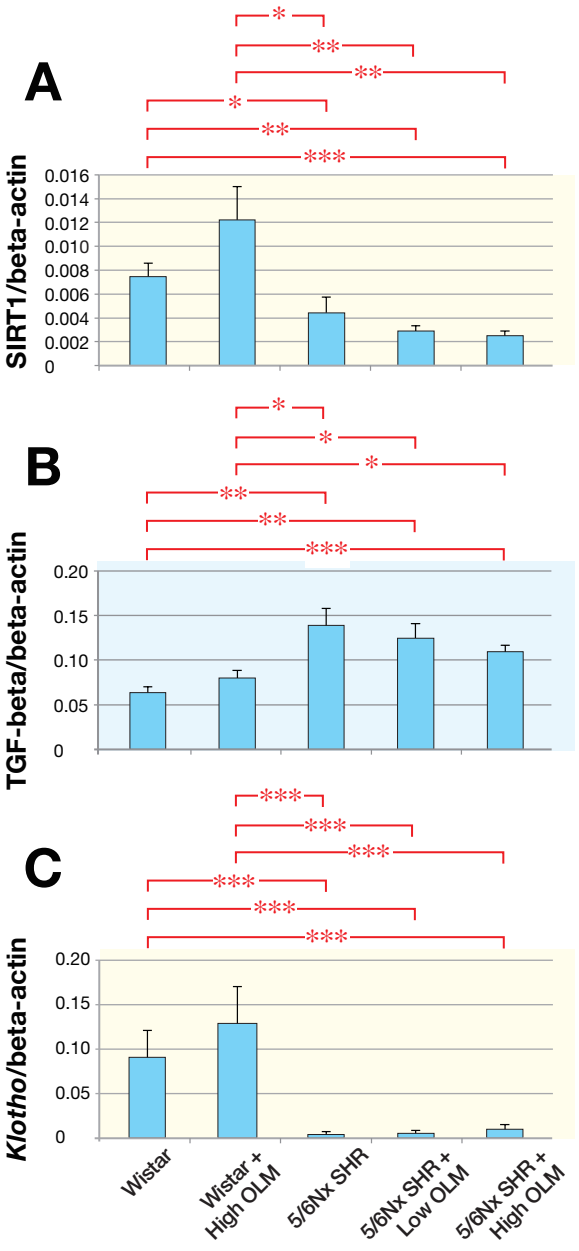


Fig. 4. Levels of mRNA for sirtuin 1 (SIRT1), transforming growth factor (TGF)-beta and *klotho* gene expression in renal tissue. The levels were standardized using the beta-actin level.

A: SIRT1 mRNA levels in the SHRs are significantly decreased than in the Wistar rats. Among SHRs, olmesartan does not attribute to any change in the level of SIRT1 mRNA expression.

B: Levels of TGF-beta mRNA expression in the SHRs are significantly higher than in the Wistar rats. Among SHRs, olmesartan does not affect the level of TGF-beta mRNA expression.

C: Levels of *klotho* mRNA expression in the SHRs are significantly lower than in the Wistar rats. Among SHRs, olmesartan does not affect the level of *klotho* mRNA expression.

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. Abbreviations, refer to the legends of Fig. 1.

DISCUSSION

In the present study, olmesartan decreased proteinuria and ameliorate histological renal damage in SHRs. SIRT1 expression in the kidney decreased in SHRs compared to normotensive Wistar rats, and olmesartan did not increase SIRT1 expression in the kidneys of SHRs.

SHRs have been used as a model for essential hypertension in humans, and 5/6Nx rats have commonly been used as an experimental models for chronic renal failure in humans.[10] In the present study, we combined these two models, and prepared 5/6Nx SHRs. During the 12-week experimental period, blood pressure, urinary protein and glomerular sclerosis in the 5/6Nx SHRs increased progressively. After 12 weeks, the glomerular sclerosis was remarkable.

Expression of SIRT1 increases with caloric restriction and in conditions that cause oxidative stress and DNA damage,[12, 13] while it decreases with a high fat diet, insulin resistance, high glucose and senescence.[1] In the kidney, SIRT1 acts as a renal survival factor by conferring resistance to cellular stress such as hypoxia, reducing interstitial fibrosis, inhibiting tubular and glomerular cell apoptosis and inflammation and regulating sodium handling, blood pressure and renal lipid metabolism.[5] In the present study, the expression of SIRT1 in the kidney was decreased in SHRs compared to Wistar rats. In a previous study, however, the expression of SIRT1 was increased in the heart of SHRs.[14]

The renin-angiotensin system plays an important role in the development of renal injury. There are convincing data that the renin-angiotensin system is a major mediator of renal injuries. Angiotensin II induces the generation of cytotoxic products such as superoxide and hydrogen peroxide via angiotensin type 1 receptor stimulation. Olmesartan and other ARBs reduce oxidative stress.[15, 16] In addition to the effect on glomerular hypertension, ARBs are effective in reducing interstitial fibrosis and tubular atrophy, each of which is closely correlated to progressive renal dysfunction. In the present study, olmesartan showed an effect in decreasing blood pressure and preventing glomerular sclerosis. Fan et al. reported that ultrahigh doses of olmesartan showed greater renoprotective effects, and that efficacy was independent of blood pressure.[11]

Miyazaki reported that overexpression of SIRT1 in vascular smooth muscle cells or treatment with resveratrol, an activator of SITR1, suppressed angiotensin type 1 receptor expression.[6] In the present study, however, olmesartan did not show any effect on the expression of SIRT1 in both SHRs and Wistar rats. Shiota et al. reported that telmisartan, an angiotensin II type 1 receptor blocker, increased the expression of SITR1 in the skel-

etal muscle of obese smice.[9] Telmisartan acts as a peroxisome proliferator-activated receptor (PPAR)-gamma activator.[17] Elena suggested that PPARs participate in the retardation of aging mediated by angiotensin II inhibition.[18] Imayama showed that telmisartan suppressed angiotensin II type 1 receptor expression through the PPAR-gamma mediated pathway.[19] It is supposed that the renoprotective effect of olmesartan is related to blood pressure regulation and oxidative stress, and that this effect is independent of SIRT1 pathway.

Klotho is known as a circulating hormone that represses the intracellular signals of insulin and insulin-like growth factor 1 and acts as an aging-suppressor.[18] Downregulation of the *klotho* gene in the kidney has been reported in SHRs, and in an experimental study, olmesartan prevented *klotho* downregulation resulting in a renoprotective effect.[20] In the present study, however, olmesartan did not show any effect on *klotho* mRNA expression in SHR groups.

In conclusion, olmesartan suppressed glomerular sclerosis, but did not increase the expression of SIRT1, suggesting that the renoprotective effect of olmesartan is independent of the SIRT1 pathway. Further studies are required to investigate the correlation between angiotensin and sirtuins.

The authors declare no conflict of interest.

REFERENCES

1 Hao CM, Haase VH. Sirtuins and relevance to the kidney. J Am Soc Nephrol. 2010;21:1620-7. PMID: 20595677.

2 Sinclair DA, Guarente L. Extrachromosomal rDNA circles-A cause of aging in yeast. Cell. 1997;91:1033-42. PMID: 9428525.

3 Kume S, Haneda M, Kanasaki K, Sugimoto T, Araki S, Isshiki K, et al. SIRT1 inhibits transforming growth factor beta-Induced apoptosis in glomerular mesangial cells via Smad7 deacetylation. J Biol Chem. 2007;282:151-8. PMID: 17098745.

4 Galle J. Reduction of proteinuria with angiotensin receptor blockers. Nat Clin Pract Cardiovasc Med. 2008;5 Suppl 1:S36-43. PMID: 18580865.

5 Kitada M, Kume S, Watanabe A, Kanasaki K, Koya D. Sirtuins and renal diseases: relationship with aging and diabetic nephropathy. Clin Sci (Lond). 2013;124:153-64. PMID: 23075334.

6 Miyazaki R, Ichiki T, Hashimoto T, Inanaga K, Imayama I, Sadoshima J, Sunagawa K. SIRT1, a longevity gene, downregulates angiotensin II type 1 receptor expression in vascular smooth muscle cells. Arterioscler Thromb Vasc Biol. 2008;28:1263-9. PMID: 18420994.

7 Zhang D, Li S, Cruz P, Kone BC. Sirtuin 1 functionally and physically interacts with disruptor of telomeric silencing-1 to regulate alpha-ENaC transcription in collecting duct. J Biol Chem. 2009;31:284:20917-26. PMID: 19491102.

8 Mattagajasingh I, Kim CS, Naqvi A, Yamamori T, Hoffman TA, Jung SB, et al. SIRT1 promotes endothelium-dependent vascular relaxation by activating endothelial nitric oxide synthase. Proc Natl Acad Sci USA. 2007;104:14855-60. PMID: 17785417.

9 Shiota A, Shimabukuro M, Fukuda D, Soeki T, Sato H, Uematsu E, et al. Telmisartan ameliorates insulin sensitivity by activating the AMPK/SIRT1 pathway in skeletal muscle of obese db/db mice. Cardiovasc Diabetol. 2012;11:139. PMID: 23137106.

10 Kanazawa M, Kohzuki M, Yoshida K, Kurosawa H, Minami N, Saito T, et al. Combination therapy with an angiotensin-converting enzyme (ACE) inhibitor and a calcium antagonist: beyond the renoprotective effects of ACE inhibitor monotherapy in a spontaneous hypertensive rat with renal ablation. Hypertens Res. 2002;25:447-53. PMID: 12135325.

11 Fan YY, Baba R, Nagai Y, Miyatake A, Hosomi N, Kimura S, et al. Augmentation of intrarenal angiotensin II levels in uninephrectomized aldosterone/salt-treated hypertensive rats; renoprotective effects of an ultrahigh dose of olmesartan. Hypertens Res. 2006;29:169-78. PMID: 16755152.

12 Cohen HY, Miller C, Bitterman KJ, Wall NR, Hekking B, Kessler B, et al. Calorie restriction promotes mammalian cell survival by inducing the SIRT1 deacetylase. Science. 2004;305:390-2. PMID: 15205477.

13 Rodgers JT, Lerin C, Haas W, Gygi SP, Spiegelman BM, Puigserver P. Ntrient control of glucose homeostasis through a complex of PGC-1alpha and SIRT1. Nature. 2005;434:113-8. PMID: 15744310.

14 Li L, Zhao L, Yi-Ming W, Yu YS, Xia CY, Duan JL, et al. Sirt1 hyperexpression in SHR heart related to left ventricular hypertrophy. Can J Physiol Pharmacol. 2009;87:56-62. PMID: 19142216.

15 Jo F, Morimoto S, Nakahigashi M, Kusabe M, Someya K, Morita T, et al. Olmesartan induces renoprotective effects by stimulating angiotensin type 2 receptors and reducing oxidative stress in diabetic nephropathy. Kidney Blood Press Res. 2011;34:418-23. PMID: 21709422

16 Arozal W, Watanabe K, Veeraveedu PT, Ma M, Thandavarayan RA, Suzuki K, et al. Effects of angiotensin receptor blocker on oxidative stress and cardiorenal function in streptozotocin-induced diabetic rats. Biol Pharm Bull. 2009;32:1411-6. PMID: 19652382.

17 Clasen R, Schupp M, Foryst-Ludwig A, Sprang C, Clemenz M, Krikov M, et al. PPARgamma-activating angiotensin type-1 receptor blockers induce adiponectin. Hypertension. 2005;46:137-43. PMID: 15939809.

18 de Cavanagh EM, Inserra F, Ferder L. Angiotensin II blockade: a strategy to slow ageing by protecting mitochondria? Cardiovasc Res. 2011;89:31-40. PMID: 20819950.

19 Imayama I, Ichiki T, Inanaga K, Ohtsubo H, Fukuyama K, Ono H, et al. Telmisartan downregulates angiotensin II type 1 receptor through activation of peroxisome proliferator-activated receptor gamma. Cardiovasc Res. 2006;72:184-90. PMID: 16938288.

20 Maeta S, Munemura C, Fukui T, Ishida C, Murawaki Y. Combination therapy with olmesartan and temocapril ameliorates renal damage and upregulates the klotho gene in 5/6 nephrectomized spontaneously hypertensive rats. Yonago Acta Med. 2009;52:27-35.