Spironolactone ameliorates arterial medial calcification in uremic rats: the role of mineralocorticoid receptor signaling in vascular calcification

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1Department of Medicine and Clinical Science, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan; 2Department of Integrated Therapy for Chronic Kidney Disease, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan; and 3Division of Internal Medicine, Fukuoka Dental College Medical and Dental Hospital, Fukuoka, Japan

Submitted 22 December 2014; accepted in final form 27 August 2015

Tatsumoto N, Yamada S, Tokumoto M, Eriguchi M, Noguchi H, Torisu K, Tsuruya K, Kitazono T. Spironolactone ameliorates arterial medial calcification in uremic rats: the role of mineralocorticoid receptor signaling in vascular calcification. Am J Physiol Renal Physiol 309: F967–F979, 2015. First published September 2, 2015; doi:10.1152/ajprenal.00669.2014.—Vascular calcification (VC) is a critical complication in patients with chronic kidney disease (CKD). The effects of spironolactone (SPL), a mineralocorticoid receptor (MR) antagonist, on VC have not been fully investigated in CKD. The present in vivo study determined the protective effects of SPL on VC in CKD rats. Rats were divided into a control group and four groups of rats with adenine-induced CKD. Three groups were treated with 0, 50, and 100 mg·kg⁻¹·day⁻¹ SPL for 8 wk, and one group was treated with 100 mg·kg⁻¹·day⁻¹ SPL for the last 2 wk of the 8-wk treatment period. After 8 wk, CKD rats developed azotemia and hyperphosphatemia, with increases in the expression of serum and glucocorticoid-regulated kinase-1 and sodium-phosphate cotransporter, in inflammation and oxidative stress level, in osteogenic signaling and apoptosis, and in aortie calcification, compared with control rats. SPL dose dependently decreased these changes in the aorta, concomitant with improvements in renal inflammation, tubulointerstitial nephritis, and kidney function. SPL neither lowered blood pressure level nor induced hyperkalemia. Treatment of CKD rats for the last 2 wk with 100 mg·kg⁻¹·day⁻¹ SPL attenuated VC compared with CKD rats with the same degree of kidney function and hyperphosphatemia. In conclusion, SPL dose dependently inhibits the progression of VC by suppressing MR signaling, local inflammation, osteogenic transition, and apoptosis in the aortas of CKD rats.

chronic kidney disease; hyperphosphatemia; mineralocorticoid receptor; spironolactone; vascular calcification

VASCULAR CALCIFICATION (VC) is highly prevalent in patients with chronic kidney disease (CKD) and is strongly associated with cardiovascular morbidity and mortality (5, 22). VC, previously considered the passive deposition of calcium (Ca) phosphate (P) crystals, is now regarded as an active cell-mediated process of complex pathology, involving the transdifferentiation of vascular smooth muscle cells (VSMCs) into osteoblast-like cells, apoptosis of VSMCs, loss of calcification inhibitors, matrix vesicle release, and extracellular matrix degradation (14, 26, 35).

Various CKD-related factors, including hyperphosphatemia, uremic toxins, and oxidative stress, have been shown to induce VC, and inhibition of these factors has been found to have protective effects in experimental studies (1, 20, 21, 24, 44). However, preventing VC in CKD patients is difficult (4). Because the pathogenesis of VC is multifactorial and mediated by complex cascades (42), novel agents that interfere with the progression of VC are needed in patients with CKD.

The renin-angiotensin-aldosterone system plays critical roles in the pathogenesis of cardiovascular disease in CKD (34), with the contribution of aldosterone to the development of cardiovascular disorders being greater than expected (8). Spironolactone (SPL), a mineralocorticoid receptor (MR) antagonist, has been found to improve cardiovascular outcomes in clinical trials (9, 32, 38). The beneficial effects of MR antagonists on cardiovascular outcomes are owing not only to their lowering of blood pressure but to their direct roles in vascular function, in their suppression of phenotypic changes in VSMCs, and to their reductions in oxidative stress and fibrogenesis (7, 25). A recent study showed that aldosterone was elevated in the calcified areas of the aortas of rats without renal failure (43). Furthermore, SPL was shown to prevent the transdifferentiation of VSMCs and to reduce tissue Ca deposition in vitro (19), suggesting that SPL has direct protective effects on VC. Only one clinical study showed the protective effects of SPL on VC in hemodialysis patients (29). However, whether SPL can prevent the progression of VC is unclear, as is the mechanism by which SPL intervenes in the pathogenesis of VC in CKD.

The adenine-induced CKD rat is a model of chronic progressive tubulointerstitial nephritis with a small amount of proteinuria, caused by accumulation of 2,8-dihydroxyadenine crystals in the renal tubules and interstitium (46). This model is similar to obstructive uropathy in the clinical setting, and to unilateral ureteral obstruction models in animals. Because adenine-fed uremic rats develop extensive arterial medial calcification with hyperphosphatemia and high serum parathyroid hormone in 6–8 wk of feeding, these rats were often used as a useful model of uremic VC in experimental studies (20, 44, 45).

This study was therefore designed to clarify the protective effects of SPL on VC in CKD, by analyzing the effects of SPL on arterial medial calcification in adenine-induced uremic rats, focusing on the effects of SPL on MR signaling and related inflammation in the aorta.

MATERIALS AND METHODS

Ethical considerations and animal care. All experimental protocols were reviewed and approved by the Committee on Ethics of Animal

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Fig. 1. Study protocol. Rats were divided into 5 groups and fed their respective diets for 8 wk. CNT, rats fed a control diet; chronic kidney disease (CKD), rats fed a diet containing 0.3% adenine; CKD-SPL50, CKD rats treated with 50 mg·kg⁻¹·day⁻¹ spironolactone (SPL) for 8 wk; CKD-SPL100, CKD rats treated with 100 mg·kg⁻¹·day⁻¹ SPL for 8 wk. CKD-SPL100L, CKD rats treated with 100 mg·kg⁻¹·day⁻¹ SPL for the last 2 of the 8 wk. L, later treatment.

Experimentation at Kyushu University Faculty of Medicine (A25-073-1). Male Sprague-Dawley (SD) rats (10 wk old) were purchased from Kyudo (Saga, Japan) and housed in a climate-controlled space with 12:12-h day-night cycles and allowed free access to food and water. Rats were acclimated to a standard diet (1.0% Ca, 1.2% Pi; Oriental Yeast, Tokyo, Japan) for 7 days. All synthetic rodent diets were purchased from Oriental Yeast.

Experimental protocol. Following acclimatization to a standard diet for 7 days, 10-wk-old male SD rats (n = 48) were randomly subdivided into five groups. Control rats (n = 8) were fed a control diet (CNT) for 56 days. The remaining 40 rats were fed a diet containing 0.3% adenine for 8 wk. One group of 16 rats was not otherwise treated, while 8 rats each were fed 50 or 100 mg·kg⁻¹·day⁻¹ SPL (197-16873; Wako, Osaka, Japan) for 8 wk. The fifth group was fed the adenine-containing diet for 8 wk plus 100 mg·kg⁻¹·day⁻¹ SPL for the last 2 wk (n = 8). The control diet included 1.0% Ca, 1.2% Pi, and 19% casein-based protein. Adenine diets in the four uremic groups contained 1.0% Ca, 1.2% Pi, 19% casein-based protein, 20% lactose, and 0.3% adenine. Adenine was used to induce chronic progressive renal failure (45). The study protocol is shown in Fig. 1.

Every 2 wk, the rats were housed in metabolic cages for 24 h, and food and water intake and urine volume were recorded. Systolic blood pressure was measured in conscious rats at weeks 2, 4, 6, and 8 using the tail-cuff method (BP-monitor MK-2000; Muromachi Kikai, Tokyo, Japan). Collected urine was centrifuged at 3,000 g for 15 min, and the supernatants were stored at −30°C until analyzed. All rats were euthanized on day 56, and their blood, aorta, heart, liver, and kidneys were collected. Serum was separated by centrifugation at 3,000 g for 15 min and stored at −30°C until analyzed. The aorta, heart, liver, and kidney were dissected into several samples; one sample each was immersed in formalin for histological analysis, and the others were stored at −80°C until analyzed.

Biochemical parameters. Serum and urine concentrations of albumin, urea nitrogen, creatinine (Cr), Ca, Pi, and potassium (K) were measured using an automated analyzer (Hitachi, Tokyo, Japan). Cr clearance was calculated using the equation urinary Cr concentration × urine volume/serum Cr concentration/1.440 (ml/min). Serum calcitriol was measured by radioimmunoassay (SRL, Tokyo, Japan). Plasma aldosterone concentration and angiotensin II (ANG II) concentration were determined by solid-phase radioimmunoassay and double-antibody radioimmunoassay, respectively (Aloka ARC-950; Hitachi-Aloka Medical, Tokyo, Japan) (17). Commercially available rat ELISA kits were used to measure concentrations of intact parathyroid hormone (Immutopics International, San Clemente, CA), serum TNF-α (R&D Systems, Minneapolis, MN), serum intact fibroblast growth factor 23 (Kainos Laboratories, Tokyo, Japan), and 8-hydroxy-2'-deoxyguanosine (8-OHdG; Japan Institute for the Control of Aging, Nikken Seil, Shizuoka, Japan). All kits were used according to the manufacturer’s instructions, and their qualities were within analytic levels.

Examination of aorta and kidney specimens. Four-micrometer sections from paraffin-embedded aortas and kidneys were deparaffinized and processed for von Kossa staining using standard methods. To quantitatively evaluate the degree of aortic and kidney calcification, frozen aortic and kidney tissues were weighed and hydrolyzed in 1 ml of hydrochloric acid (6 mol/l) for 24 h. The Ca concentrations of the supernatants were determined by the o-cresolphthalein complexone method using commercially available kits (Calcium E-test Wako; Wako Pure Chemical Industry, Osaka, Japan), and the Pi concentrations of the supernatants were measured using commercially available kits (Phospha C-test Wako; Wako Pure Chemical Industry). Ca and Pi concentrations were normalized to dry tissue weight (μg/mg wet weight) (44). Digital micrographs of stained aortas were captured on an Eclipse E800 microscope (Nikon, Tokyo, Japan).

Two-micrometer sections from paraffin-embedded kidneys were deparaffinized and processed for Sirius Red staining using standard methods. Fibrotic areas, determined by Sirius Red staining, were quantitatively assessed as described (3, 17). Representative histological images were captured by light microscopy (Eclipse E800 microscope, Nikon).

Two survival curves were compared using the log-rank test. Fig. 2 shows the survival of each group of rats. The survival of each group of rats was determined using the Kaplan-Meier method and compared using the log-rank test. A two-sided P value <0.05 was considered statistically significant.
RNA isolation and real-time PCR. Total RNA was extracted from frozen rat tissue in liquid nitrogen using the guanidinium thiocyanate phenol-chloroform method (TRIzol reagent, Invitrogen Life Technologies, Carlsbad, CA), according to the manufacturer’s instructions. Complementary DNA was prepared by reverse transcription using a PrimeScript reagent kit (Perfect Real Time; Takara Bio, Otsu, Japan). Real-time quantitative PCR was performed using the SYBR Premix Ex Taq (Takara Bio), Applied Biosystems 7500 Real-time PCR systems (Applied Biosystems, Foster City, CA), and primers for GAPDH (RA015380); runt-related transcription factor 2 (Runx2; RA045439); serum- and glucocorticoid-regulated factor 2 (Runx2; RA045439); and Klotho (RA058439) with all primers purchased from Takara Bio. The amplification protocol consisted of an initial denaturation at 95°C for 30 s, followed by 40 cycles of denaturation at 95°C for 5 s and annealing and extension at 65°C for 35 s. The specificity of the PCR products was confirmed by analysis of their melting curves and by agarose gel electrophoresis. All measurements were performed in duplicate, and fold-changes in mRNAs were calculated by the $2^{-\Delta\Delta Ct}$ method, using GAPDH as an internal reference.

Immunohistochemistry. Four-micrometer sections from paraffin-embedded aortas and two-micrometer sections from paraffin-embedded kidney were deparaffinized, rehydrated, and prepared for antigen retrieval. Antigen retrieval was performed by heating in a microwave with 0.5% skim milk for 30 min at room temperature and then incubated in a humidified chamber for 12 h at room temperature with primary antibodies to Runx2 (diluted 1:25; Santa Cruz Biotechnology), phosphorylated p65 (diluted 1:50; Santa Cruz Biotechnology), ED-1 (diluted 1:200; Chemicon International, Temecula, CA), osteopontin (diluted 1:100; Santa Cruz Biotechnology), and 8-OHdG (diluted 1:200; Japan Institute for the Control of Aging). After three washes in phosphate-buffered saline/Tween 20 (0.2% vol/vol) for 5 min each, the sections were incubated with a horseradish peroxidase-conjugated secondary antibody (4 μg/ml; Nichirei, Tokyo, Japan) for 30 min at room temperature. Horseradish peroxidase was visualized by reaction with 3,3′-diaminobenzidine tetrahydrochloride and hydrogen peroxide. Representative digital micrographs were captured on an Eclipse E800 microscope (Nikon). To detect apoptosis in aortic tissue, TdT-mediated dUTP nick-end labeling (TUNEL) was performed using a commercial kit (In situ cell death detection kit, Roche Life Science), according to the manufacturer’s instructions.

Western blot analysis. Abdominal aorta, heart, liver, and renal cortex in lysis buffer (Tissue Protein Extraction Reagent; Thermo Scientific, Rockford, IL) containing protease inhibitor (Protease Inhibitor Cocktail for General Use; Nacalai Tesque, Kyoto, Japan) and phosphatase inhibitor (Phosphatase Inhibitor Cocktail; Nacalai Tesque) were homogenized using a gentleMACS (Miltenyi Biotec, Bergisch Gladbach, Germany). The homogenate was centrifuged at 15,000 g for 5 min at 4°C.

### Table 1. Effects of diets containing adenine and SPL on physical parameters over 24 h

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<td>101.5 ± 8.9*</td>
<td>71.2 ± 6.0*</td>
<td>69.7 ± 3.1*</td>
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Values are means ± SE. Blood pressure was measured by the tail-cuff method. CKD, chronic kidney disease; L, late treatment; ND, not determined; CNT, rats fed a control diet; CKD, rats fed a diet containing 0.3% adenine; CKD-SPL50, rats fed a diet containing 0.3% adenine and 50 mg kg$^{-1}$day$^{-1}$ (0.1%) spironolactone (SPL); CKD-SPL100, rats fed a diet containing 0.3% adenine and 100 mg kg$^{-1}$day$^{-1}$ (0.2%) SPL; CKD-SPL100L, rats fed a diet containing 0.3% adenine and 200 mg kg$^{-1}$day$^{-1}$ (0.4%) SPL. All measurements were performed in duplicate, and fold-changes in mRNAs were calculated by the $2^{-\Delta\Delta Ct}$ method, using GAPDH as an internal reference.
SPL PREVENTS AORTIC CALCIFICATION IN CKD

RESULTS

SPL affects survival rate and physical parameters independently of blood pressure level in uremic rats. Uremia was induced in rats by feeding a diet containing 0.3% adenine, with these rats used to test the protective effects of SPL on VC (Fig. 1). Four of the 16 rats fed the adenine diet alone (CKD group), and 1 of 8 rats treated with 50 mg·kg⁻¹·day⁻¹ SPL for 8 wk (CKD-SPL50 group) died during the study period (log-rank test, \( P = 0.016 \)) (Fig. 2). None of the rats in the other three groups died during the 8-wk study period. Systolic blood pressure levels were comparable among the five groups at each time point (Table 1).

Serial changes in body weight, food intake, urine volume, and water intake are shown in Table 1. Body weight, food intake, and water intake were lower and urine volume was greater in CKD rats than those in CNT rats at each time point. SPL treatment abrogated these changes induced by CKD.

SPL improves mineral homeostasis in uremic rats. Serum Pi levels were significantly higher in CKD than in CNT rats, but were significantly lower in CKD rats treated with 50 and 100 mg·kg⁻¹·day⁻¹ SPL for 8 wk than in CKD rats (Table 2). Serum Pi levels were comparable in CKD rats and those treated with 100 mg·kg⁻¹·day⁻¹ SPL for 2 wk. Urinary Pi excretion in both of these groups was significantly lower than in CNT rats, but was similar in CNT rats and those treated with 50 and 100 mg·kg⁻¹·day⁻¹ SPL for 8 wk (Table 3). Serum Ca levels were comparable in the five groups. The serum level of parathyroid hormone was significantly higher in CKD than in CNT rats, and water intake are shown in Table 1. Body weight, food intake, urine volume, and water intake are shown in Table 1. Body weight, food intake, and water intake were lower and urine volume was greater in CKD rats than those in CNT rats at each time point. SPL treatment abrogated these changes induced by CKD.

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was lower in rats treated with 50 and 100 mg·kg\(^{-1}\)·day\(^{-1}\) SPL for 8 wk, but was not altered in rats treated with 100 mg·kg\(^{-1}\)·day\(^{-1}\) SPL for 2 wk (Table 2). The serum calcitriol level was lower in CKD than in CNT rats, but did not differ significantly among the four CKD groups. Serum fibroblast growth factor 23 was increased in CKD compared with CNT, which was prevented by SPL treatment (CKD vs. CKD-SPL100) (Table 2).

SPL attenuates VC and phenotypic changes in the aortas of uremic rats. Representative photomicrographs of von Kossa-stained sections and quantification of aortic Ca and Pi content are shown in Fig. 3. CKD rats developed arterial medial calcification, whereas CNT rats did not. SPL treatment dose dependently decreased Ca and Pi content in the aortas of rats treated with treated with 50 and 100 mg·kg\(^{-1}\)·day\(^{-1}\) SPL for 8 wk and 100 mg·kg\(^{-1}\)·day\(^{-1}\) SPL for the last 2 wk (Fig. 3, B and C).

**Fig. 3.** Effects of SPL on calcification and phenotype in the aorta. **A**: representative photomicrographs of abdominal aortas stained with von Kossa stain (original magnification ×40). Quantification of calcium (Ca; **B**), and phosphate (Pi; **C**) contents in abdominal aortas is also shown. **D–G**: relative expression of runt-related transcription factor 2 (Runx2; **D**), sodium-phosphate cotransporter-1 (Pit-1; **E**), TNF-α (**F**), and serum- and glucocorticoid-regulated kinase-1 (Sgk-1; **G**) mRNAs normalized to that of GAPDH mRNA. Values are means ± SE and compared by 1-way ANOVA followed by the Tukey-Kramer test. A 2-sided \(P\) value <0.05 was considered statistically significant. *\(P\) < 0.05 vs. CNT. #\(P\) < 0.05 vs. CKD.
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To determine whether SPL has protective effects on VC irrespective of improvement in renal function and P1 excretion, we performed multivariable analysis using data from CNT rats and CKD rats treated with 0, 50, and 100 mg·kg⁻¹·day⁻¹ SPL for 8 wk. SPL treatment dose dependently decreased aortic Ca content, even after adjustment for Cr clearance and serum levels of Ca and P1 (Table 4).

In assessing VSMC phenotype in aortas, we found that the levels of Runx2, Pit-1, TNF-α, and Sgk-1 mRNAs were higher in CKD than in CNT rats, but were decreased by SPL treatment (Fig. 3, D–G). Linear regression analyses showed that Ca content in the aorta was significantly correlated with P1 content in the aorta ($R^2 = 0.776$, $P < 0.001$) and the levels of Runx2 ($R^2 = 0.431$, $P < 0.001$), Sgk-1 ($R^2 = 0.539$, $P < 0.001$), TNF-α ($R^2 = 0.210$, $P = 0.002$), and Pit-1 ($R^2 = 0.162$, $P = 0.007$) mRNAs. The Sgk-1 mRNA level showed significant correlations with TNF-α ($R^2 = 0.142$, $P = 0.013$), Runx2 ($R^2 = 0.562$, $P < 0.001$), and Pit-1 ($R^2 = 0.532$, $P < 0.001$) mRNA levels.

Immunohistochemical assays showed strong staining for Runx2 in the calcified areas of the aortas of CKD rats, staining ameliorated with treatment with 50 and 100 mg·kg⁻¹·day⁻¹ SPL for 8 wk (Fig. 4A). Similarly, positive staining for phosphorylated p65, a marker of activation of the NF-kB pathway, TUNEL, a marker of apoptosis, and 8-OHdG, a marker of oxidative stress were observed in the calcified areas of the aorta in CKD rats, with SPL treatment, attenuating the expression of these markers (Fig. 4, B–D). Western blot analysis showed that Runx2 was increased in CKD rats, which was ameliorated by SPL treatment (Fig. 4E).

SPL ameliorates systemic inflammation in uremic rats. Serum levels of TNF-α were higher in CKD than in CNT rats, but were lower in CKD rats treated with SPL than in CKD rats (Fig. 5A). Similar results were observed when serum 8-OHdG levels were measured (Fig. 5B).

SPL attenuates kidney damage and preserves kidney function in uremic rats. CKD rats showed high serum levels of urea nitrogen and Cr, and low levels of Cr clearance (Table 2 and Table 3). SPL dose dependently decreased serum levels of urea nitrogen and Cr, and increased Cr clearance. The fibrotic areas in the renal cortex, as determined by Sirius Red staining, were higher in CKD than in CNT rats, but were decreased by treatment of CKD rats with 50 and 100 mg·kg⁻¹·day⁻¹ SPL for 8 wk (Fig. 6, A and E). There were no significant differences in fibrotic areas between CKD rats and those treated with 100 mg·kg⁻¹·day⁻¹ SPL for 2 wk (CKD vs. CKD-SPL100L). CKD rats showed greater global sclerosis of glomeruli than CNT rats, but sclerosis was ameliorated by treatment with 50 and 100 mg·kg⁻¹·day⁻¹ SPL for 8 wk (data not shown). No significant serial changes in the level of proteinuria were observed among the five groups every 2 wk (data not shown). Urinary protein excretion was comparable among the five groups at week 8, and urinary K excretion and serum K level were comparable in the five groups (Table 2 and Table 3).

Ca contents in the kidney were higher in CKD than in CNT rats, with both being decreased by the treatment of CKD rats with 50 and 100 mg·kg⁻¹·day⁻¹ SPL for 8 wk (Fig. 6, B and F), but not by treatment with 100 mg·kg⁻¹·day⁻¹ SPL for 2 wk (Fig. 6F).

The levels of TGF-β1, osteopontin, TNF-α, and Runx2 mRNAs were higher in the kidneys of CKD than CNT rats, with three of these increases being ameliorated by SPL treatment for 8 wk, but not 100 mg·kg⁻¹·day⁻¹ SPL for 2 wk (Fig. 7, A, B, D, and E). Although the level of Sgk-1 mRNA was higher in CKD than in CNT rats, there were no differences among the four CKD groups (Fig. 7C). Klotho mRNA levels in the kidney were lower in the four CKD groups than in the CNT group, while there were no significant differences among the four CKD groups (Fig. 7F).

Linear regression analyses showed that interstitial fibrosis was significantly correlated with TGF-β1 ($R^2 = 0.399$, $P < 0.001$), osteopontin ($R^2 = 0.458$, $P < 0.001$), collagen type I ($R^2 = 0.291$, $P < 0.001$), Sgk-1 ($R^2 = 0.301$, $P < 0.001$), and TNF-α ($R^2 = 0.488$, $P < 0.001$) mRNA levels. The level of Sgk-1 mRNA was significantly correlated with the levels of TGF-β1 ($R^2 = 0.375$, $P < 0.001$), osteopontin ($R^2 = 0.354$, $P < 0.001$), collagen type I ($R^2 = 0.228$, $P < 0.001$), and TNF-α ($R^2 = 0.234$, $P < 0.001$) mRNAs. Immunohistochemistry revealed that expression levels of ED-1 and osteopontin were higher in CKD than in CNT rats, with these increases prevented by early, but not late, SPL treatment (Fig. 6, C and D). Western blot analysis showed that osteopontin was also increased in CKD rats, an increase ameliorated by treatment with SPL (Fig. 6G).

Effects of SPL on the renin-angiotensin-aldosterone system in uremic rats. Plasma ANG II concentration was increased in CKD rats and was dose dependently enhanced by SPL treatment (Table 2). Plasma aldosterone concentration was increased in CKD, which was enhanced by SPL treatment dose dependently (Table 2). However, neither plasma aldosterone concentration ($P = 0.847$) nor plasma ANG II concentration ($P = 0.712$) was linearly correlated with Ca content in the aorta.

To determine the expression of MR protein in the aorta and liver, we performed Western blotting. The expression of MR protein in K-562 whole cell lysate, aorta, kidney, heart, and liver are shown in Fig. 8A. Two bands (one band was located at molecular weight 100–120 kDa, and the other was at 60–80 kDa) were clearly observed in K-562 whole cell lysate (arrows, Fig. 8B), being consistent with the sample image provided by the manufacturer. Those two bands were
also identified in the lanes of aorta and kidney. Hence we regarded these two bands as true MR bands. Western blot analysis showed that the expression of MR protein in the aorta was comparable among the five groups of rats, while MR protein in the kidney was significantly lower in CKD and CKD-SPL100L than in CNT rats, being different from the patterns of aortic Ca content and kidney function (Fig. 8, C and D). These results indicate that the effects of SPL on VC and kidney function were independent of MR protein level.

**DISCUSSION**

Although SPL has been shown to improve cardiovascular outcomes in patients with heart diseases (7, 9, 25, 32, 38), the protective effects of SPL on VC in CKD remain unclear. The present study revealed three novel findings. First, SPL dose dependently attenuated VC and kidney damage in rats with adenine-induced uremia. Second, inhibition of VC was mediated by suppression of osteogenic transdifferentiation and apoptosis of VSMCs in the aorta. Third, amelioration of osteo-
genetic transition and apoptosis of VSMCs were due to blocking of MR signaling, followed by inhibition of local inflammation and oxidative stress and downregulation of Pit-1 and Sgk-1. These results indicate that MR signaling plays important roles in the pathogenesis of VC in CKD, and that SPL may retard the progression of VC in CKD.

Mounting evidence has demonstrated the critical roles of MR signaling in the progression of renal fibrosis (10, 12, 31). Aldosterone and cortisol activate MR, which regulates a variety of genes with MR-responsive elements, including Sgk-1 in the kidney. More recently, however, MR signaling was shown to be involved in the pathogenesis of vascular diseases, especially of VC (28, 39). For example, recent studies have shown that a set of genes, including those encoding Sgk-1 and inflammatory proteins, are regulated by MR signaling in the vasculature and that this set of genes differs from genes in the kidneys regulated by MR signaling (6, 19, 28). Our findings suggest that Pit-1 and Sgk-1 are among the candidate proteins regulated by MR and are critically involved in the protective effects of SPL on VC.

Pit-1, expressed in various cells, is a sodium-Pi cotransporter that regulates Pi entry into cells (16). Pi entry into VSMCs through Pit-1 is the first critical step in the VC cascade (14). A recent experimental study showed that SPL inhibits transdifferentiation of VSMCs into osteoblast-like cells by downregulating Pit-1 and prevents VC in klotho-hypomorphic mice (39). In addition, Pit-1 silencing suppressed all the VC-related genes in the cultured VSMCs (16, 39). In our study, CKD rats showed upregulation of Pit-1 and Runx2, whereas SPL attenuated these increases independently of plasma aldosterone and ANG II concentration. Importantly, Pit-1 has putative MR-responsive elements. These findings collectively indicate that activation of MR signaling followed by Pit-1 upregulation plays a pivotal role in the pathogenesis of VC in CKD in a plasma aldosterone- and ANG II concentration-independent manner. Furthermore, SPL suppresses MR activation, upregulation of Pit-1, and transdifferentiation of VSMCs, leading to the amelioration of VC in CKD.

In the present study, SPL prevented local inflammation (increased p65 in the aorta), oxidative stress (8-OHdG in the aorta), and apoptosis of VSMCs in the aorta. Inflammation and oxidative stress promote osteogenic transdifferentiation and apoptosis of VSMCs (2, 44, 48). Both TNF-α and oxidative stress stimulate the NF-κB pathway (15), with phosphorylation of p65 being closely linked to the activation of the NF-κB pathway (13). Furthermore, SPL prevents the apoptosis of vascular cells induced by MR activation, by inhibiting protein kinase B/Akt activation (41). These results suggest that SPL prevents VC by inhibiting the NF-κB pathway (inflammation), leading to the amelioration of the multistep cascade associated with complex VC pathology in CKD.

Another important downstream target of MR signaling in the VC process is Sgk-1, which possesses a putative MR-responsive element (16, 39) and has been shown to regulate epithelial sodium channel expression in the kidney (37). Importantly, Sgk-1 was shown to activate the NF-κB pathway and induce inflammation, apoptosis, and fibrosis in the heart, breast cancer cells, and glomerular mesangial cells (36, 37a, 47). A more recent study showed that Sgk-1 is important in the development of vascular inflammation and atherogenesis (6). These suggest that blockade of MR signaling prevents Sgk-1-dependent activation of NF-κB signaling in the aorta, leading to the amelioration of the multistep cascade associated with complex VC pathology in CKD.

SPL may also protect against VC by maintaining Pi excretion through the preservation of kidney function. Pi retention in CKD was shown to be the upstream trigger that initiates and accelerates the sequential cascade of VC (14, 35). Regardless of the severity of CKD, rats treated with a Pi binder do not develop VC, suggesting that Pi loading plays a critical role in VC development (45). Furthermore, mice with double-knockout of klotho and renal sodium-Pi IIa do not develop VC, because the Pi in-out balance is achieved through maintenance of urinary Pi excretion (30). Indeed, this study found that urinary Pi excretion was higher and serum Pi level lower in CKD-SPL100 than in CKD rats. These results collectively indicate that maintaining urinary Pi excretion and the serum Pi level is important in preventing VC in CKD.

Our study showed that SPL treatment for 2 wk following 6 wk of CKD decreased Ca content in rat aortas. The levels of Sgk-1, Pit-1, TNF-α, and Runx2 mRNAs were lower in CKD-SPL100L than in CKD rats. Furthermore, aortic Ca content was comparable between rats with CKD-SPL100L and 6 wk of CKD rats, while kidney function was comparable between

Fig. 5. Effect of SPL on systemic inflammation. A and B: serum levels of TNF-α (A) and 8-OHdG (B). A 2-sided P value < 0.05 was considered statistically significant. *P < 0.05 vs. CNT. #P < 0.05 vs. CKD.
Fig. 6. Effects of SPL on the histology and phenotype of the kidney. A–D: representative photomicrographs of kidney sections stained with Sirius Red (A) and von Kossa stains (B) and with antibodies against ED-1 (C) and osteopontin (OPN; D). Quantification of fibrotic areas stained with Sirius Red (E) and Ca content (F) in the kidney is also shown. G: Western blot analysis of osteopontin protein in the kidney. Values are means ± SE and compared using 1-way ANOVA followed by the Tukey-Kramer test. A 2-sided P value < 0.05 was considered statistically significant. *P < 0.05 vs. CNT. #P < 0.05 vs. CKD.
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these two groups (data not shown). Previous in vitro studies showed that SPL has direct protective effects on VC in cultured VSMCs (19, 39). Our study suggests that SPL has a direct protective effect on VSMCs in the aorta independently of the effects of SPL on kidney function and that treatment with SPL is a practical and promising strategy in patients with established CKD and VC.

SPL treatment of various animal models with acute kidney injury and CKD have renoprotective effects on renal fibrosis and inflammation through the inhibition of TGF-β1, collagen type I, and macrophage infiltration (10, 12, 31). Our study showed that SPL attenuated macrophage infiltration and fibrosis through suppression of TGF-β1 and osteopontin expression in rats with adenine-induced CKD. As shown in other rat models, the SPL-mediated improvements in renal fibrosis may be the result of blocking of Sgk-1 signaling (11). Interestingly, recent studies have shown that aldosterone exerts both MR-dependent and -independent effects (26). Additional studies are therefore needed to clarify the precise mechanisms by which SPL exerts renoprotective effects in CKD.

It remains unclear whether the effects of SPL on plasma ANG II and aldosterone levels are involved in SPL amelioration of VC. Several experimental studies showed that blockage of ANG II receptor I reduced vascular calcification in uremic rats (18). This study found that neither plasma ANG II nor aldosterone concentration correlated with Ca content in the aorta. However, the cross talk between ANG II and aldosterone is complex, and treatment with SPL has been shown to affect these hormones (17, 33). Thus the protective effects of SPL on VC in our rat model may be associated with changes in the plasma renin-angiotensin system.

This study had several limitations. First, we did not examine the effect of SPL on the phosphorylation of Sgk-1 and Pit-1 proteins (23). Because phosphorylated, not total, Sgk-1 is more important in Sgk-1-mediated reactions (49), further studies are needed to determine the role of phosphorylated Sgk-1. Second, we did not clarify the effects of SPL on fibroblast growth factor 23 and klotho and their effects on Pi metabolism and VC. Third, we could not determine the mechanism by which SPL improved survival, nor could we determine the cause of death of rats in the CKD and CKD-SPL groups. Because rat body weight at death was drastically decreased due to malnutrition and malnutrition was closely related to serum Cr level, SPL may have reversed malnutrition by preventing progressive kidney dysfunction, leading to improved mortality in rats treated with SPL.

In conclusion, the present study demonstrated that SPL attenuates VC in rats with adenine-induced CKD, in manners both dependent and independent of renal function. These results suggest that SPL may be a candidate drug to simultaneously prevent the progression of CKD and VC in patients with CKD, by mitigating MR signaling and local and systemic inflammation. Further studies are needed to determine the
precise mechanism by which MR signaling affects the progression of VC in CKD patients.

ACKNOWLEDGMENTS

We thank Edanz Editing (http://www.edanzediting.co.jp/) for their English editing of our manuscript.

GRANTS

This study was supported in part by the Japanese Association of Dialysis Physicians, the Ministry of Education, Culture, Sports, Science, and Technology-Supported Program for the Strategic Research Foundation at Private Universities, and a grant from The Clinical Research Promotion Foundation 2014.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Author contributions: N.T., S.Y., M.T., and K. Tsuruya provided conception and design of research; N.T., S.Y., and H.N. performed experiments; N.T., S.Y., and M.E. analyzed data; N.T., S.Y., M.T., M.E., K. Torisu, and K. Tsuruya interpreted results of experiments; N.T. and S.Y. prepared figures;
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