ORIGINAL ARTICLE

L/N-type calcium channel blocker cilnidipine ameliorates proteinuria and inhibits the renal renin–angiotensin–aldosterone system in deoxycorticosterone acetate-salt hypertensive rats

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Cilnidipine, an N/L-type calcium channel blocker, has been reported to inhibit sympathetic nerve activity and has a greater renoprotective effect than L-type calcium channel blockers. To investigate the hypothesis that cilnidipine might ameliorate advanced hypertensive nephropathy and inhibit the renal renin–angiotensin–aldosterone system, cilnidipine (1 mg per kg per day) or amlodipine (1 mg per kg per day) was administered to uninephrectomized deoxycorticosterone (DOCA)-salt hypertensive rats (DOCA-salt) for 4 weeks by gavage. Although the blood pressure in the DOCA-salt group was higher than that of control, neither cilnidipine nor amlodipine had any effect on the increase in blood pressure in the DOCA-salt group. The DOCA (40 mg per kg per week, subcutaneously (s.c.)) and salt (1% NaCl in drinking water) treatment significantly aggravated the levels of urinary protein excretion and creatinine clearance and increased glomerulosclerosis and collagen deposition in the tubulointerstitial area of the kidney. These effects were attenuated by cilnidipine treatment. Reverse transcription-polymerase chain reaction analysis revealed that the renal expression of mRNA for collagen I/IV and transforming growth factor- β was enhanced in the DOCA-salt group and that the overexpression of these molecules was suppressed by cilnidipine. Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase-derived superoxide production in the kidney and urinary norepinephrine excretion, which were enhanced in the DOCA-salt group, were suppressed by cilnidipine. Cilnidipine also decreased the activity and expression of angiotensin-converting enzyme (ACE) and the aldosterone concentration in the renal homogenate. Although neither cilnidipine nor amlodipine had any effect on the increased blood pressure in the DOCA-salt group, these renal changes were not induced by treatment with amlodipine. In conclusion, cilnidipine inhibited renal dysfunction, sympathetic nerve activity and renal renin–angiotensin–aldosterone system in the DOCA-salt group.

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INTRODUCTION

Because the kidney is a major target organ for the complications in hypertension, one of the essential aims of antihypertensive therapy should be to suppress the occurrence and progression of hypertensive renal damage.¹ The guidelines for the management of hypertension recommend that blood pressure should be strictly controlled in hypertensive patients with kidney disease.^{2–4} Glomerular hypertension is thought to be one of the main causes of hypertensive renal dysfunction.^{5,6} Angiotensin-converting enzyme inhibitors and angiotensin II type 1 receptor blockers are of specific benefit in retarding the progression of chronic kidney disease.^{7,8} These agents control glomerular capillary pressure by dilating the afferent and efferent arterioles.^{9–11} On the other hand, it has also been indicated that

sympathetic overactivity plays a pivotal role in the progression of renal disease in patients with chronic kidney disease and in experimental animals.^{12–15} Therefore, it is expected that treatment aimed to modulate sympathetic nerve activity would be of benefit to renal tissue.

Cilnidipine, a dihydropyridine calcium channel blocker, has dual-channel inhibitory actions at both N- and L-type calcium channels.¹⁶ N-type calcium channels, which are predominantly distributed in the sympathetic nervous system, control neurotransmitter release from the nerve endings of sympathetic neurons.¹⁷ Early studies have shown that cilnidipine exhibited a prolonged antihypertensive effect without increasing heart rate or circulating plasma norepinephrine concentrations in spontaneously hypertensive rats (SHR) and in patients with

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essential hypertension.^{18–21} The dual-channel inhibitory actions of cilnidipine raise the possibility that cilnidipine would have a greater renoprotective effect than L-type calcium channel blockers, as L-type calcium channels are not expressed in glomerular efferent arterioles.²² In small-sized clinical studies, cilnidipine had greater renoprotective effects than amlodipine.^{23–25} Furthermore, it has been shown that cilnidipine was more beneficial than amlodipine as additional medication for hypertensive patients who had kidney disease associated with significant proteinuria and who were already under treatment with a renin–angiotensin system inhibitor.²⁶

The deoxycorticosterone acetate (DOCA)-salt rat is a useful model for the investigation of renal injury in the hypertensive setting. DOCA-salt treatment triggers a malignant hypertension that leads to renal injury, characterized by renal fibrosis, glomerulosclerosis and renal hypertrophy.^{27,28} Kidney damage in DOCA-salt rats is accompanied by a significant increase in the production of reactive oxygen species, proinflammatory cytokines and profibrotic factors.²⁹ In this study, we investigated the renoprotective effects of cilnidipine in DOCA-salt rats and compared the effects with those of amlodipine. In addition, we also examined the effects of cilnidipine on the renal renin–angiotensin–aldosterone system.

METHODS

Animal preparation

Male Wistar rats (Shimizu Laboratory Supply, Kyoto, Japan) weighing 100 g were housed in an environmentally controlled room with a 12-h light/dark cycle and given standard rodent chow and tap water *ad libitum*. The rats were acclimated to handling. Right unilateral nephrectomy was performed with rats under ether-induced anesthesia. After surgery, rats were divided into four groups in the beginning of the study: (1) the DOCA-salt group received weekly subcutaneous injections of DOCA (40 mg per kg body weight; Nacalai Tesque, Kyoto, Japan), which was suspended in carboxymethylcellulose and were provided with 1% NaCl in drinking water (DOCA-salt, *n*=6); (2) the DOCA-salt+cilnidipine group, received DOCA, 1% NaCl drinking water and cilnidipine (kindly provided by Mochida Pharmaceutical, Tokyo, Japan) at 1 mg per kg body weight per day by gavage (cilnidipine, *n*=6); (3) the DOCA-salt+amlodipine group received DOCA, 1% NaCl drinking water and amlodipine at 1 mg per kg body weight per day by gavage (amlodipine, *n*=6); and (4) the control group received the vehicle (control, *n*=6). Rats were housed in individual metabolic cages to record urine output and to collect urinary samples. Systolic blood pressure was measured in conscious animals by the tail-cuff method (Model UR-5000; Ueda, Nagano, Japan). After 4 weeks of treatment, each rat was anesthetized with an intraperitoneal injection of pentobarbital sodium (35 mg per kg). The left femoral artery was exposed and then cannulated with polyethylene tubing (PE10 fused into PE50; Becton Dickinson, Franklin Lakes, NJ, USA) for collecting blood samples. The free ends of the catheters were tunneled subcutaneously to the back. After a recovery period, the femoral catheter was connected to a pressure transducer (MP5100; Edwards Lifescience, Tokyo, Japan) to measure mean arterial pressure with rats in an awakened condition. Blood samples were collected before urethane anesthesia. At the end of the treatment period, the kidneys were removed under deep anesthesia with urethane (1.2 g kg⁻¹, intraperitoneally), weighed and kept at -80 °C for subsequent analysis. All rats were handled in accordance with the Institutional Animal Care and Use Committee of Kyoto Pharmaceutical University with respect to the National Research Council's guidelines and National Institutes of Health guidelines.

Functional studies

The concentrations of serum creatinine, blood urea nitrogen, 24-h urinary protein and creatinine excretion were determined with the use of commercially available kits (Creatinine-Test Wako, Micro TP-Test Wako; Wako, Osaka, Japan).

Histological analysis

After methacarn fixation, the kidney tissues were embedded in paraffin. The 4-µm-thick slices were stained with Masson's trichrome. Light microscopy was used to semiquantitatively evaluate kidney sections. Sections were assessed using computer software. The tubulointerstitial collagen volume fractions were calculated as the percentage of total area within a field. Glomerulosclerosis was scored in 50 glomeruli in each section as follows: Grade 0 for a normal glomerulus; Grade 1, mild sclerosis (<25%); Grade 2, moderate segmental sclerosis (25–50%); Grade 3, severe segmental sclerosis (50–75%); and Grade 4, global sclerosis. The diameter of Bowman's capsule was measured. In addition, to investigate atrophic changes in the glomerulus, the diametric ratio of the glomerulus to Bowman's capsule was also determined.

Immunohistochemistry

The dissected kidney was embedded in OCT compound (Tissue-Tek, Sakura Finetechnical, Tokyo, Japan), snap frozen and then subsequently stored at -80 °C before sectioning. Cross-cryosections (4-µm thick) were fixed in 4% phosphate-buffered formaldehyde. Endogenous peroxidase activity was quenched by incubation in methanol containing 0.3% hydrogen peroxide. Sections were incubated with anti-podocin antibody overnight at 4 °C (1:100; Abcam, Cambridge, UK). Sections were then washed with phosphate-buffered saline and incubated with a fluorescein isothiocyanate-conjugated immunoglobulin G secondary antibody (Vector, Temecula, CA, USA) for 1 h at room temperature. The podocin-positive glomerulus was evaluated using a confocal laser scan microscope (LSM510 Ver.4.2; Carl Zeiss Micro Imaseng, Jena, Germany) and assessed using computer software. The expression of podocin was determined in 20 glomeruli in each section.

Measurement of urinary norepinephrine excretion

Urinary norepinephrine excretion is widely used as an indicator of sympathetic nerve activity.^{30,31} Urinary concentrations of norepinephrine were determined by high-performance liquid chromatography with electrical chemical detection (HPLC-ECD, EP-10; Eicom, Kyoto, Japan). In brief, 6 mol l⁻¹ HCl (1 ml) was added to the 24-h urinary samples to prevent the decomposition of norepinephrine. The urinary samples were centrifuged at 700 g for 5 min at 4 °C, and the supernatants were collected. After alumina adsorption and ion pairing, the urinary samples were injected into the high-performance liquid chromatography system.

RNA isolation and reverse transcription-polymerase chain reaction

The RNA isolation and reverse transcription-polymerase chain reaction analysis were performed as described previously.³² In brief, the total RNAs were isolated from rat kidney by the acid guanidium thiocyanate-phenol-chloroform method using Isogen reagent (Nippon Gene, Tokyo, Japan). First-strand cDNA was synthesized from total RNA (1 µg) with a commercially available RT kit (High Capacity cDNA Reverse Transcription Kit; Applied Biosystems, Foster City, CA, USA) using random primers. The PCR products were performed according to the protocol shown in Table 1. The PCR cycles so obtained were separated by electrophoresis on a 1.5% agarose gel and then visualized by ethidium bromide staining under ultraviolet light. The results were presented relative to the expression of the control *glyceraldehyde-3-phosphate dehydrogenase* gene.

Protein extraction and western blotting

Protein extraction and western blotting analysis were performed as described previously.³² In brief, total protein was extracted from kidneys using iced Tris buffer (5 mmol l⁻¹, pH 7.4) containing the protease inhibitors. The protein extracts were separated with sodium dodecyl sulfate-polyacrylamide gel electrophoresis, electrotransferred to membranes and blocked overnight with 4% non-fat dry milk. The membranes were incubated with rabbit polyclonal antibody against angiotensin-converting enzyme (ACE, 1:500; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and then incubated with appropriate horseradish peroxidase-conjugated secondary antibody (1:2500, anti-rabbit immunoglobulin G, horseradish peroxidase-linked antibody; Cell Signaling Technology, Beverly, MA, USA). The amount of each product was normalized with respect to the amount of actin (1:5000, mouse anti-actin monoclonal

antibody (Chemicon International, Temecula, CA, USA); 1:2500, anti-mouse IgG (H+L) horseradish peroxidase conjugate (Promega Corporation, Madison, WI, USA)).

Measurement of ACE activities

ACE activity in the kidney was measured using a synthetic substrate, hippuryl-His-Leu, specifically designed for ACE (Peptide Institute, Osaka Japan). The kidney was homogenized in 10 volumes (wv^{-1}) of 20 mmol l⁻¹ Tris-HCl buffer, pH 8.3, containing 5 mmol l⁻¹ Mg(CH₃COO)₂, 30 mmol l⁻¹ KCl, 250 mmol l⁻¹ sucrose and 0.5% NP-40. The homogenate was centrifuged at 8000 g for 15 min at 4 °C. Next, 25 µl of supernatant was incubated for 10 min at 37 °C with 5 mmol l⁻¹ hippuryl-His-Leu in 100 µl of 100 mmol l⁻¹ phosphate buffer, pH 8.3, containing 0.3 mol l⁻¹ NaCl. The reaction was terminated by the addition of 375 µl of 3% metaphosphoric acid, and then the mixture was centrifuged at 20 000 g for 10 min at 4 °C. The supernatant was applied to a reversed-phase column (4.6 mm i.d. × 250 mm; Tosoh Corporation, Kanagawa, Japan), which had been equilibrated with 10 mmol l⁻¹ KH₂PO₄ and CH₃OH

(1:1, pH 3.0), and eluted with the same solution at a rate of 0.5 ml min⁻¹. Hippuric acid was detected by ultraviolet absorbance at 218 nm. One unit of ACE activity was defined as the amount of enzyme that cleaved 1 µmol of hippuric acid per min.

NADPH oxidase activity

Kidney was homogenized in cold lysis buffer (20 mmol l⁻¹ K₂H₂PO₄, pH 7.0, 1 mmol l⁻¹ EGTA, 10 µg ml⁻¹ aprotinin, 0.5 µg ml⁻¹ leupeptin and 1.0 mmol l⁻¹ phenylmethylsulfonyl fluoride). The homogenate was centrifuged at 800 g for 10 min at 4 °C to remove the unbroken cells and debris. Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity was measured by a luminescence assay in a 50 mmol l⁻¹ phosphate buffer, pH 7.0, containing 1 mmol l⁻¹ EGTA, 150 mmol l⁻¹ sucrose, 5 µmol l⁻¹ dark-adapted lucigenin as the electron acceptor and 100 µmol l⁻¹ NADPH as the substrate, in a final volume of 900 µl. The reaction was started by the addition of 100 µl of homogenate, and luminescence measurements were obtained every 15 s for 5 min. Protein content was determined by the bicinchoninic acid method (Pierce, Rockford, IL, USA), and the results were standardized to this measurement.

Table 1 PCR primers and PCR protocols

Product size	PCR primer sequences	PCR protocols
GAPDH 308 bp	Forward: 5'-TCCCTCAAGATTGTCAGCAA-3' Reverse: 5'-AGATCCACACGATACATT-3'	94 °C 30 s ⁻¹ 57 °C 40 s ⁻¹ 72 °C min ⁻¹ 26 cycles
TGF-β 400 bp	Forward: 5'-CGAGGTGACCTGGGCACCATCCATGAC-3' Reverse: 5'-CTGCTCCACCTTGGGCTTGCACCCAC-3'	94 °C 30 s ⁻¹ 62 °C 40 s ⁻¹ 72 °C min ⁻¹ 28 cycles
Collagen I 315 bp	Forward: 5'-CGAGGTGATAGCTTGGCTTA-3' Reverse: 5'-ACTATCGATCGCATCCGACT-3'	94 °C 30 s ⁻¹ 64 °C 40 s ⁻¹ 72 °C min ⁻¹ 28 cycles
Collagen IV 185 bp	Forward: 5'-TTGGCTATTCCTTTGTGATG-3' Reverse: 5'-TCTCGTCTCTCTATGGTG-3'	94 °C 30 s ⁻¹ 56 °C 40 s ⁻¹ 72 °C min ⁻¹ 40 cycles
CYP11B2 346 bp	Forward: 5'-ACTCCGTGGCCTGAGACG-3' Reverse: 5'-TTCAAGTCCACCACACAG-3'	94 °C 30 s ⁻¹ 60 °C 40 s ⁻¹ 72 °C min ⁻¹ 30 cycles

Abbreviations: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PCR, polymerase chain reaction; TGF-β, transforming growth factor-β.

Aldosterone assay

Each sample of plasma and the extract from kidney tissue were added to the assay wells (50 µl per well). Each well received aldosterone acetylcholinesterase inhibitor tracer (50 µl) and antiserum (50 µl); samples were then incubated at 4 °C overnight. Finally, the plate was developed by Ellman's reagent and read at a wavelength of 405 nm. The concentration was calculated according to the assay manufacturer's standard protocol (Cayman Chemical, Ann Arbor, MI, USA).

Statistics

Data are expressed as means ± s.e.m. Results were analyzed using one-way analysis of variance for multiple comparisons followed by Fisher's Protected Least Significant Difference test. A value of $P < 0.05$ was considered statistically significant.

RESULTS

Biochemical parameters in experimental animals

The biochemical parameters in the three groups of rats at the end of the study period are shown in Table 2. Systolic blood pressure and mean arterial pressure were greater in the DOCA-salt rats than that in the control rats, and the body weight in the control group was greater than that in the DOCA-salt group. Treatment with cilnidipine or amlodipine had no significant effect on systolic blood pressure, mean arterial pressure or body weight. Urinary protein excretion, blood urea nitrogen and serum creatinine concentrations in the DOCA-salt group were significantly higher than that in the control group, and creatinine clearance was significantly lower in the DOCA-salt group than that in the control group. Cilnidipine, but not amlodipine, significantly ameliorated these changes in proteinuria and blood urea nitrogen induced by DOCA and 1% NaCl. In the amlodipine group, serum

Table 2 General characteristics and renal function in control rats (control), DOCA-salt hypertensive rats, DOCA-salt hypertensive rats receiving cilnidipine (cilnidipine) and DOCA-salt hypertensive rats receiving amlodipine (amlodipine)

Experimental group	BW (g)	KW (g)	KW/BW (mg g ⁻¹ BW)	SBP (mm Hg)	MAP (mm Hg)	U-pro (mg day ⁻¹)	CCr (ml min ⁻¹)	S-Cr (mg dl ⁻¹)	Blood urea nitrogen (mg dl ⁻¹)
Control	289.3 ± 4.85	1.82 ± 0.08	0.61 ± 0.02	127.3 ± 1.5	110.5 ± 2.9	6.43 ± 0.61	1.51 ± 0.14	0.30 ± 0.02	20.1 ± 1.8
DOCA-salt	200.7 ± 7.5*	3.10 ± 0.22*	1.76 ± 0.22*	216.9 ± 6.2*	206.2 ± 6.8*	601.1 ± 81.0*	0.52 ± 0.07*	1.18 ± 0.12*	75.6 ± 10.7*
Cilnidipine	200.0 ± 9.86*	3.72 ± 0.38*	1.78 ± 0.13*	205.3 ± 4.8*	191.0 ± 8.2*	419.6 ± 30.1*,#	1.35 ± 0.10*,†	0.35 ± 0.03#	38.4 ± 2.17*,#
Amlodipine	193.8 ± 10.8*	3.32 ± 0.26*	1.74 ± 0.16*	206.7 ± 6.6*	190.4 ± 8.8*	518.8 ± 28.1*	1.00 ± 0.05*,#	0.50 ± 0.03*,#	52.0 ± 6.16*

Abbreviations: BW, body weight; CCr, creatinine clearance; DOCA, deoxycorticosterone acetate; KW, kidney weight; MAP, mean arterial pressure; SBP, systolic blood pressure; S-Cr, serum creatinine; U-Pro, urinary protein.

* $P < 0.05$ vs. control; # $P < 0.05$ vs. DOCA-salt; † $P < 0.05$ vs. amlodipine. Values are means ± s.e.m. ($n = 6$).

creatinine level was higher and creatinine clearance was lower than that in the cilnidipine group. In rats given DOCA and 1% NaCl, kidney weight to body weight ratio was significantly greater than that in the control rats. Treatment with cilnidipine or amlodipine had no effect on the increase in kidney weight/body weight of the DOCA-salt group.

Histopathological changes in experimental animals

Figure 1 presents representative images of the glomerular and tubulointerstitial pathology in the experimental groups at the end of

the study period. Kidneys from control animals were histologically normal, whereas kidneys from animals treated with DOCA-salt showed severe glomerular injury. The DOCA-salt group of rats showed more severe glomerular and tubulointerstitial fibrosis. In animals receiving cilnidipine, lesions were markedly reduced compared with the DOCA-salt rats. On the other hand, although amlodipine slightly attenuated histological changes in the DOCA-salt rats, there was no significant difference in the grades of glomerulosclerosis or fibrotic area between the DOCA-salt and the amlodipine groups. The diameters of Bowman's capsule were

Figure 1 Representative renal histological findings (a–d), glomerulosclerosis index (e) and collagen volume fraction in tubulointerstitium (f), as determined by Masson's trichrome staining of transverse sections in control rats (control; a), deoxycorticosterone acetate (DOCA)-salt-treated hypertensive rats (DOCA-salt; b) and DOCA-salt hypertensive rats receiving cilnidipine (cilnidipine; c) or amlodipine (amlodipine; d). (g,h) are the diameter of Bowman's capsule and the diametric ratio of each glomerulus to Bowman's capsule, respectively. Bars indicate 100 μ m. Values are means $\pm$ s.e.m. ($n=6$). * $P<0.05$ vs. control; # $P<0.0001$ vs. DOCA-salt; † $P<0.0005$ vs. amlodipine.

significantly larger in the DOCA-salt rats than that in the control rats. The diametric ratio of each glomerulus to Bowman's capsule was smaller in the DOCA-salt group than that in the control. Cilnidipine, but not amlodipine, normalized the expansion of Bowman's capsule and glomerular atrophy in the DOCA-salt rats.

Expression of podocin in the kidney

The expression of podocin was decreased in the DOCA-salt rats compared with the control. This decrease was attenuated in the cilnidipine-treated rats. On the other hand, amlodipine had no effect on podocin expression (Figure 2).

Expression of mRNA for transforming growth factor- β and collagen I/IV

Compared with the control group, the DOCA-salt group had higher levels of expression of transforming growth factor- β (TGF- β), collagen I and collagen IV mRNA. Treatment with cilnidipine prevented any increase in the renal expression of these molecules in the DOCA-salt rats. On the other hand, amlodipine had no effect on the mRNA expression of any of these molecules (Figure 3).

NADPH oxidase-dependent superoxide production

NADPH oxidase-derived superoxide production in the kidney was significantly increased in the DOCA-salt group compared with levels

Figure 2 Immunohistochemical photomicrographs of renal sections from control rats (control; **a**), deoxycorticosterone acetate (DOCA)-salt-treated hypertensive rats (DOCA-salt; **b**) and DOCA-salt hypertensive rats receiving cilnidipine (cilnidipine; **c**) or amlodipine (amlodipine; **d**). Representative renal sections stained immunohistochemically for podocin (**a–d**). Bar indicates 100 μ m. The podocin-positive area in the glomeruli (**e**). Values are means $\pm$ s.e.m. ($n=6$). * $P<0.05$ vs. control; # $P<0.05$ vs. DOCA-salt.

Figure 3 Representative expression of the mRNA for transforming growth factor- β (TGF- β) (a), collagen I (c) and collagen IV (e) in kidney from control rats (control), deoxycorticosterone acetate (DOCA)-salt-treated hypertensive rats (DOCA-salt) and DOCA-salt hypertensive rats receiving cilnidipine (cilnidipine) or amlodipine (amlodipine), assayed by reverse transcription-polymerase chain reaction (RT-PCR). Quantitative analysis of the mRNA expression for TGF- β (b), collagen I (d) and collagen IV (f), normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) by scanning densitometry. Values are means $\pm$ s.e.m. ($n=6$). * $P<0.05$ vs. control; # $P<0.05$ vs. DOCA-salt; † $P<0.05$ vs. amlodipine.

in the control group. The activation of NADPH oxidase was suppressed by treatment with cilnidipine. Amlodipine had no effect on the activation of NADPH oxidase in the DOCA-salt rats (Figure 4).

Figure 4 Activity of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase in the kidney from control rats (control), deoxycorticosterone acetate (DOCA)-salt-treated hypertensive rats (DOCA-salt) and DOCA-salt hypertensive rats receiving cilnidipine (cilnidipine) or amlodipine (amlodipine), assayed by lucigenin-enhanced chemiluminescence. Results are converted to the percentage of control. Values are means $\pm$ s.e.m. ($n=6$). * $P<0.05$ vs. control; # $P<0.05$ vs. DOCA-salt; † $P<0.05$ vs. amlodipine.

Figure 5 Urinary norepinephrine excretion in control rats (control), deoxycorticosterone acetate (DOCA)-salt-treated hypertensive rats (DOCA-salt), and DOCA-salt hypertensive rats receiving cilnidipine (cilnidipine) or amlodipine (amlodipine). Values are means $\pm$ s.e.m. ($n=6$). * $P<0.05$ vs. control; # $P<0.05$ vs. DOCA-salt; † $P<0.05$ vs. amlodipine.

Changes in urinary norepinephrine excretion

Figure 5 shows the urinary excretion of norepinephrine in each treated group. The urinary level of norepinephrine in the DOCA-salt group was significantly higher than that in the control. Cilnidipine reduced the urinary excretion of norepinephrine in the DOCA-salt rats, whereas amlodipine did not.

Activity and expression of ACE in the kidney

Renal ACE activity level and expression are shown in Figure 6. In the DOCA-salt rats, ACE activities in the kidney were significantly higher than that in the control rats. Cilnidipine treatment significantly suppressed the activation of ACE in the DOCA-salt hypertensive rat kidney (Figure 6a). The expression of ACE in the DOCA-salt kidney was elevated, and the overexpression of ACE was suppressed by the administration of cilnidipine (Figures 6b and c). On the other hand, amlodipine had no effect on the increased activity level or expression of ACE in the DOCA-salt rat kidney (Figures 6a–c).

Renal aldosterone levels and expression of aldosterone synthase (CYP11B2)

In the DOCA-salt hypertensive animals, there was a significant increase in aldosterone content and CYP11B2 expression in the kidney

Figure 6 Activity (a) and expression (b) of angiotensin-converting enzyme (ACE) in the kidney from control rats (control), deoxycorticosterone acetate (DOCA)-salt-treated hypertensive rats (DOCA-salt) and DOCA-salt hypertensive rats receiving cilnidipine (cilnidipine) or amlodipine (amlodipine). Quantitative analysis of ACE protein expression, normalized to actin by scanning densitometry (c). Values are means $\pm$ s.e.m. ($n=6$). * $P<0.05$ vs. control; # $P<0.05$ vs. DOCA-salt; † $P<0.05$ vs. amlodipine.

compared with the control animals. These increases were restored to the control level by treatment with cilnidipine. On the other hand, there was no significant difference in renal aldosterone levels or CYP11B2 expression between the DOCA-salt- and amlodipine-treated groups (Figure 7).

DISCUSSION

This study showed that administration of the L/N-type calcium channel blocker cilnidipine to the DOCA-salt hypertensive rat reduced proteinuria, normalized the levels of creatinine clearance and attenuated glomerulosclerosis and interstitial fibrosis, as well as the expression of collagen I/IV and TGF- β , despite the absence of any reduction in blood pressure. On the other hand, the L-type calcium channel blocker amlodipine failed to improve biochemical and pathological parameters, unlike cilnidipine. In this study, we showed for the first

Figure 7 Aldosterone levels (a) and the expression of CYP11B2 mRNA (b) in the kidney tissue from control rats (control), deoxycorticosterone acetate (DOCA)-salt-treated hypertensive rats (DOCA-salt) and DOCA-salt hypertensive rats receiving cilnidipine (cilnidipine) or amlodipine (amlodipine). (b) Representative CYP11B2 mRNA expression in the kidney and its quantitative analysis. Values are means $\pm$ s.e.m. ($n=6$). * $P<0.05$ vs. control; # $P<0.05$ vs. DOCA-salt; † $P<0.05$ vs. amlodipine.

time that cilnidipine was more beneficial than amlodipine in the DOCA-salt hypertensive rats.

Tubulointerstitial damage can be the result of hypertension, oxidative stress or renal inflammation, which are evident in the DOCA-salt animal model. Fibrosis leads to chronic impairment of renal function. As shown in this study, the DOCA-salt rats developed proteinuria, glomerulosclerosis, interstitial fibrosis and renal dysfunction. A recent study reported that cilnidipine attenuated interstitial fibrosis, ectodermal dysplasia-1-positive cell infiltration and albuminuria in Dahl salt-sensitive rats fed a high-sucrose diet, which mimics metabolic syndrome, whereas amlodipine had no effect on these parameters.³³ This study extended these findings to show that cilnidipine significantly attenuated renal elevation of collagen I/IV and TGF- β mRNA levels and normalized creatinine clearance, as well as serum creatinine and blood urea nitrogen in the DOCA-salt hypertensive rats. Thus, our results, taken together with the previous reports, strongly support the idea that the L/N-type calcium channel blocker cilnidipine would exhibit beneficial effects against renal injury related to hypertension. The DOCA-salt-treated rats exhibit severe hypertension, which would cause irreversible damage in the kidney, explaining why the amelioration of collagen I/IV, TGF- β or oxidative stress by cilnidipine did not extend to the complete normalization of proteinuria or BUN.

Oxidative stress can accompany hypertension in many animal models, including SHR, angiotensin II-infused rats, renovascular

hypertension and DOCA-salt hypertension.³⁴ In addition, NADPH oxidase is a major source of reactive oxygen species production in the kidney.³⁵ We showed that NADPH oxidase activity was increased in the kidneys of DOCA-salt hypertensive rats. Moreover, an important result of this study was that cilnidipine prevented the activation of NADPH oxidase. The latest report by Hishikawa *et al.*,³⁶ in which they compared antioxidant activity of cilnidipine and amlodipine by measuring ionomycin-stimulated superoxide production in cultured mesangial cells, showed that cilnidipine exhibited significantly greater antioxidant activity than amlodipine. These results indicate that superior antioxidant effects compared with amlodipine might explain these results observed for cilnidipine treatment of renal injury in DOCA-salt hypertensive rats.

Hypertensive renal dysfunction is caused by glomerular damage owing to glomerular hypertension. In the kidney, norepinephrine constricts both the afferent and the efferent arterioles in the glomeruli,^{37,38} leading to glomerular hypertension. A recent report using an *ex vivo* SHR hydronephrotic model showed that cilnidipine diluted both the afferent and efferent arterioles, whereas nifedipine had no effect on efferent arterioles.³⁹ Sympathetic nerve activity was increased in the DOCA-salt hypertensive rats.^{40,41} In this study, a significant decrease in urinary norepinephrine excretion was observed in the cilnidipine-treated group, whereas amlodipine had no effect on the excretion of norepinephrine in the DOCA-salt hypertensive rats. Cilnidipine caused a significant decrease in renal cortical norepinephrine levels in SHR/Izumo, although treatment with amlodipine did not decrease the tissue level of norepinephrine.⁴² These results suggest that the N-type calcium channel-blocking action of cilnidipine suppresses reflex sympathetic hyperactivity.

Increasing evidence suggests that podocyte injury and loss from the glomerulus is strongly associated with the process leading to glomerulosclerosis.⁴³ In addition, it has been reported that aldosterone induced podocyte injury.⁴⁴ Therefore, we investigated the effects of cilnidipine on the expression of podocin, the major component of podocytes. We found that cilnidipine restored the expression of podocin, which was decreased in the DOCA-salt animals. This result raises the possibility that renoprotective effects mediated by cilnidipine could be derived from the inhibition of podocyte injury.

The renin–angiotensin–aldosterone system plays an important role in the pathogenesis and progression of renal disease. The sympathetic nervous system is closely related to the renin–angiotensin–aldosterone system. In a recent report, Konda *et al.*⁴² investigated the different effects of cilnidipine and amlodipine on plasma aldosterone levels in SHR/Izumo, and they showed that the cilnidipine-treated group had significantly lower plasma aldosterone levels. Furthermore, it has been reported that cilnidipine treatment tended to decrease renal tissue angiotensin II levels in Dahl-salt-sensitive rats.⁴⁵ In this study, we also showed that cilnidipine, but not amlodipine, reduced renal ACE activity and renal aldosterone content in the DOCA-salt hypertensive rats. To confirm that cilnidipine inhibited locally produced aldosterone in the kidney, we investigated the expression of renal CYP11B2, aldosterone synthase. The results showed that cilnidipine inhibited the overexpression of renal CYP11B2 and that cilnidipine inhibited aldosterone production in the kidney, whereas changes in plasma aldosterone concentration were similar to those of the renal homogenate (data not shown). To our knowledge, this is the first investigation into the effects of cilnidipine on ACE activity and aldosterone content in the kidney. In addition, dihydropyridine calcium channel blockers have been reported to exert mineralocorticoid receptor antagonist activity.⁴⁶ Although further studies are needed, the effects of cilnidipine on the renin–angiotensin–aldosterone system have

become a focus of attention. Our results and previous reports suggest that inhibition of the renal renin–angiotensin–aldosterone system might underlie the renoprotective effects observed.

The mechanisms by which cilnidipine protects the kidney remain unclear. We suggest that inhibition of sympathetic nerve activity via N-type calcium channel blockade would protect the kidney, similar to cilnidipine. The renin–angiotensin–aldosterone system is upregulated by the activation of the sympathetic nervous system. Angiotensin II and aldosterone induce oxidative stress (e.g., NADPH oxidase activation) and secretion of inflammatory cytokines and growth factors (e.g., TGF- β). Therefore, we suppose that sympathoinhibitory effects of cilnidipine, as shown by the decrease in urinary NE excretion, would lead to the inhibition of renal activity, expression of ACE and CYP11B2, and aldosterone production. In addition, we suppose that not only sympathoinhibition, but also direct antioxidant properties would be associated with the renoprotective effect of cilnidipine. Hishikawa *et al.*³⁶ showed that cilnidipine exerted stronger antioxidant effects than amlodipine using cultured mesangial cells.

In conclusion, we showed the direct renoprotective effects (including attenuation of proteinuria and inhibition of renal dysfunction), fibrosis and oxidation of cilnidipine in the DOCA-salt hypertensive rat model. We also provided novel evidence that the L/N-type calcium channel blocker cilnidipine inhibited the renal renin–angiotensin–aldosterone system, including measurements of renal ACE activity and aldosterone content in the DOCA-salt hypertensive rats. Further clarification of the precise mechanisms by which cilnidipine inhibits renal renin–angiotensin–aldosterone system is required.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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