Spironolactone Suppresses Peritubular Capillary Loss and Prevents Deoxycorticosterone Acetate/Salt-Induced Tubulointerstitial Fibrosis

Yoshitaka Iwazu, Shigeaki Muto, Genro Fujisawa, Eiko Nakazawa, Koji Okada, Shun Ishibashi, Eiji Kusano

Abstract—We examined whether and how peritubular capillary (PTC) loss in the renal cortex contributes to the development of deoxycorticosterone acetate (DOCA)/salt-induced tubulointerstitial fibrosis. Uninephrectomized rats provided with 0.9% NaCl/0.3% KCl drinking solution ad libitum were divided into control, DOCA, and spironolactone groups, which were administered vehicle, DOCA alone, and DOCA plus spironolactone for 1 (initial phase) and 4 weeks (delayed phase), respectively. Exposure to DOCA initiated a sequence of events that initially involved reduced PTC density, followed by a delayed response that involved further reduced PTC density, development of tubulointerstitial fibrosis and hypertension, enhanced expression of transforming growth factor-β1 and connective tissue growth factor, and impaired renal function. Concomitant with the reduced PTC density, the 2 hypoxia-responsive angiogenic factors (vascular endothelial growth factor and hypoxia-inducible factor-1α) and the antiangiogenic factor (thrombospondin-1) were upregulated in cortical tubular cells of the DOCA group during the 2 phases and only in the delayed phase, respectively. In the DOCA group, PTC endothelial cell apoptosis was enhanced during the 2 phases, and PTC endothelial cell proliferation was inhibited in the delayed phase. In accordance with upregulation of thrombospondin-1, p53 expression was enhanced in the DOCA group in the delayed phase. The initial and delayed effects of DOCA were blocked in the spironolactone group. We conclude that exposure to DOCA initially caused the reduced PTC density associated with enhanced apoptosis independent of thrombospondin-1, which induced tubulointerstitial fibrosis via p53-mediated thrombospondin-1 activation, and spironolactone conversely corrected the effects of DOCA to prevent fibrosis. (Hypertension. 2008;51:1-6.)

Key Words: tubular hypoxia ■ peritubular capillary ■ endothelial cell apoptosis ■ thrombospondin-1 ■ p53

Excess mineralocorticoids, because of administration of aldosterone or deoxycorticosterone acetate (DOCA) together with salt induce malignant hypertension that gradually leads to damage of the kidney, heart, and vasculature.1,2 The kidney damage of this model is characterized by renal vascular injury, tubulointerstitial fibrosis, tubular dilatation, glomerulosclerosis, and renal hypertrophy.3,4 The effect of mineralocorticoid/salt on renal fibrosis can be blocked by the selective mineralocorticoid receptor (MR) antagonist, eplerenone.3,4 However, the mechanisms responsible for mineralocorticoid/salt-induced MR-mediated renal fibrosis remain unknown.

Peritubular capillaries (PTCs) play a major role in maintaining renal function and hemodynamics because they surround tubules and provide oxygen and nutrients to tubular cells.5 Loss of PTCs or reduced PTC density (rarefaction) is now believed to result in impaired delivery of oxygen and nutrients to the tubules, producing chronic tubular hypoxia, which, in turn, induces tubular damage, extracellular matrix synthesis and cytokine release, and fibroblast proliferation, leading to tubulointerstitial fibrosis.6 The reduced PTC density is now an important factor of the progression of tubulointerstitial fibrosis to kidney failure.7 In different animal models of renal diseases, including remnant kidney,7 patients with chronic allograft nephropathy6 and in different animal models of renal diseases, including remnant kidney,7 patients with chronic allograft nephropathy6 and in different animal models of renal diseases, including remnant kidney,7 experimental chronic kidney disease and aging,8 the reduced PTC density is associated with enhanced apoptosis.9 Recently, it has been recognized that MR activation contributes to the pathophysiology of heart failure by initiating cardiomyocyte apoptosis,10–13 whereas MR blockade with spironolactone (SPL) or eplerenone limits apoptosis.11,12 However, it is unclear whether the reduced PTC density is actually related to enhanced apoptosis and contributes to the development of DOCA/salt-induced tubulointerstitial injury. Therefore, we explored whether and how PTC loss in the renal cortex contributes to development of DOCA/salt-induced tubulointerstitial fibrosis.

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Materials and Methods

Animals
The study protocol was approved by the Jichi Medical University Animal Ethics Committee. Male Sprague-Dawley rats (Clea; 180 to 220 g) were anesthetized with Brevital sodium, and the left kidney was removed. Immediately after nephrectomy, animals were randomly divided into 3 groups (n=6 per group): control, DOCA, and SPL groups, which were respectively administered vehicle; DOCA (20 mg/kg SC) alone; and DOCA plus SPL (50 mg/kg per day SC) for 1 and 4 weeks just after the surgery. The rats were then pair-fed with standard rat chow and a drinking solution containing 0.9% NaCl/0.3% KCl ad libitum for 1 or 4 weeks.

Systolic blood pressure (SBP) was measured in conscious rats by the tail-cuff method. Rats were placed in metabolic cages for 24-hour urine collection to measure creatinine and NAG concentrations. Animals were decapitated at weeks 1 and 4. Trunk blood was collected for the determination of serum creatinine concentration, and creatinine clearance (CCR) was then calculated. Immediately after the right kidney was removed, the upper third portion of the kidney was cut transversely and preserved in 3% formaldehyde solution. Specimens were embedded in paraffin and cut transversely into 5-μm-thick slices on a microtome.

Renal Histology
The kidney sections were used for picro-Sirius red staining and immunohistochemical staining using the following primary antibodies: monoclonal mouse anti-rat endothelial cell antibody (RECA-1) (1:50; Oxford Biotechnology; polyclonal rabbit anti-human von Willebrand factor (vWF) antibody (1:100; Chemicon International); affinity-purified rabbit vascular endothelial growth factor (VEGF); IgG (1:50; Immuno-Biological Laboratories); monoclonal mouse anti-human hypoxia-inducible factor-1α (HIF-1α) antibody (1:200; Novus Biologicals); affinity-purified rabbit cleaved caspase-3 (Casp3) antibody (1:50; Calbiochem); monoclonal mouse anti-human thrombospondin-1 (TSP-1) antibody (1:10; Santa Cruz Biotechnology); mouse monoclonal antibody to rat proliferating cell nuclear antigen (PCNA; 1:100; Santa Cruz); affinity-purified polyclonal rabbit anti-human vWF antibody (1:100; Chemicon International); affinity-purified rabbit cleaved caspase-3 (Casp3) antibody (1:50; Calbiochem); monoclonal mouse anti-human hypoxia-inducible factor-1α (HIF-1α) antibody (1:200; Novus Biologicals); affinity-purified rabbit cleaved caspase-3 (Casp3) antibody (1:50; Calbiochem); monoclonal mouse anti-human thrombospondin-1 (TSP-1) antibody (1:10; Santa Cruz Biotechnology); mouse monoclonal antibody to rat proliferating cell nuclear antigen (PCNA; 1:100; Santa Cruz); affinity-purified polyclonal rabbit anti-human vWF antibody (1:100; Chemicon International); affinity-purified polyclonal goat anti-human transforming growth factor-β1 (TGF-β1) antibody (1:100; Santa Cruz); and affinity-purified polyclonal goat anti-human connective tissue growth factor (CTGF) antibody (1:100; Santa Cruz). Immunohistochemistry was performed using the Vectastain Elite ABC kit (Vector Laboratories), as described previously. Sections to be assayed for Casp3, TSP-1, and TGF-β1 were heated in a microwave oven (500 W) for 5 minutes in 0.01 mol/L of citrate buffer (pH 6.0). Negative controls without primary antibodies were carefully examined for each reaction. Nuclei were counterstained with hematoxylin or methyl green.

To examine whether there was any evidence of endothelial cell proliferation, double-immunostaining was performed with the anti-vWF antibody and the anti-PCNA antibody. After staining with the anti-PCNA antibody, residual peroxidase activity was extinguished with 3% H2O2 in methanol for 5 minutes. The remaining biotin was blocked by incubation with avidin D and biotin solution (Vector). Samples were incubated with the anti-vWF antibody overnight at 4°C, following sequentially by biotinylated rabbit IgG antibody (Vector) and peroxidase-conjugated avidin D, with color development using diaminobenzidine and NiCl.

Identification of Apoptosis
Apoptotic cells were identified based on the presence of fragmented nuclear DNA in histological sections labeled using the TUNEL method. Briefly, deparaffinized 5-μm–thick sections were microwave irradiated, as described above. Immediately after irradiation, sections were immersed in 0.01% citrate buffer at 4°C for 20 minutes. After blocking endogenous peroxidase activity by immersion in 3% H2O2 in methanol, sections were rinsed in 0.1% H2O2 in Tris/HCl buffer (pH 7.2), 140 mmol/L of Na cacodylate, and 1 mmol/L of CoCl2 and then incubated for 60 minutes at 37°C with terminal deoxynucleotidyltransferase (1:100) and biotinylated dUTP (1:200) in terminal deoxynucleotidyltransferase buffer. The biotinylated nuclei were visualized using avidin-peroxidase and NiCl-containing diaminobenzidine.

PTC endothelial cell apoptosis was identified by double labeling using TUNEL and RECA-1. Initially, sections were labeled using the TUNEL protocol described above. Next, the sections were blocked for 15 minutes each in avidin D and biotin (Vector) in PBS. The sections were incubated with biotinylated mouse RECA-1, biotinylated normal mouse IgG, and avidin–biotin–peroxidase complex and were visualized with diaminobenzidine. Negative controls were produced by omitting dUTP or terminal deoxynucleotidyltransferase from the TUNEL protocol. PTC endothelial cell apoptosis was also detected by immunohistochemical staining with the anti-Casp3 antibody and the RECA-1 in serial sections of the kidney tissues.

Quantification of Morphology and Immunohistochemical Staining
Using computerized image analysis software (Image Pro 5.1, Media Cybernetics), we measured the collagen volume fraction in the tubulointerstitial, perivascular, and glomerular areas, as described previously. In each sample, ≥20 randomly selected cortical tubulointerstitial fields (0.141 mm2 per field) were assessed at x400 magnification for quantification of PTCs and apoptotic PTCs. For PTCs, the number of PTC lumen surrounded by RECA-1– or anti-vWF antibody–positive cells with <8-μm lumen size and with 1 nucleus per tubulointerstitial field was counted, and PTC density was expressed as the number per tubulointerstitial field. For apoptotic and proliferative PTCs, the number of both apoptotic PTCs (RECA-1- and TUNEL-positive cells) and PTCs (RECA-1–positive cells) per tubulointerstitial field, as well as the number of both proliferative PTCs (anti-vWF antibody– and anti-PCNA antibody–positive cells) and PTCs (anti-vWF antibody–positive cells) per tubulointerstitial field, were counted, and the percentage of apoptotic PTC and percentage of proliferative PTC were expressed as the percentage of apoptotic and proliferative PTCs compared with the total number of PTCs, respectively. For quantification of the expres-
sion of HIF-1α and p53 in cortical tubules, the number of HIF-1α- and p53-positive nuclei in ≥200 randomly selected cortical tubules was counted, and the data were expressed as the number of HIF-1α- and p53-positive nuclei per tubule, respectively. For quantification of the expression of VEGF, TSP-1, TGF-β1, and CTGF in cortical tubules, their percentage areas in 20 randomly selected cortical tubulointerstitial areas of the stained tissue within a given field without vessels or glomeruli were measured using the computerized image analysis system (Image Pro 5.1). Data were expressed as the percentage of the total tubulointerstitial field. All of the quantification was performed in a blinded manner.

Statistics
Results are presented as the means±SEMs, and data were analyzed using Student unpaired t test or ANOVA for repeated measures, followed by posthoc analysis with Fisher test, where appropriate. \( P<0.05 \) was considered statistically significant.

Results
We first compared SBP, urine NAG levels, and CCr among the 3 groups of rats (Figure 1A through 1C, respectively). None of the 3 parameters at baseline or week 1 were different among the groups. However, both SBP and urine NAG levels were significantly greater and CCr was significantly lower at week 4 in the DOCA group compared with the control group. The DOCA effects were completely inhibited in the SPL group.

At week 4, the tubulointerstitial collagen volume fraction by picro-Sirius red staining in the DOCA group was significantly greater than in the control group, although it was not elevated compared with the control group at week 1 (Figure S1A, see the online data supplement available at http://hyper.ahajournals.org). Similar findings were observed with respect to the glomerular and perivascular collagen volume fractions (data not shown). Because it has been suggested that the profibrotic growth factors (TGF-β1 and CTGF) play a pivotal role in renal fibrogenesis,14,16 we examined whether TGF-β1 and/or CTGF were involved in the development of tubulointerstitial fibrosis induced by DOCA. At week 4, expression of both TGF-β1 and CTGF in the DOCA group was increased compared with that in the control group, and this was completely suppressed in the SPL group (Figure S1B and S1C and S1D and S1E, respectively), although it was not different among the groups at week 1.

We then assessed the integrity of the microvasculature in the renal cortex of the 3 groups by immunostaining for RECA-1 (Figure 2A). At weeks 1 and 4, PTC density was significantly lower in the DOCA group than in the control group, and the DOCA effects were completely and partially normalized in the SPL group, respectively (Figure 2B). Furthermore, in the DOCA group, the reduced PTC density was significantly greater at week 4. Similar changes in the microvasculature were found by labeling with a second endothelial cell marker, anti-vWF antibody (data not shown).

We next examined whether enhanced apoptotic cell death contributes to the reduced PTC density in the DOCA group. In double-labeling assays using TUNEL and RECA-1, at week 1, apoptotic PTCs were rarely identified in the control and SPL groups but were prominent in the DOCA group (Figure 3A). Statistical analyses showed a significant increase in the percentage of apoptotic PTC in the DOCA group at weeks 1 and 4, which was completely suppressed in the SPL group, although the increase was less at week 4 (Figure 3B).

To demonstrate the possibility that the reduced PTC density in the kidney cortex of the DOCA group at weeks 1
and 4 may lead to chronic tubular hypoxia, we compared expression of the 2 hypoxia-responsive angiogenic factors (VEGF and HIF-1α) among the 3 groups of tubular cells. At weeks 1 and 4, VEGF expression was elevated significantly in the DOCA group compared with the control group, and this was completely suppressed in the SPL group, although VEGF expression was greater at week 4 in the DOCA group (Figure 4A and 4B). Similarly, at week 4, the number of HIF-1α-staining nuclei per cortical tubule in the DOCA group was significantly greater than in the control group and was completely reversed in the SPL group, although it was not elevated compared with the control group at week 1 (data not shown).

The above findings demonstrating that the PTC density at weeks 1 and 4 was reduced despite upregulation of the angiogenic factors in cortical tubules of the DOCA group suggest the possibility that the expression of angiogenic factors may be enhanced in the tubules and oppose the stimulatory effects of the angiogenic factors on endothelial cell growth. To assess this possibility, we examined TSP-1 expression, because this can induce endothelial cell apoptosis and inhibit angiogenesis by activating Casp3. At week 4, TSP-1 expression in cortical tubule cells of the DOCA group was significantly greater than in those of the control group, and this was reversed by SPL treatment, although it was not different among the groups at week 1 (Figure 4C and 4D). Because the tumor suppressor p53 is known to transactivate TSP-1 and causes apoptosis in fibroblasts, we examined whether p53 was involved in the DOCA-induced TSP-1 upregulation at week 4. Enhanced expression of p53 was observed in cortical tubule cells of the DOCA group during the delayed phase, which was blocked in the SPL group (Figure 4E and 4F).

The finding that the degree of reduced PTC density in the DOCA group was greater at week 4 than at week 1 but the increase in the percentage of apoptotic PTC was less at week 4 suggests that the reduced PTC density in the group at week 4 cannot be explained only by the enhanced apoptosis. Because it has been shown that TSP-1 also inhibits VEGF-mediated endothelial cell proliferation, we examined the PTC endothelial cell proliferation during this phase by double immunostaining with antibodies against vWF and PCNA (Figure 5A). At week 4, the percentage of proliferative PTC in the DOCA group did not increase significantly compared with the control group (Figure 5B). Therefore, the reduced PTC density in the DOCA group at week 4 was attributable to both enhanced apoptosis and inhibition of cell proliferation, both of which were associated with TSP-1 upregulation.

### Discussion

We demonstrated that the initial phase of chronic exposure to DOCA (week 1) exhibited reduced PTC density, whereas the delayed phase (week 4) involved the development of tubulointerstitial fibrosis and hypertension, further reduced PTC density, elevated NAG, and reduced CCr. In parallel with the development of tubulointerstitial fibrosis, the expression of the profibrotic growth factors (TGF-β1 and CTGF) was enhanced in cortical tubule cells of the DOCA group, indicating that the 2 growth factors are highly associated with the development of tubulointerstitial fibrosis in this model. The expression of the 2 hypoxia-responsive angiogenic factors (VEGF and HIF-1α) was also enhanced in cortical tubule cells of the DOCA group, although the expression of VEGF to the reduced PTC density appears earlier than that of HIF-1α. Therefore, in the DOCA group, the reduced PTC density and the resulting tubular hypoxia were indeed detected during the initial phase without impaired renal function or histological tubulointerstitial fibrosis, and both impaired renal function and tubulointerstitial fibrosis occurred subsequently. In other words, the reduced PTC density associated with tubular hypoxia was an initiator for the development of tubulointerstitial fibrosis in this model.

We also demonstrated that, in the DOCA group, the reduced PTC density during the initial phase resulted from enhanced apoptosis, whereas that during the delayed phase was because of both enhanced apoptosis and inhibition of proliferation. The effects of DOCA on PTC endothelial cell apoptosis and proliferation were also blocked by SPL treatment. Therefore, this is the first study to demonstrate that MR activation promotes PTC endothelial cell apoptosis via mechanisms involving the upregulation of Casp3 during the 2 phases and inhibits PTC endothelial cell proliferation only during the delayed phase. In the heart, MR activation induces cardiomyocyte apoptosis both in vitro and in vivo.
Moreover, aldosterone also enhances apoptosis in skeletal muscles and in tubule cells. Similar to our findings, increased Casp3 activity has been reported to be involved as a mechanism of myocyte apoptosis. Apoptosis is also recognized in tubule cells subjected to injury by ischemia or toxic drugs. Savill noticed that, in these circumstances, there may be a close relationship between tubular cell regeneration and increased apoptosis and suggested a beneficial role for apoptosis in the remodeling of hyperplastic tubules. Whether this concept can be applied to the enhanced PTC apoptosis in DOCA-induced tubulointerstitial injury requires further investigation.

In the DOCA group at the 2 phases, we observed reduced PTC density despite upregulation of the angiogenic factors, although the degree of both the reduced PTC density and enhanced expression of the angiogenic factors was greater during the delayed phase. It has been shown that TSP-1 not only induces endothelial cell apoptosis but can also exert its antiangiogenic effect by inhibiting endothelial cell proliferation. TSP-1 is expressed in tubule cells and in tubulointerstitial fibroblasts, and its expression correlates with loss of the microvascular endothelium. We found that only at delayed phase was TSP-1 expression in cortical tubule cells greater in the DOCA group than in the control group.
Therefore, the reduced PTC density in the DOCA group during the initial and delayed phases is mediated by TSP-1-independent and -dependent mechanisms, respectively. The TSP-1 upregulation during the delayed phase could both induce PTC apoptosis and inhibit PTC proliferation and thereby effectively overcome the stimulatory effect of the angiogenic factors on PTC growth, resulting in reduced PTC density. TSP-1 expression in cancerous tissues is under the regulation of p53.20 Similarly, we observed that, in accordance with the upregulation of TSP-1, expression of p53 was markedly enhanced in the DOCA group at delayed phase, and upregulation of both proteins was reversed in the SPL group. Thus, upregulation of p53 is coupled with that of TSP-1 during the delayed phase, and p53 may regulate TSP-1 expression via MR activation.

We also demonstrated that both the initial and delayed effects of DOCA were inhibited by SPL, indicating that both effects were mediated by MR activation. Although we evaluated the effects of SPL on SBP by the indirect tail-cuff method, those of SPL during the delayed phase may occur via its BP lowering effect, because during the phase, the DOCA group during the phase exhibited tubulointerstitial fibrosis and hypertension, both of which were completely inhibited in the SPL group. In sharp contrast, those of SPL during the initial phase may be independent of BP effect, because during the phase, the DOCA-induced and SPL-normalized only exhibited reduced PTC density without any changes in SBP.

Perspectives

Exposure to DOCA initially caused reduced PTC density associated with enhanced apoptosis of endothelial cells independent of TSP-1, which secondarily induced tubulointerstitial fibrosis via p53-mediated TSP-1 activation. Furthermore, SPL conversely corrected the effects of DOCA to prevent the fibrosis. These findings may provide novel insights into the pathogenesis of mineralocorticoid/salt-induced renal injury and may help to explain the beneficial effects of MR inhibition in clinical trials for patients with hyperaldosteronism.

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Disclosures

None.

References

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SPIRONOLACTONE SUPPRESSES PERITUBULAR CAPILLARY LOSS AND PREVENTS DOCA/SALT-INDUCED TUBULOINTERSTITIAL FIBROSIS

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**Figure S1.** (A) Tubulointerstitial collagen volume fraction determined by picro-Sirius red staining in the kidney cortex of the control, DOCA, and SPL groups. Values are presented as the mean ± SEM (n=6 per group). **P<0.001 vs. control; ++P<0.001 vs. DOCA.** Representative micrographs of immunostaining for TGF-β1 (B) and CTGF (D) in the kidney cortex of the control, DOCA, and SPL groups at W4. Magnification, x400. The immunostaining areas for TGF-β1 (C) and CTGF (E) in cortical tubulointerstitial areas of the 3 groups. Values are presented as the mean ± SEM (n=6 per group). *P<0.01 vs. control; +P<0.01 vs. DOCA.