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Combination Therapy with Olmesartan and Temocapril Ameliorates Renal Damage and Upregulates the *klotho* Gene in 5/6 Nephrectomized Spontaneously Hypertensive Rats

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Recent studies suggest that chronic kidney disease may induce cardiovascular disease through oxidative stress, and that the aging suppressor gene *klotho* reduces oxidative stress in the kidney. In this study, we examined the changes in *klotho* gene expression, and the renoprotective effects of olmesartan (OLM), angiotensin II receptor blocker (ARB) alone or in combination with temocapril (TEM), angiotensin-converting enzyme inhibitor (ACEI) in 5/6-nephrectomized (5/6-Nx) spontaneously hypertensive rats. Male 5/6-Nx spontaneously hypertensive rats were randomly assigned to 5 groups as follows: control group; 5/6-Nx group, 5/6-Nx rats; low OLM group, 5/6-Nx rats administered low-dose OLM (3 mg/kg/day); high OLM group, 5/6-Nx rats administered high-dose OLM (10 mg/kg/day); OLM+TEM group, 5/6-Nx rats administered high-dose OLM and TEM (10 mg/kg/day each). These drugs were administered for 12 weeks. Systolic blood pressure, glomerular sclerosis and transforming growth factor beta 1 mRNA in high OLM and OLM+TEM groups were significantly lower than that in the 5/6-Nx group. Only the OLM+TEM group showed improvement of serum creatinine and urinary 8-hydroxy-2′-deoxyguanosine. Expression of *klotho* mRNA, which was downregulated in the 5/6-Nx group, was upregulated in the high OLM and OLM+TEM groups. OLM dose-dependently prevented *klotho* mRNA downregulation in 5/6-Nx rats, thus confirming a renoprotective effect. In addition, combination therapy of OLM and TEM was more effective than OLM alone. In conclusion, the combination of OLM and TEM inhibits the progression of renal damage in 5/6-Nx rats through the upregulation of *klotho* gene.

Key words: angiotensin II receptor blocker; angiotensin-converting enzyme; cardiovascular disease; chronic kidney disease; inhibitor; *klotho* gene

In chronic kidney disease, in addition to end-stage renal disease, mild renal failure can cause cardiovascular disease as a result of oxidative stress (Descamps-Latscha et al., 2005). Angiotensin II, through the angiotensin II type 1 receptor, increases the expression of p47phox, which is a component protein of NADPH oxidase, and leads to overproduction of free radicals (Tojo et al., 2002). Angiotensin receptor blocker (ARB) and angiotensin-converting enzyme inhibitor (ACEI) inhibit the action of angiotensin II, which results in the reduction of oxidative stress (Dohi et al., 2003; Fliser et al., 2005).

The aging suppressor gene *klotho*, which is predominantly expressed in the kidney, prevents

Abbreviations: ACEI, angiotensin-converting enzyme inhibitor; ARB, angiotensin II receptor blocker; ELISA, enzyme-linked immunosorbent assay; IGF1, insulin-like growth factor 1; 5/6-Nx, 5/6-nephrectomized; 8-OHdG, 8-hydroxy-2′-deoxyguanosine; OLM, olmesartan; TEM, temocapril

insulin and insulin-like growth factor 1 (IGF1) signals to reduce oxidative stress (Kurosu et al., 2005). It has been reported that angiotensin II, through the activation of angiotensin II type 1 receptor, downregulates the expression of the *klotho* gene in the kidney, and the introduction of exogenous *klotho* ameliorates renal injury (Mitani et al., 2002). In addition, another study showed that the renal expression of *klotho* gene was downregulated in patients with chronic renal failure (Koh et al., 2001). Furthermore, in chronic kidney disease, it has been suggested that the downregulation of renal *klotho* expression is involved in the onset of cardiovascular disease. Taken together, it appears that angiotensin II, oxidative stress and the *klotho* gene are closely related to one another, and contribute to the progression of renal failure and onset of cardiovascular disease.

In this study, we examined the therapeutic effects of ARB and ACEI on renal damage in 5/6-nephrectomized (5/6-Nx) spontaneously hypertensive rats. In addition, we determined the changes in urinary 8-hydroxy-2′-deoxyguanosine (8-OHdG) and *klotho* mRNA.

Materials and Methods

Animals

Male spontaneously hypertensive rats of the Izumo strain were purchased from Japan SLC (Shizuoka, Japan), and were maintained at a temperature of 24 ± 2°C with a 12-h light-dark cycle. Animals were given standard pellet chow and tap water. Anesthesia was performed by intraperitoneally injecting pentobarbital (Dainippon Pharmaceutical, Osaka, Japan) at dose of 50 mg/kg. All experiments were carried out in accordance with the Animal Experimentation Guidelines of Tottori University.

Establishment of model

Male 6-week-old spontaneously hypertensive rats underwent a 5/6-nephrectomy. First, the surgical excision of approximately 2/3 of the renal cortex of the left kidney was performed. One week later, the right kidney was removed. At 1 week after surgery, baseline measurement of body weight, blood pressure, urine volume and urinary protein was performed. Blood pressure was measured in conscious rats using the tail-cuff method with a sphygmomanometer (Softron, Tokyo, Japan). Urine was collected from individual rats housed in metabolic cages for 24 h. Administered drugs were olmesartan (OLM) as ARB and temocapril (TEM) as ACEI. Rats were divided into 5 experimental groups: control group, non-nephrectomized rats (n = 8); 5/6-Nx group, 5/6-Nx rats (n = 8); low OLM group, 5/6-Nx rats administered low-dose OLM (3 mg/kg/day); high OLM group, 5/6-Nx rats administered high-dose OLM (10 mg/kg/day); and OLM+TEM group, 5/6-Nx rats administered high-dose OLM and high-dose TEM (10 mg/kg/day each). Dosage of each drug was selected based on earlier reports so that they would exhibit comparable antihypertensive effects (Kanazawa et al., 2002; Fan et al., 2006). Drugs were administered once a day for 12 weeks beginning 1 week after nephrectomy. Every 4 weeks, we measured body weight, blood pressure, urine volume and urinary protein in each group.

After 12 weeks of administration, rats were killed under pentobarbital anesthesia. Blood was collected from the heart. Serum samples were frozen and stored at –80°C, and serum creatinine was measured. Remnant kidneys were removed and 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 the RNeasy 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

Table 1. Primer sequences

	Forward primer	Reverse primer
TGF-β_1	*5′-CCTGCCCCTACATTTGGA-3′*	*5′-TGGTTGTAGAGGGCAAGGAC-3′*
klotho	*5′-CAAGAAGTTCATAATGGAAAGCTTAAA-3′*	*5′-ATGCGGTGTACCCAATGAC-3′*
β-actin	*5′-CTGGCTCCTAGCACCATGA-3′*	*5′-TAGAGCCACCAATCCACACA-3′*

in a final volume of 11.5 μL containing 4 μL of 5× standard buffer, 2 μL of 0.1 M dTT, 1 μL of Super-Script II RNase H-reverse transcriptase (Invitrogen, Carlsbad, CA), 2 μL of 10 mM dNTPs (Promega, Madison, WI), 1 μL of 50 pmol/μL Random Primer (Promega), 0.5 μL of 100 pmol/μL Oligo (dt) 15 Primer (Promega) and 1 μL of 40 units/μL Ribonuclease Inhibitor (Wako Pure Chemical Industries, Osaka). 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: PCR grade water, 4.1 μL; Universal ProbeLibrary probe, 1 μL (Roche, Tokyo); Forward primer (10 μM), 0.2 μL; Reverse primer (10 μM), 0.2 μL; LightCycler TaqMan Master, 2 μL (Roche); and cDNA sample, 2.5 μL. mRNA levels of transforming growth factor beta 1 (TGF-β_1) and *klotho* were assessed by real-time PCR assays using β-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 10 min, repeat 45 cycles of 95°C for 30 s and 60°C for 1 min.

Histological analysis

Three-micrometer sections of formalin-fixed, paraffin-embedded kidneys 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 (Fig. 1): 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 area; Grade 4, sclerosis of more than 3/4 of the glomerulus. The index of glomerular sclerosis was calculated using the following formula (Saito et al., 1987): index of glomerular sclerosis = $(1 \times N1 + 2 \times N2 + 3 \times N3 + 4 \times N4)/(N0 + N1 + N2 + N3 + N4)$, where N is the number of glomeruli at each grade of sclerosis.

Urinary 8-OHdG measurement

Urinary 8-OHdG concentrations were measured by enzyme-linked immunosorbent assay (ELISA) using a commercially available competitive 8-OHdG ELISA kit (Japan Institute for Control of Aging, Shizuoka). The kit can measure 8-OHdG values ranging from 0.5 to 200 ng/mL using a specific monoclonal antibody, N45.1.

Statistical analysis

Statistical significance of intergroup differences in quantitative data was assessed by Student's *t*-test (Stat View for Windows; SAS Institute, Cary, NC). $P < 0.05$ was considered significant.

Results

Blood pressure

Mean systolic blood pressure in the rats during the 12-week experimental period is shown

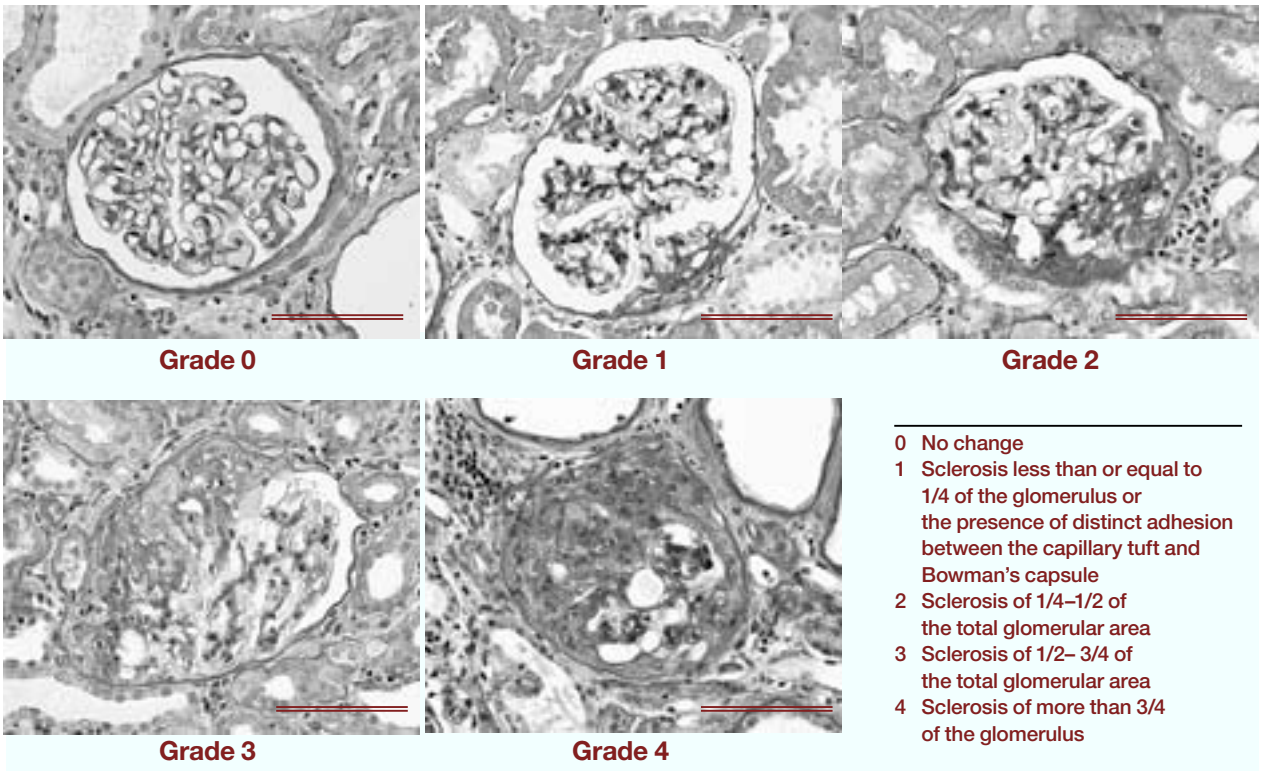

Fig. 1. Classification of glomerular sclerosis by periodic acid-Schiff stain. Bar = 100 μm.

in Fig. 2. Systolic blood pressure after nephrectomy increased progressively throughout the experimental period in the 5/6-Nx group. Drug administration induced a significant decrease in the treated groups (low OLM, high OLM and OLM+TEM groups) when compared with in the 5/6-Nx group. At the end of the 12-week administration period, the level was significantly lower in the treated groups than in the 5/6-Nx group: 228 ± 22 for the 5/6-Nx group, 191 ± 15 for the low OLM group, 178 ± 27 for the high OLM group, 153 ± 16 mmHg for the OLM+TEM group. The OLM+TEM group showed significantly lower levels than the low OLM group ($P < 0.001$).

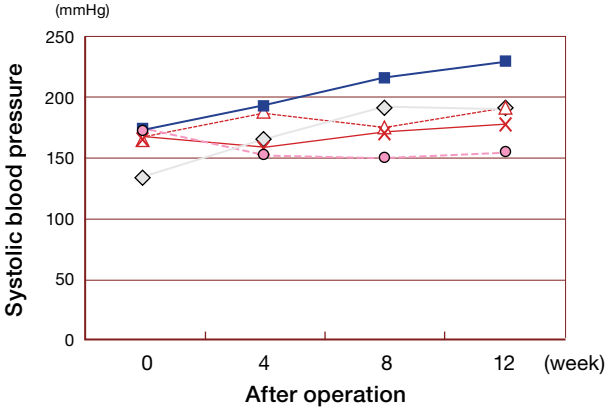

Fig. 2. Course of mean systolic blood pressure in the 5 groups. The systolic blood pressure of rats is significantly decreased at 12 weeks of administration in groups of low OLM (△), high OLM (×) and OLM+TEM (●) when compared with in the 5/6-Nx group (■). ◇, control group. High OLM, high-dose olmesartan; 5/6-Nx, 5/6-nephrectomized; low OLM, low-dose OLM; OLM+TEM, high-dose OLM combined with high-dose temocapril.

Proteinuria

Figure 3 shows total urinary protein excretion in each group during the 12 weeks. The control group showed no change, while the 5/6-Nx group exhibited progressive increase throughout the experimental period. During the period, urinary protein levels were lower in the treated groups than

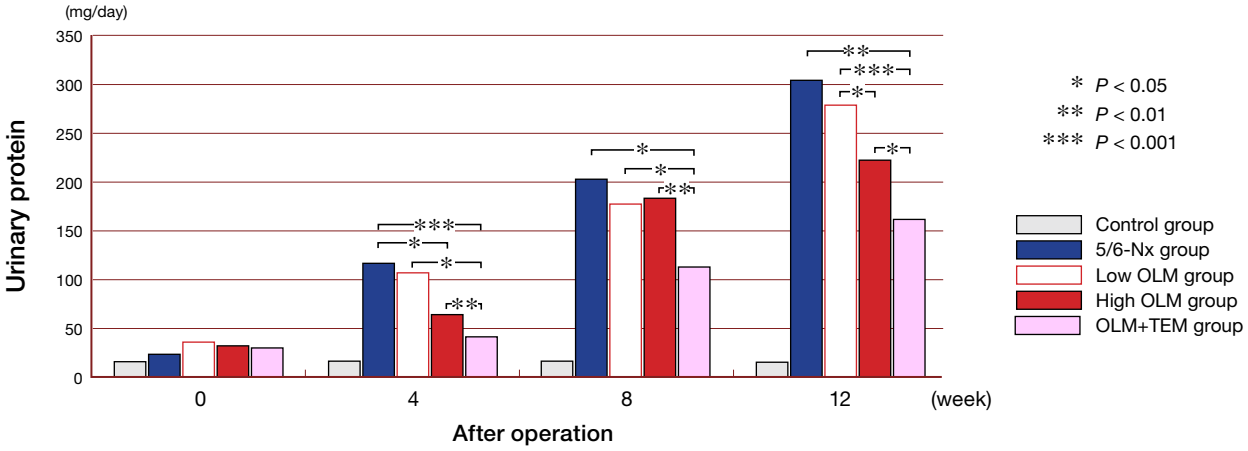

Fig. 3. Effects of treatments on urinary protein in the 5 groups. High OLM, high-dose olmesartan; 5/6-Nx, 5/6-nephrectomized; low OLM, low-dose OLM; OLM+TEM, high-dose OLM combined with high-dose temocapril.

in the 5/6-Nx group: especially in the OLM+TEM group, the level was significantly lower at 4, 8 and 12 weeks after the operation.

Serum creatinine

Figure 4 shows serum creatinine in the 5 groups of rats at 12 weeks. The serum creatinine level was significantly higher in the 5/6-Nx group than in the control group ($P < 0.01$). The treated groups (low OLM, high OLM and OLM+TEM groups) showed lower levels than the 5/6-Nx group; especially, the difference between the OLM+TEM and 5/6-Nx groups was significant ($P < 0.05$).

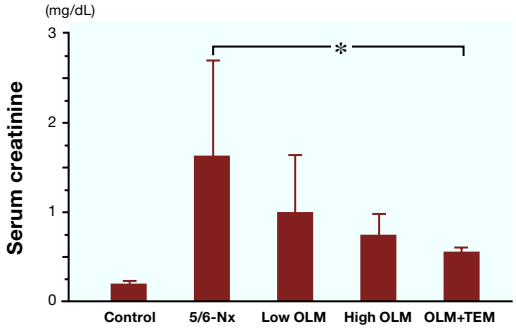

Fig. 4. Serum creatinine concentrations in the 5 groups at 12 weeks. Values are expressed as mean ± SD. *$P < 0.05$. High OLM, high-dose olmesartan; 5/6-Nx, 5/6-nephrectomized; low OLM, low-dose OLM; OLM+TEM, high-dose OLM combined with high-dose temocapril.

Histological findings in the kidney

In the control group, only a few sclerotic changes of glomeruli and interstitial fibrosis were observed. The values of the index of glomerular sclerosis in the treated groups were significantly lower than in the 5/6-Nx group: $P < 0.05$ between the low OLM and 5/6-Nx groups; $P < 0.05$ between the high OLM and 5/6-Nx groups; $P < 0.01$ between the OLM+TEM and 5/6-Nx groups. In comparison among the treated groups, the index was significantly lower in the OLM+TEM group than in the low OLM group ($P < 0.05$) (Fig. 5).

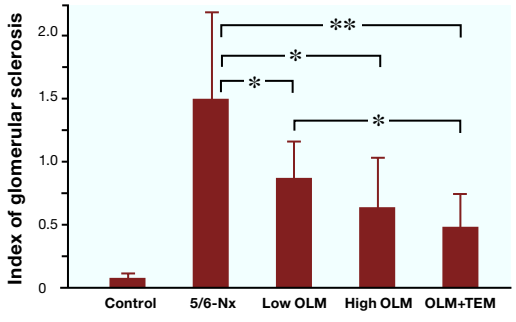

Fig. 5. Index of glomerular sclerosis in the 5 groups at 12 weeks. Values are expressed as mean ± SD. *$P < 0.05$, **$P < 0.01$. High OLM, high-dose olmesartan; 5/6-Nx, 5/6-nephrectomized; low OLM, low-dose OLM; OLM+TEM, high-dose OLM combined with high-dose temocapril.

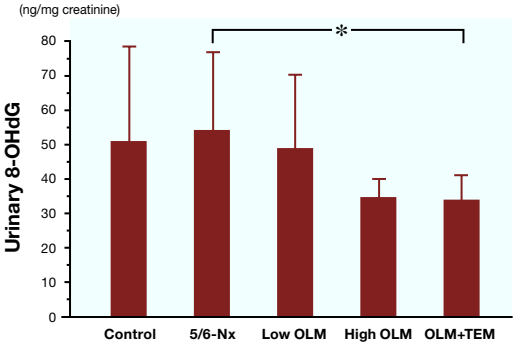

Fig. 6. Urinary 8-hydroxy-2'-deoxyguanosine (8-OHdG)/urinary creatinine ratio in the 5 groups at 12 weeks. Values are expressed as mean ± SD. *$P < 0.05$. High OLM, high-dose olmesartan; 5/6-Nx, 5/6-nephrectomized; low OLM, low-dose OLM; OLM+TEM, high-dose OLM combined with high-dose temocapril.

Urinary 8-OHdG/urinary creatinine ratio

Levels of urinary 8-OHdG/creatinine in the 5/6-Nx group at 12 weeks were slightly higher than in the control group: the difference was not significant. Levels of urinary 8-OHdG/creatinine in the treated groups were lower than in the 5/6-Nx group: the difference between the OLM+TEM and 5/6-Nx groups was significant ($P < 0.05$) (Fig. 6).

mRNA quantification of TGF-β_1 and klotho

The TGF-β_1 mRNA level in the 5/6-Nx group was markedly increased when compared with in the control group (Fig. 7a). This increase was significantly alleviated in the high OLM and OLM+TEM groups, but not in the low OLM group. The level in the OLM+TEM group was significantly lower than that in the low OLM group ($P < 0.05$).

The *klotho* mRNA level was significantly lower in the 5/6-Nx group than in the control group (Fig. 7b). The *klotho* mRNA levels in the treated groups were higher in the 5/6-Nx group: the high OLM and OLM+TEM groups showed significant difference to the 5/6-Nx group ($P < 0.01$). Among the treated groups, levels were significantly higher in the high OLM and OLM+TEM groups than in the low OLM group ($P < 0.05$).

Discussion

The rennin-angiotensin system plays an important role in the development of hypertension and the progression of renal failure. Numerous studies have shown that ARB or ACEI is able to prevent the progression of renal failure (Parving et al., 2001; Nakao et al., 2003; Schmieder et al., 2007). ARB or ACEI alleviates glomerular hypertention by reducing the constructive effects of angiotensin II on the efferent arterioles, which leads to inhibition of glomerular sclerosis. Angiotensin II also activates the production of free radicals, promotes cell growth, and increases the synthesis of inflammatory and profibrotic cytokines (Wolf et al., 2000; Brewster and Perazella., 2004; Long et al., 2004). Furthermore, there is increasing evidence

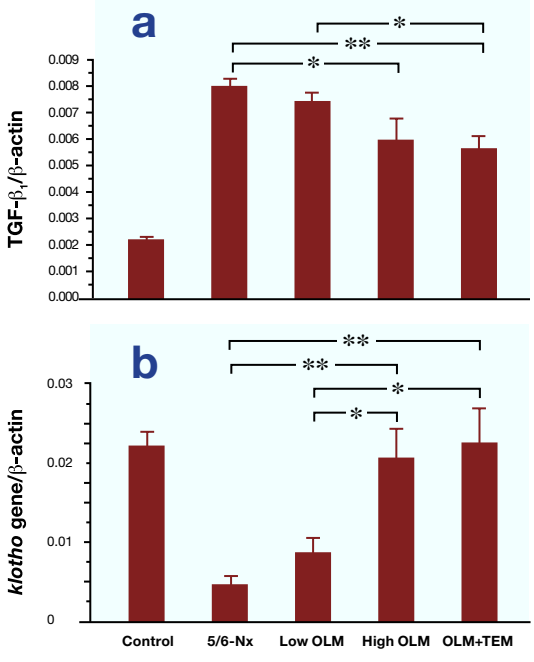

Fig. 7. Levels of mRNA for transforming growth factor-β_1 (TGF-β_1) and *klotho* gene in renal tissue.
(**a**) Expression of TGF-β_1 mRNA.
(**b**) Expression of *klotho* mRNA.
Values are expressed as mean ± SEM. *$P < 0.05$, **$P < 0.01$. High OLM, high-dose olmesartan; 5/6-Nx, 5/6-nephrectomized; low OLM, low-dose OLM; OLM+TEM, high-dose OLM combined with high-dose temocapril.

supporting a potential role for aldosterone, which is stimulated by angiotensin II, in the pathophysiology of renal injury (Hollenberg, 2004; Remuzzi et al., 2008).

Spontaneously hypertensive rats have been used as a model for essential hypertension in humans, and 5/6-Nx rats have commonly been used as an experimental model for chronic renal failure in humans. In the present study, we combined the 2 rat models, and prepared 5/6-Nx spontaneously hypertensive rats. Systolic blood pressure, urinary protein and glomerular sclerosis in 5/6-Nx spontaneously hypertensive rats increased progressively throughout the experimental period. After 12 weeks, each of the ARB-treated groups (low and high OLM groups) showed a significant decrease in systolic blood pressure and index of glomerular sclerosis when compared with the 5/6-Nx group. These data indicate that OLM ameliorates renal injury in a dose-dependent manner. Indeed, Fan et al. also reported that ultrahigh doses of OLM showed greater renoprotective effects than typical doses of OLM, and that efficacy was independent of blood pressure (Fan et al., 2006).

In our study, the OLM- and TEM-administered rats showed greater renoprotective effects than only the high OLM-administered rats did, indicating that combination therapy with ARB and ACEI has a beneficial effect on renoprotection as compared with ARB alone. It is known that ACE inhibition results in reduced degradation of bradykinin (Gavras, 1992), and that bradykinin may cause selective efferent arteriolar dilatation and stimulate endothelial NO formation. Jacobsen et al. showed that dual blockade of the rennin-angiotensin system offers additional renal protection in type 1 diabetic patients with diabetic nephropathy (Jacobsen et al., 2003).

TGF-β_1 has been shown to play a predominant role in mediating angiotensin II-induced extracellular matrix production and promotes the fibrosis of cardiovascular and renal tissues (Saito et al., 2004; Dabek et al., 2006). In the present study, renal TGF-β_1 mRNA levels were markedly lower in the high OLM group and OLM+TEM groups. This suggests that the reduction in TGF-β_1 is closely correlated with the prevention of progressive renal damage by inhibition of the renin-angiotensin system.

Defects in *klotho* gene expression in mice have been reported to lead to a syndrome closely resembling human aging, including shortened life span, infertility, arteriosclerosis, osteoporosis and pulmonary emphysema (Kuro-o et al., 1997). The *klotho* gene is expressed in limited tissues, notably the distal convoluted tubules in the kidney and the choroid plexus in the brain.

klotho protein functions as a circulating hormone that binds to a cell-surface receptor and represses the intracellular signals of insulin and IGF1. It appears that the anti-aging effects of *klotho*-induced inhibition of insulin/IGF1 signaling are associated with increased resistance to oxidative stress (Kurosu et al., 2005). It has been shown that *klotho* protein inhibits the phosphorylation of FoxO forkhead transcription factors, which induce expression of manganese superoxide dismutase, thereby facilitating removal of reactive oxygen species and conferring resistance to oxidative stress (Yamamoto et al., 2005). Mitani et al. have demonstrated in experimental models that continuous administration of angiotensin II reduces renal *klotho* gene expression (Mitani et al., 2002).

Downregulation of *klotho* mRNA in the kidney has been reported in animal hypertention models, such as spontaneously hypertensive rats and deoxycorticosterone acetate-salt hypertensive rats (Aizawa et al., 1998). In the present study, expression of *klotho* mRNA in the 5/6-Nx group was markedly downregulated when compared with the control group. This reduction of *klotho* mRNA was ameliorated in the high OLM and OLM+TEM groups, but not in the low OLM group. Losartan completely blocked angiotensin II-induced *klotho* mRNA downregulation, which is independent of its blood pressure-lowering effects (Mitani et al., 2002). This is the 1st report showing that OLM dose-dependently prevents *klotho* mRNA downregulation in 5/6-Nx sponta-

neously hypertensive rats.

Urinary 8-OHdG is a biological marker of in vivo oxidative DNA damage (Shigenaga et al., 1989), and the average urinary 8-OHdG excretion in the OLM+TEM group was significantly lower than in the 5/6-Nx group: this fact indicates that OLM- and TEM-induced *klotho* overexpression reduces oxidative DNA damage. In addition to ACEI (de Cavanagh et al., 2000, 2001), OLM (Yao et al., 2004) has been reported to improve endothelin-induced hypertension and oxidative stress in rats. Fujimoto et al. also reported that OLM inhibits superoxide production and oxidative stress, independent of its blood pressure-lowering effects (Fujimoto et al., 2008).

A recent study reported that chronic kidney disease is an independent risk factor for cardiovascular disease, which involves excessive oxidative stress (Descamps-Latscha et al., 2005). Furthermore, it has been reported that expression of the *klotho* gene in the kidney is downregulated in chronic renal failure in vivo (Aizawa et al., 1998) and in vitro (Koh et al., 2001). Saito et al. demonstrated that *klotho* gene transfer in an experimental rat model of atherosclerotic disease improved endothelial dysfunction (Saito et al., 2000), and that *klotho* protein protects the cardiovascular system through endothelium-derived NO production by humoral pathways (Saito et al., 1998). These data suggest that the downregulation of renal *klotho* expression in patients with chronic kidney disease is involved in the onset of cardiovascular disease. ARB reduced the incidence of cardiovascular disease (Yusuf et al., 2000; de Zeeuw et al., 2004), which raises the relationship between ARB and inhibition of *klotho* gene downregulation. In the present study, the combination of OLM and TEM significantly decreased urinary 8-OHdG, and showed renoprotective effects. These data suggest that the combination therapy is more effective for cardiovascular protection.

In conclusion, the present study demonstrated that OLM ameliorates *klotho* gene downregulation in 5/6-Nx rats in a dose-dependent manner, and this has a renoprotective effect. In addition, combination therapy of OLM and TEM was more effective than OLM alone. This suggests that *klotho* gene upregulation by the renin-angiotensin system inhibitor leads to a reduction in cardiovascular disease.

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