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The Effects of Olmesartan and Alfacalcidol on Renoprotection and *klotho* Gene Expression in 5/6 Nephrectomized Spontaneously Hypertensive Rats

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Recently, an angiotensin inhibitor has been shown to upregulate the *klotho* mRNA level in chronic renal failure. In addition, the administration of vitamin D has been reported to improve the mortality of patients with chronic renal failure. In this study, we examined the effects of an angiotensin inhibitor and/or vitamin D on the progression of chronic renal failure by using male 5/6 nephrectomized (5/6Nx) spontaneously hypertensive rats. Male 5/6Nx spontaneously hypertensive rats were assigned to 4 groups as follows: 5/6Nx group, 5/6Nx rats; Alf group, 5/6Nx rats administered alfacalcidol (0.2 μg/kg/day); Olm group, 5/6Nx rats administered olmesartan (15 mg/kg/day); Alf + Olm group, 5/6Nx rats administered alfacalcidol (0.2 μg/kg/day) and olmesartan (15 mg/kg/day). These drugs were administered for 12 weeks. Systolic blood pressure in the Alf, Olm and Alf + Olm groups were significantly decreased relative to that in the 5/6Nx group during the 12-week experimental period. As a result, all treated groups showed renoprotection based on improvement of the systolic blood pressure, urinary protein excretion and histological renal fibrosis. Combination therapy of alfacalcidol and olmesartan was more effective than either alfacalcidol or olmesartan alone. Expression of *klotho* mRNA was significantly upregulated in the Alf + Olm group in comparison with in the 5/6Nx group. Serum levels of fibroblast growth factor 23 in the Alf group and the Alf + Olm group were significantly higher than those in the 5/6Nx group and the Olm group. In conclusion, the combination of Olm and Alf inhibited the progression of renal damage in the 5/6Nx group through the strong antihypertensive effect as well as the upregulation of the *klotho* gene.

Key words: angiotensin II receptor blocker; chronic renal failure; fibroblast growth factor 23; *klotho* gene; vitamin D

It was discovered approximately a decade ago that a deletion of the *klotho* gene results in phenotypes resembling those of human aging-associated disorders (arteriosclerosis, osteoporosis, ectopic calcification and skin atrophy, together with short life-span). Transcripts of this gene are expressed predominantly in the kidney, choroids plexus and parathyroid gland (Kuro-o et al., 1997). The complication of chronic renal failure (CRF) closely resembles phenotypes seen in *klotho* mutants.

Abbreviations: Alf group, group of alfacalcidol-administered 5/6Nx rats; Alf + Olm group, group of alfacalcidol- and olmesartan-administered 5/6Nx rats; ARB, angiotensin II receptor blocker; CRF, chronic renal failure; FGF23, fibroblast growth factor 23; IGS, index of glomerular sclerosis; Nx, nephrectomized; Olm group, group of olmesartan-administered 5/6Nx rats; TGF, transforming growth factor

Therefore, the severe reduction of *klotho* expression might be related to the pathophysiology of CRF (Haruna et al., 2007). Indeed, the expression of the *klotho* gene in the kidney of the nephrectomized (Nx) rats, a model of CRF in humans, was significantly downregulated (Aizawa et al., 1998). In addition, it is known that the production of *klotho* in the kidney of human CRF patients was markedly reduced (Koh et al., 2001). Previously, we have shown that a renin-angiotensin system inhibitor such as angiotensin-converting enzyme inhibitor or angiotensin II receptor blocker (ARB) prevented the *klotho* mRNA downregulation in the 5/6Nx rats, indicating that renin-angiotensin system inhibitors have a renoprotective effect (Maeta et al., 2009).

In *klotho*-deficient mice, the levels of serum P, Ca and active vitamin D [1,25(OH)$_2$ vitamin D$_3$] were elevated. Ectopic calcification and vascular calcification in *klotho*-deficient mice might be due to elevated blood levels of Ca, P and active vitamin D. Reduction of serum 1,25(OH)$_2$ vitamin D$_3$ concentration by dietary restriction resulted in alleviation of most of the phenotypes (Tsujikawa et al., 2003).

In addition, there is a report that active vitamin D therapy is a possible factor in the increased serum fibroblast growth factor 23 (FGF23) levels in chronic kidney disease (Nakanishi et al., 2005; Saito et al., 2005). FGF23 was identified as an endocrine factor produced by bone with known functions to regulate urinary P excretion, as well as the production of 1,25(OH)$_2$ vitamin D$_3$ and parathyroid hormone (Kurosu et al., 2006). Serum FGF 23 levels were significantly elevated in chronic kidney disease patients in a close correlation with serum creatinine (Komba and Fukagawa, 2009). Increased FGF 23 level appears to be independently associated with mortality among patients undergoing hemodialysis (Gutiérrez et al., 2008). By contrast, observational studies of long-term hemodialysis patients showed that vitamin D treatment has been associated with improved survival (Teng et al., 2005). In addition, vitamin D use is associated with lower mortality in nondialysis patients with chronic kidney disease (Shoben et al., 2008). Therefore, the administration of 1,25(OH)$_2$ vitamin D$_3$ has therapeutic value for patients with chronic kidney disease.

In this study, we examined the renoprotective effects of an ARB and/or 1,25(OH)$_2$ vitamin D$_3$ on renal damage in 5/6Nx spontaneously hypertensive rats.

Materials and Methods

Materials

Male spontaneously hypertensive rats of the Izumo strain were obtained from Japan SLC (Shizuoka, Japan), and maintained in a room at controlled temperature of 24 ± 2˚C with a 12-h light-dark cycle. All experiments were carried out in accordance with the Animal Experimention Guidelines of Tottoti University.

Olmesartan was generously provided by Daiichi-Sankyo Pharmaceutical (Tokyo, Japan), was suspended in 0.5% of methylcellulose solution to a given concentration. Alfacalcidol was synthesized in Chugai Pharmaceutical (Fuji-Gotemba, Japan), was dissolved in medium-chain triglyceride and diluted to given concentration.

Establishment of the model

Anesthesia was performed by intraperitoneally injecting pentobarbital (Dainippon Pharmaceutical, Osaka, Japan) at dose of 50 mg/kg. The 6-week-old spontaneously hypertensive rats undergone a 5/6 nephrectomy, consisting of the surgical excision of approximately 2/3 of the renal cortex of the left kidney. One week later, the right kidney was removed. Then, the rats were divided into 4 experimental groups: 5/6Nx group, 5/6Nx rats (n = 8); Alf group, 5/6Nx rats administered by gavage with 0.2 μg/kg/day alfacalcidol (n = 8); Olm group, 5/6Nx rats administered by gavage with 15 mg/kg/day olmesartan, an ARB (n = 8); and Alf + Olm group, 5/6Nx rats administered by gavage with 0.2 μg/kg/day alfacalcidol and 15 mg/kg/day olmesartan (n = 8).

The dose of olmesartan was selected on the basis of an earlier study reporting that the rats would

Table 1. Primer sequences

	Forward primer	Reverse primer
TGF-*β1*	*5′-CCTGCCCCTACATTTGGA-3′*	*5′-TGGTTGTAGAGGGCAAGGAC-3′*
klotho	*5′-CAAGAAGTTCATAATGGAAAGCTTAAA-3′*	*5′-ATGCGGTGTACCCAATGAC-3′*
β-actin	*5′-CTGGCTCCTAGCACCATGA-3′*	*5′-TAGAGCCACCAATCCACACA-3′*

TGF, transforming growth factor.

exhibit comparable antihypertensive effects (Porteri et al., 2005). The dose of alfacalcidol was examined previously in an ovariectomized rat model of osteoporosis for human (Shiraishi et al., 2000).

Drugs were administered once a day beginning 1 week after the nephrectomy for 12 weeks. Every 4 weeks, we measured body weight, blood pressure, urinary volume and urinary protein of each groups. Blood pressure was measured in conscious rats by the tail-cuff method with a sphygmomanometer (Softron, Tokyo). Urine was collected from individual rats housed in metabolic cages for 24 h. At 12 weeks after the administration, the rats were killed under pentobarbital anesthesia. Blood was collected from their hearts, and those serum samples were frozen and stored at –80˚C.

Concentrations of serum creatinine, Ca, P, 1,25$(OH)_2$ vitamin D_3, urinary protein and urinary creatinine were measured by routine laboratory methods. The level of proteinuria excretion is affected by the physique of animals. So, we measured urinary protein levels using the ratio of urinary protein/creatinine, in which creatinine compensates the measurement of proteinuria. Serum FGF23 concentration was determined by using an FGF23 enzyme-linked immunosorbent assay kit from Kainos Laboratories (Tokyo). The 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 by 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 in a final volume of 11.5 μL containing 4 μL of 5 × standard buffer, 2 μL of 0.1 M dithiothreitol, 1 μL of SuperScriptII RNase H-reverse transcriptase (Life Technologies, Carlsbad, CA), 2 μL of 10 mM dNTPs (Promega, Madison, WI), 1 μL of 50 pmol/μL Random Primers (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, Japan). The samples were incubated at 37˚C for 60 min, and then 95˚C for 5 min and cooling to 4˚C for 5 min.

Real-time PCR

To prepare a reverse transcribed sample, we mixed 4.1 μL of PCR grade water, 1 μL of Universal PribeLibrary probe (Roche, Tokyo), 0.2 μL of 10-μM Reverse primer, 2 μL of LightCycler TaqMan Master (Roche) and 2.5 μL of a cDNA sample, and used 10 μL of the mixture for quantitative real-time PCR. The mRNA levels of transforming growth factor (TGF)-*β1* and *klotho* genes were assessed by the real-time PCR assays, using *β*-actin as an internal standard. 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-thick sections of formalin-fixed, paraffin-embedded kidneys were stained with periodic acid-Schiff and periodic acid-methenamine silver. For calculating 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 area; Grade 4, sclerosis of more than 3/4 of the glomerulus. The index of glomerular sclelosis (IGS) was calculated by using the following formula (Kanazawa et al., 2002):

$$\text{IGS} = \frac{(1 \times N_1 + 2 \times N_2 + 3 \times N_3 + 4 \times N_4)}{(N_0 + N_1 + N_2 + N_3 + N_4),}$$

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 (Stat View for Windows; SAS Institute, Cary, NC). $P < 0.05$ was considered significant.

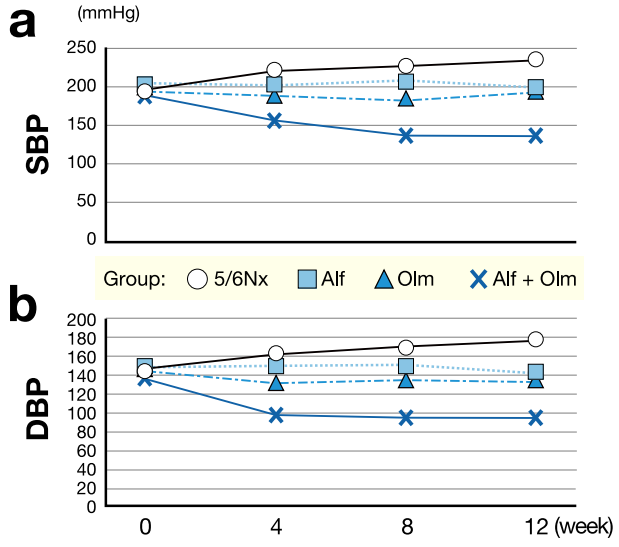


Fig. 1. Courses of the mean SBP (**a**) and DBP (**b**) during the 12-week experimental period in the 4 groups of rats. Both pressures are significantly decreased at week 12 in groups of Alf, Olm and Alf + Olm when compared with in the 5/6Nx group. 5/6Nx, group of rats subjected to 5/6 nephrectomy; Alf, group of 0.2 μg/kg/day alfacalcidol-administered 5/6Nx rats; Olm, group of 15 mg/kg/day olmesartan-administered 5/6Nx rats; Alf + Olm, group of 0.2 μg/kg/day alfacalcidol- and 15 mg/kg/day olmesartan-administered 5/6Nx rats. DPB, diastolic blood pressure; SBP, systolic blood pressure.

Results

Blood pressure

The systolic blood pressure of the rats during the 12-week experimental period is shown in Fig. 1. The systolic blood pressure after nephrectomy was increased progressively throughout the experimental period in the 5/6Nx group. After the initiation of treatment, the systolic blood pressures in the Alf, Olm and Alf + Olm groups were gradually decreased. At the end of the 12-week administration period, each systolic blood pressure in the treated groups was significantly lower than that in the 5/6Nx group (233.0 ± 9.6 mmHg for the 5/6Nx group, 199.4 ± 18.6 mmHg for the Alf group, 191.6 ± 14.2 mmHg for the Olm group, 136.0 ± 19.9 mmHg for the Alf + Olm group). The Alf + Olm group showed significantly lower levels of the systolic blood pressur than any other group did at week 12. In addition, we found a similar trend for diastolic blood pressure (Fig. 1). At the end of the 12-week administration period, each diastolic blood pressure was also significantly lower than that in the 5/6Nx group (177.3 ± 8.8 mmHg for the 5/6Nx group, 140.6 ± 18.3 mmHg for the Alf group, 134.0 ± 12.1 mmHg for the Olm group, 94.1 ± 15.4 mmHg for the Alf + Olm group).

Proteinuria

Figure 2 shows urinary protein excretion at every 4 weeks for each group. During the 12 weeks, urinary protein excretion in the 5/6Nx group was increased progressively throughout the experimental period. In all treated groups, urinary protein excretion was significantly lower than that in the 5/6Nx group at week 12. Especially, urinary protein excretion in the Alf + Olm group was significantly decreased compared with that in the Alf group and Olm group.

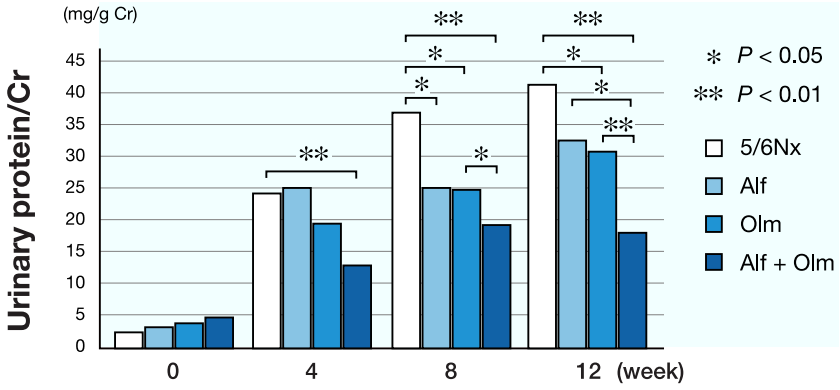


Fig. 2. Mean urinary protein levels at 0, 4, 8 and 12 weeks in the 4 groups. Urinary protein excretion in the Alf + Olm group is significantly decreased compared with that in the 5/6Nx group, Alf group and Olm group at week 12.

Serum creatinine

Although olmesartan did not significantly decrease the serum creatinine level at week 12, the serum creatinine level in the Alf group was significantly lower than that in the 5/6Nx group ($P < 0.01$), but not significantly lower than that in the Olm group ($P = 0.33$). The level of serum creatinine in the Alf + Olm group was significantly lower than that in the 5/6Nx group ($P < 0.01$) and in 2 other alfacalcidol-treated groups ($P < 0.05$) (Fig. 3a). Figure 3b showed the creatinine clearance in the 4 groups of rats at week 12. The creatinine clearance in the Alf + Olm group was significantly improved in comparison with the 5/6Nx group ($P < 0.05$).

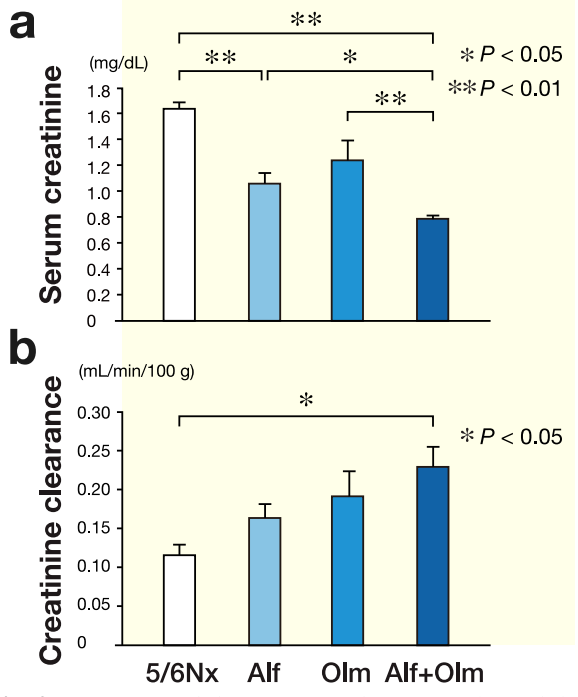


Fig. 3. Serum creatinine concentrations (**a**) and creatinine clearance levels (**b**) in the 4 groups at week 12. The level of serum creatinine in the Alf + Olm group is significantly lower than the other groups. The creatinine clearance in the Alf + Olm group is significantly improved in comparison with the 5/6Nx group. Values are expressed as means ± SD.

Serum Ca, P and 1,25(OH)$_2$ vitamin D$_3$

Figure 4a shows serum Ca concentrations in the 4 groups of rats at week 12. The alfacalcidol-treated groups (Alf, Alf + Olm) had significantly higher Ca values than the 5/6Nx and Olm groups had ($P < 0.01$). By contrast, the serum P values in the 4 groups did not differ significantly (Fig. 4b). We also examined the values of serum 1,25(OH)$_2$ vitamin D$_3$ in the 4 groups at week 12. The alfacalcidol-treated groups (Alf, Alf + Olm) had remarkably higher serum levels of 1,25(OH)$_2$ vitamin D$_3$ than the non-treated groups (5/6Nx, Olm) had (Fig. 4c).

Serum FGF23

Levels of serum FGF23 in the alfacalcidol-treated groups (Alf, Alf + Olm) at week 12 were significantly higher than those in the 5/6Nx and Olm groups ($P < 0.01$) (Fig. 5).

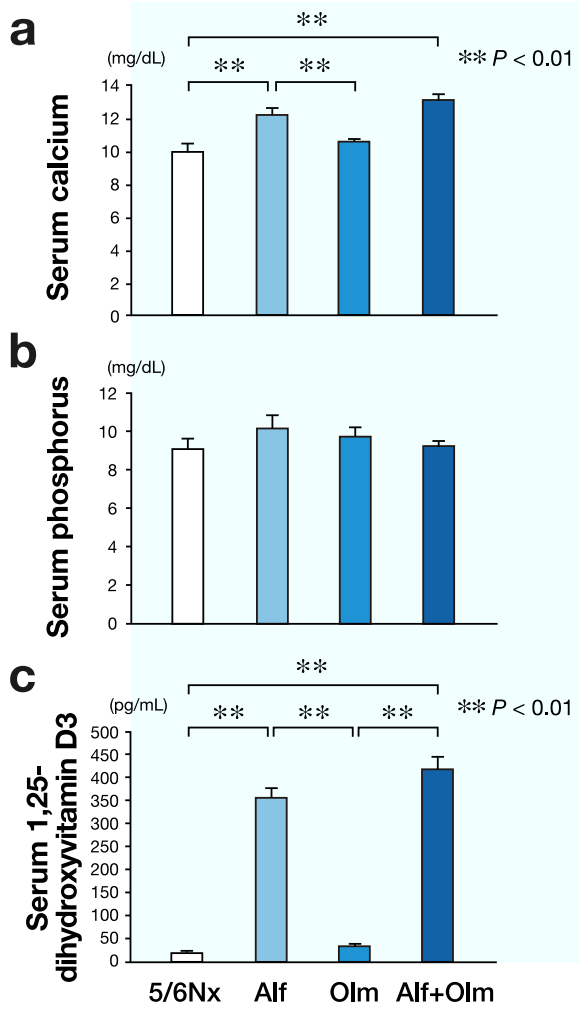


Fig. 4. Serum concentrations of Ca (**a**), P (**b**) and 1,25(OH)$_2$ vitamin D$_3$ (**c**). Levels of Ca and 1,25(OH)$_2$ vitamin D$_3$ are significantly higher in alfacalcidol-treated groups than in non-treated groups. Differences in P levels are not significant among the 4 groups.

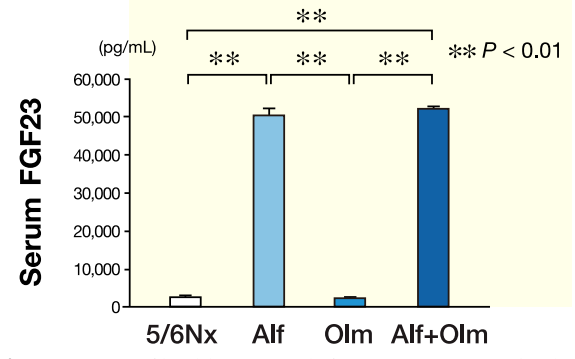


Fig. 5. Serum fibroblast growth factor 23 (FGF23) in the 4 groups at week 12. Levels of serum FGF23 in alfacalcidol-treated groups at week 12 are significantly higher than those in non-treated groups.

Histological findings in the kidney

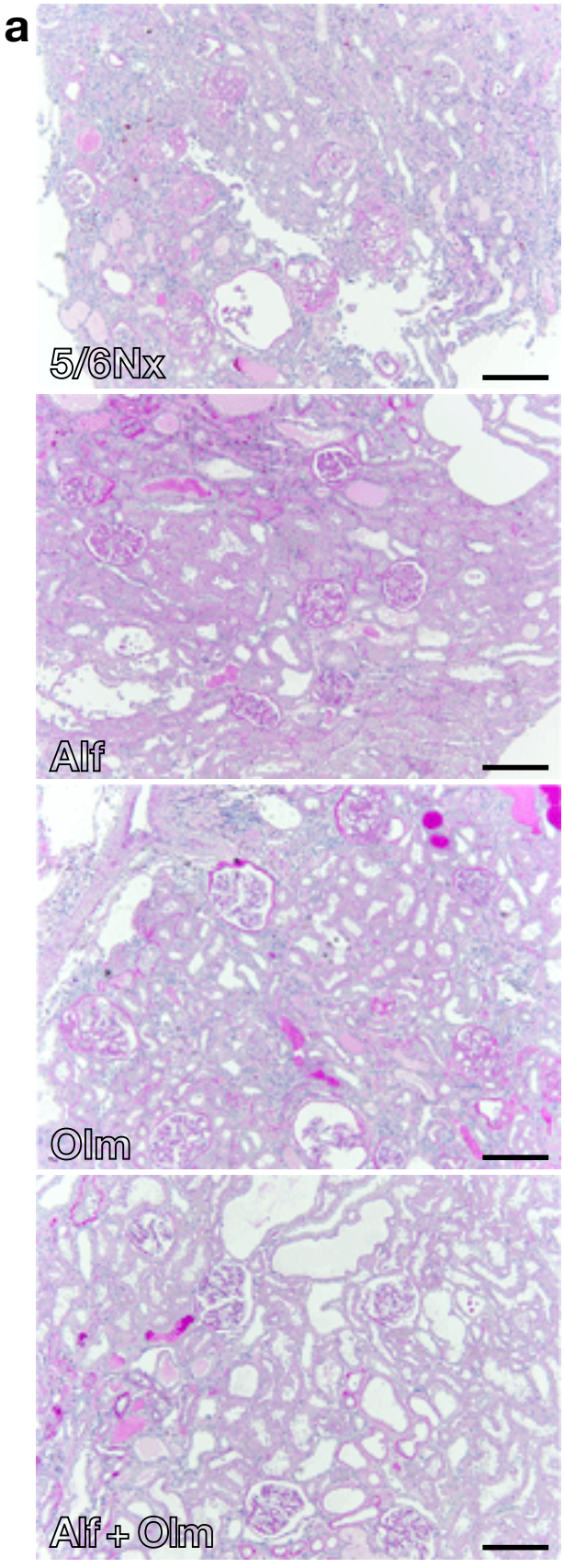
Histological examination of the kidney revealed remarkably advanced glomerular sclerosis in the 5/6Nx group at week 12. Conversely, the Alf, Olm and Alf + Olm groups had bettered glomerular sclerosis. Among the 3 groups, the Alf + Olm group markedly ameliorated glomerular sclerosis (Fig. 6a).

The IGS in all treated groups was significantly lower than that in the 5/6Nx group ($P < 0.05$ for the Alf group, $P < 0.05$ for the Olm group and $P < 0.01$ for the Alf + Olm group). The IGS in the Alf + Olm group was significantly lower than that in the Alf group ($P < 0.05$) and the Olm group ($P < 0.01$) (Fig. 6b).

mRNA quantification of TGF-β1 and klotho gene

Figure 7 shows the mRNA levels of the TGF-*β1* gene and *klotho* gene in renal tissue. The TGF-*β1* mRNA level in the 5/6Nx group was increased as compared with the levels in the other treated groups, significantly alleviated in the Alf and Alf + Olm groups ($P < 0.01$ for the Alf group, $P < 0.01$ for the Alf + Olm group), but not in the Olm group (Fig. 7a).

The *klotho* mRNA levels of the alfacalcidol-treated groups were increased in comparison with the level of the 5/6Nx group: the increase was especially significant in the Alf + Olm group. Furthermore, in comparison between the 5/6Nx group and either of alfacalcidol-treated groups, the increase was significantly higher for the Alf + Olm group than for the Alf group ($P < 0.05$ for the Alf + Olm group versus the 5/6Nx group, $P < 0.05$ for the Alf + Olm group versus the Alf group) (Fig. 7b).



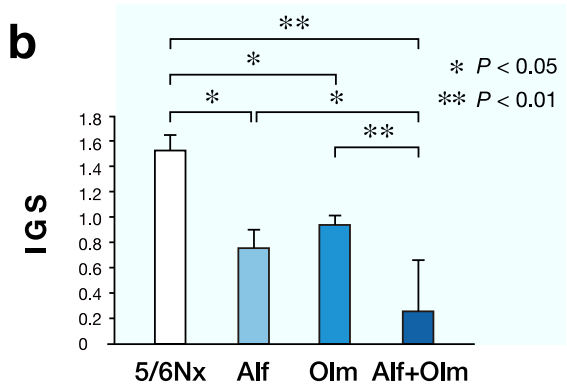


Fig. 6. Levels of glomerular sclerosis examined at week 12.

a: Histological examination of kidneys of rats in the 4 groups by periodic acid-Schiff staining. The sclerosis remarkably advances in the 5/6Nx group, and improves in the Alf, Olm and Alf + Olm groups. Especially, the sclerosis markedly ameliorates in the Alf + Olm group. Bar = 200 μm.

b: Levels of the index of glomerular sclerosis (IGS) in the 4 groups at week 12. All treated groups show significantly lower levels than the 5/6Nx group. The Alf + Olm group show significantly lower level than the Alf and Olm groups.

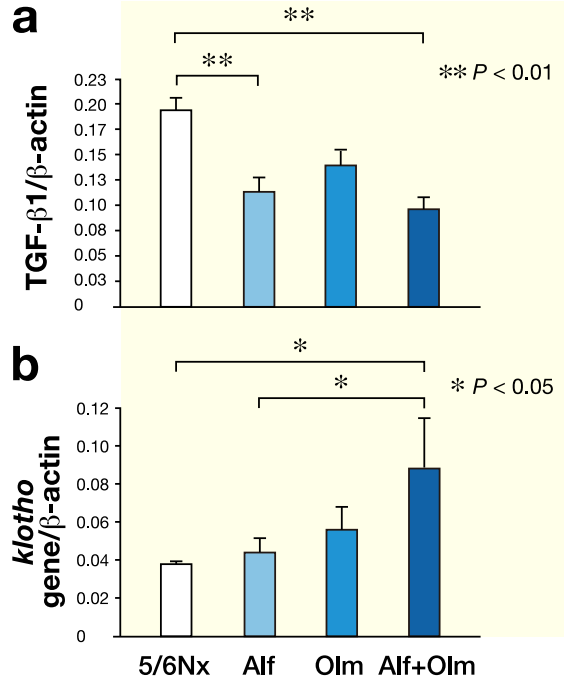


Fig. 7. Expressions of mRNA for TGF-*β1* and *klotho* genes in renal tissue.

a: Expression of TGF-*β1* mRNA. TGF-*β1* mRNA levels are significantly alleviated in the Alf and Alf + Olm groups, but not in the Olm group.

b: Expression of *klotho* mRNA. *klotho* mRNA levels in the treated groups are increased in comparison with the level in the 5/6Nx group. The level is significantly increased in the Alf + Olm group compared with that in the 5/6Nx or Alf group.

Discussion

In the present study, we demonstrated that the administration of alfacalcidol and/or olmesartan improved the CRF and up-regulated the *klotho* gene in the kidney. In particular, the combination therapy of the 2 drugs provided the most effective renoprotection. Spontaneously hypertensive rats have been used as a model for essential hypertension in humans, and 5/6Nx rats have commonly been used as an experimental model for CRF in humans. In this study, we combined these 2 models, and prepared 5/6Nx spontaneously hypertensive rats. During the 12-week experimental period, blood pressure, urinary protein and glomerular sclerosis in the 5/6Nx spontaneously hypertensive rats increased progressively. After 12 weeks, the glomerular sclerosis was remarkable.

The renin-angiotensin system plays an important role in the development of hypertension and the progression of CRF. TGF-$\beta 1$ has been shown to play a predominant role in mediating angiotensin II-induced extracellular matrix production and inhibits its degradation by increasing production of proteinase inhibitors in mesangial and tubulointerstitial cells (Border et al., 1992). Thus, TGF-$\beta 1$ plays a central role in the development glomerular sclerosis and tubulointerstitial fibrosis. In addition, continuous administration of angiotensin II resulted in the development of CRF, and decreased *klotho* mRNA expression in the rat kidney (Mitani et al., 2002). In our previous study, we have shown that the group treated with olmesartan prevented the progress of CRF and the *klotho* mRNA downregulation in the 5/6Nx rats (Maeta et al., 2009). In this study, the administration of olmesartan improved blood pressure, urinary protein, glomerular index of the kidney, and up-regulated the *klotho* mRNA.

It is well known that *klotho* gene in the kidney of CRF patients was markedly reduced (Koh et al., 2001). Recently, the roles of *klotho* function have been evaluated. Klotho protein exists in 2 forms: one is a secreted form that circulates in the blood, and the other is a transmembrane form expressed primarily in renal tubular cells (Kuro-o, 2009). It has been revealed that the transmembrane form of *klotho* functions as a co-receptor for FGF23.

FGF23 is secreted from osteocytes in response to high blood levels of P and 1,25$(OH)_2$ vitamin D_3 (Saito et al., 2005). FGF23 reduces 1, 25$(OH)_2$ vitamin D_3 by down-regulating the expression of the synthesized enzyme (Dusso et al., 2005). Thus, FGF23 induces a negative P balance by functioning as a phosphaturic hormone as well as a counter-regulatory hormone for 1,25$(OH)_2$ vitamin D_3. FGF23-deficient mice have hyperphospatemia and hypervitaminosis D associated with ectopic calcification. Besides the predictable phenotypes, FGF23-deficient mice develop multiple aging-like phenotypes that are almost identical with those observed in *klotho*-deficient mice (Larsson et al., 2003). These observations indicate that FGF23 and *klotho* may function in a common signal transduction pathway. In our present study, levels of serum FGF23 in the groups treated with alfacalcidol (Alf group, Alf + Olm group) were significantly higher than those in the 5/6Nx group and olmesartan alone treated group.

1,25$(OH)_2$ vitamin D_3 plays an important role in controlling bone metabolism through the regulation of Ca and P homeostasis. 1,25$(OH)_2$ vitamin D_3 deficiency is frequently recognized in patients with chronic kidney disease, in whom it is associated with increased mortality (Ravani et al., 2009). It was reported that 1,25$(OH)_2$ vitamin D_3 deficiency has also been involved in the progression of renal failure (González et al., 2004). Recently vitamin D analogues have been shown to have beneficial effects in patients with diabetic nephropathy (de Zeeuw et al., 2010). Several mechanisms could be involved in these renoprotective effects including direct antiproteinuric effects thorough the protection of podocyte, interactions with the renin-angiotensin system, and anti-inflammatory effects (Doorenbos et al., 2009). In our present study, we also found that the alfacalcidol administration yielded renoprotective effects and improvement in blood pressure, proteinuria and the IGS and TGF-$\beta 1$ gene expression. These renoprotective ef-

fects were the strongest in combination therapy of alfacalcidol and olmesartan. There are also some reports of investigations on the effect of vitamin D on blood pressure (Kristal-Boneh et al., 1997; Witham et al., 2009; Judd et al., 2010). Higher levels of active vitamin D are associated with lower blood pressure. Although the mechanism by which vitamin D may regulate blood pressure is unclear, it was suggested that vitamin D is negative regulator of renin (Li et al., 2002).

It has been reported that renal injury was reduced in *klotho*-overexpressing transgenic mice, because of suppression of oxidative stress (Haruna et al., 2007). In addition, *klotho* inhibited TGF-β1 signaling and suppressed renal fibrosis (Doi et al., 2011). Moreover, the administration of $1,25(OH)_2$ vitamin D_3 induced *klotho* gene expression in the kidney (Tsujikawa et al., 2003). The *klotho* mRNA level of the Alf + Olm group was significantly increased compared with that of 5/6Nx group, such that suppression of *klotho* gene downregulation might attenuate the progression of renal damage.

In the present study, we selected alfacalcidol as a vitamin D analogue. As there have been no reports of alfacalcidol being used for uremic rats, the dose of alfacalcidol referred to in the previous reports of dosage in ovariectomized rats, osteoporosis model, was administered (Shiraishi et al., 2000). The values of serum Ca and $1,25(OH)_2$ vitamin D_3 of the groups treated with alfacalcidol (Alf group, Alf + Olm group) were found to be very high. There is a possibility that hypercalcemia causes diuresis and natriuresis, which lead to dehydration and renal insufficiency (Shiraishi et al., 2003). However, the values of serum creatinine and the IGS of alfacalcidol-treated groups was lower than those in the 5/6Nx group in the present study, so we thought that hypercalcemia did not cause renal injury. In our study the serum FGF23 levels in the groups treated with alfacalcidol (Alf group, Alf + Olm group) were significantly higher those in the 5/6Nx group and Olm group, despite the improvement of the IGS and *klotho* gene expression. There was a therapeutic window for active vitamin D therapy, whereby too high a dose could be harmful by raising FGF23 excessively, but lower doses might promote less elevation of FGF23 (Isalova et al., 2009). In the present study, the groups treated with alfacalcidol showed an increase in serum FGF23, but improvement of renal failure.

In conclusion, the present study demonstrated that alfacalcidol and/or olmesartan exhibited a renoprotective effect, throughout the antihypertensive effect, as well as upregulation of *klotho* gene expression in the kidney.

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