Effects of L/N-Type Calcium Channel Antagonist, Cilnidipine on Progressive Renal Injuries in Dahl Salt-Sensitive Rats

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The sympathetic nerve activity plays an important role on the renal function through the vasoactive system and the renin-angiotensin system. Although interest in the renal protective effects of anti-sympathetic agents has been increased, there are not enough data to clarify this efficiency. Therefore, we investigated the effects of L/N-type calcium channel antagonist, cilnidipine on progressive renal injury in Dahl salt-sensitive (Dahl S) rats. Male Dahl S rats (6 weeks of age) were fed a high salt (4% NaCl) diet. They were divided into groups with similar blood pressure at 12 weeks of age and they received vehicle (n=7) or cilnidipine (30 mg/kg/d as food admix, n=9) for 8 weeks. Cilnidipine treatment suppressed the increase in systolic blood pressure. Although urinary protein excretion was not influenced, cilnidipine inhibited the increase in blood urea nitrogen and decrease in creatinine clearance. Histological investigation revealed that progression of glomerulosclerosis was inhibited in cilnidipine treatment group. Of notes, cilnidipine reduced plasma norepinephrine level and plasma rennin activity compared with vehicle-treated Dahl S rats. These data indicated that cilnidipine has suppressive effects against progressive renal injury in Dahl S rats. This effect is not only explained by the L-type calcium channel blocking action that lowered blood pressure, but also partially explained by the N-type calcium channel blocking action that lead to suppression of the sympathetic nerve activity and renin-angiotensin system.

Key words N-type calcium channel; hypertension; renal disease; Dahl salt-sensitive (Dahl S) rat

Renal sympathetic nerve activity plays an important role on renal function through the vasoactive system and the renin-angiotensin system. In the clinical study, it has been suggested that sympathetic overactivity plays a pivotal role for progression of renal disease in patients with chronic renal failure or end-stage renal disease. Recent studies suggesting that renal sympathetic denervation prevent glomerular hyperfiltration and glomerulosclerosis in some experimental models. From these indications, it is expected that treatments aimed at modulating sympathetic nerve activity will suppresses progressive renal injury.

N-Type (neuronal) calcium channels, predominantly distributed on the sympathetic nerves have been widely recognized to control the neurotransmitter releases. Cilnidipine is a dihydropyridine (DHP) calcium channel blocker (CCB), and it has been demonstrated to inhibit both N-type and L-type (long acting) calcium channels in various types of neurons. Cilnidipine also has been confirmed to suppress the norepinephrine (NE) releases from the isolated rat vascular preparation. In the experiments of anesthetized dogs, sympathetic nerve stimulation-induced increase in plasma NE level was suppressed by cilnidipine. Thus cilnidipine suppressed sympathetic hyperactivity in a variety of experimental models through the blocking action of N-type calcium channels.

The Dahl salt-sensitive (Dahl S) rats that develop hypertension and renal injuries with a high-salt diet have been widely recognized to be a model of chronic renal failure. It has been suggested that sympathetic nerve activity is increased or easy to increase by physical stress in Dahl S rats on a high-salt diet. Wu et al. reported that high-salt induced changes in intrarenal vessel structure and renal haemodynamic function in Dahl S rats are dependent on the activity of the sympathetic nervous system. These reports suggest that increase in sympathetic nerve activity is considered to be a one of precipitating cause of renal failure in Dahl S rats.

Many reports have been suggested that L-type calcium channel antagonist has renal protective effects in a renal injury model with hypertension. But there are not enough data to elucidate the effects of N-type calcium channel antagonist against progressive renal injury. Although L/N-type calcium channel antagonist, cilnidipine is expected to suppress the renal injury by both blocking action of L-type calcium channel that lead to hypotensive effect and N-type calcium channel that lead to suppression of sympathetic nerve activity, renal protective profile of cilnidipine has not ever been assessed in animal model of renal injury with hypertension. In the present study, we investigated to clarify the renal protective effects of L/N-type calcium channel antagonist, cilnidipine in Dahl S rats as a model of chronic renal failure.

MATERIALS AND METHODS

Animal Preparation All experiments were conducted according to animal ethics committee of Ajinomoto Co., Inc. Male Dahl salt-sensitive (Dahl S) rats and Dahl salt-resistant (Dahl R) rats were purchased from SEAC Yoshitomi (Fukuoka, Japan). The rats were given normal laboratory chow containing 0.3% (w/w) of sodium (CRF-1, Charles Liver, Japan) and tap water was available ad libitum before the experiments. Rats were housed alone in each cage during the periods of experiments.

Experimental Protocol A total of 18 Dahl S rats aged 6 weeks were fed a high-salt (4% NaCl) diet for 6 weeks. Thereafter, at 12 weeks of age, the rats were divided into two groups: (a) Dahl S rats fed a high-salt diet alone (vehicle group, n=7), and (b) Dahl S rats fed a high-salt diet containing cilnidipine (30 mg/kg/d as food admix, n=9). These rats were maintained for another 8 weeks as therapeutic period. Six age-matched Dahl R rats were given normal (0.3% NaCl, n=6) diet and served as a normal reference. Systolic blood pressure at 12 weeks of age and they received vehicle (n=7) or cilnidipine (30 mg/kg/d as food admix, n=9) for 8 weeks. Cilnidipine treatment suppressed the increase in systolic blood pressure. Although urinary protein excretion was not influenced, cilnidipine inhibited the increase in blood urea nitrogen and decrease in creatinine clearance. Histological investigation revealed that progression of glomerulosclerosis was inhibited in cilnidipine treatment group. Of notes, cilnidipine reduced plasma norepinephrine level and plasma rennin activity compared with vehicle-treated Dahl S rats. These data indicated that cilnidipine has suppressive effects against progressive renal injury in Dahl S rats. This effect is not only explained by the L-type calcium channel blocking action that lowered blood pressure, but also partially explained by the N-type calcium channel blocking action that lead to suppression of the sympathetic nerve activity and renin-angiotensin system.

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pressure (BP) was measured every two weeks by the tail-cuff method (Softron Co., Tokyo, Japan). At the end of the study, the rats were euthanized by exsanguinations under anesthesia. The kidneys were quickly removed for histological studies and for measurement of the cortical tissue norepinephrine (NE) and angiotensin II (Ang II) level. Cilnidipine is supplied by Ajinomoto Co. Inc., Tokyo, Japan. All reagents used in this study were of analytical grade.

Assessment of Biochemical Parameters For measurement of biochemical parameters, blood samples were taken from the subclavian vein under ether anesthesia before noon. Blood samples were centrifuged (1000 g) for 15 min and the plasma was stored at −40 °C until analysis. Then the blood urea nitrogen and plasma creatinine levels were determined by an autoanalyzer (Fuji Dri-Chem 5500; Fujifilm Medical Co. Inc., Tokyo, Japan). Plasma NE was measured by high-performance liquid chromatography. Plasma renin activity (PRA) was measured by standard radioimmunoassay methods. Extraction of NE and Ang II from renal cortical tissue and subsequent processing are described in detail elsewhere. Briefly, pieces of renal cortex were homogenized in a 3.3-fold volume of ice-cold saline containing 0.1 N HCl. After centrifugation (12000 g at 4 °C) for 30 min, the supernatant was applied to high-performance liquid chromatography for measurement of NE. For measurement of Ang II, the remaining supernatant was applied to Florisil (Sigma Chemical Co., St. Louis, MO, U.S.A.) and then centrifuged (1000 g) for 2 min. After discarding the supernatant, the pellet was washed with distilled water, 0.5 N HCl–acetone, and petroleum ether. For radioimmunoassay of Ang II, the extract was dried and then dissolved in Tris buffer (pH 8.5). Anti-Ang II rabbit antiserum (SRL, Tokyo, Japan) was added and incubation was carried out for 24 h at 4 °C. This was followed by addition of [125-I]-Ang II and further incubation for another 8 h at 4 °C. Finally, the radioactivity in the precipitate was counted with a γ-spectrometer. Plasma von Willebrand factor (vWF) level was determined according to the streptavidin biotin method using the rabbit polyclonal antibody for vWF. Briefly, a 96-well microtiter plate was coated with a rabbit anti-rat vWF polyclonal antibody (10 μg/ml, Dako) and left overnight at room temperature for 24 h. After blocking with 1% bovine serum albumin for 1 h, diluted plasma samples were added and incubated at room temperature for 2 h. After being washed, the plates were incubated with a peroxidase-conjugated rabbit anti-rat vWF polyclonal antibody (1:3000 dilution, Dako) at room temperature for 1 h. After the plates were washed again, the numbers of bound vWF molecules were quantified by measuring the optical density at 490 nm. The plasma vWF antigen level was calculated as the percentage of the value of predosing plasma sample in each rat.

Collection and Analysis of Urine The rats were individually placed in metabolic cages and urine samples were collected for 24 h from 10:00 a.m. to determine the urine protein excretion. Urine samples were centrifuged (1000 g) for 5 min to remove sediment and then stored at −40 °C until analysis. The protein concentration was measured with a commercially available assay kit (Micro TP testwako, Wako Pure Chemical Industries Ltd., Tokyo, Japan).

Histological Study The kidneys were fixed with a 10% formalin solution and embedded in paraffin for histological study. After fixation with formalin, sections of 4 μm thick-

ness were made and observed by light microscopy after staining with periodic acid-Schiff. The severity of glomerular injury was evaluated using microscopy. A minimum 30 glomeruli was examined in each specimen.

Data Analysis All values are expressed as mean±S.E.M. Unpaired t-test was employed for overall statistical analysis between vehicle-treated values and others. The statistical analysis was performed using commercially available software, Statview 5.0 (Abacus Concepts, Inc., Berkeley, CA, U.S.A.). Differences were considered to be statistically significant when the probability value was less than 0.05.

RESULTS

Hemodynamic Effects During the experiment, 2 rats of the 9 vehicle-treated Dahl S rats dropped out because of cerebral breeding and severe renal insufficiency. All cilnidipine-treated Dahl S rats and normotensive Dahl R rats completed the study. High salt diet led to progressive increases in systolic BP in Dahl S rats over the 6-week period to an average of 183±3.0 mmHg. Systolic BP levels were the same between the 2 groups at the beginning of the treatment (182±6 mmHg in vehicle-treated group, 183±4 in cilnidipine-treated group). In vehicle-treated Dahl S rats, systolic BP continued to increase during the experiment, however, the cilnidipine (30 mg/kg/d as food admix) significantly decreased systolic blood pressure during the treatment period (224±4 mmHg in vehicle-treated group, 162±6 in cilnidipine-treated group at the end of the study). Dahl R rats showed no change in the systolic blood pressure throughout the study (Fig. 1).

Effects on Renal Function and Biochemical Parameters A high-salt diet increased urinary protein excretion, blood urea nitrogen (BUN) and plasma creatinine level and markedly decreased creatinine clearance (Ccre) compared with Dahl R rats. Although urinary protein excretion was not affected by cilnidipine, increase in BUN, plasma creatinine level and decrease in Ccre were inhibited (Table 1). Figure 2 shows that plasma norepinephrine (NE) level in cilnidipine-treated Dahl S rats (696±91 pg/ml) was significantly lower than vehicle-treated Dahl S rats (1160±186 pg/dl). Plasma renin activity (PRA) in the vehicle-treated Dahl S rats (4.6±1.2 ng/ml/h) was lower than Dahl R rats (26.5±5.9 ng/ml/h). As shown in Fig. 2, further lower PRA was observed in cilnidipine-treated Dahl S rats (1.2±0.4 ng/ml/h) compared with vehicle-treated Dahl S rats. Plasma von Willebrand Factor (vWF) level was significantly increased in vehi-
cle-treated Dahl S rats (100±14%) compared with Dahl R rats (30±2%). In contrast, as shown in Fig. 3, cilnidipine significantly lowered plasma vWF level (63±4%).

**Histological Changes**  Figure 4 shows light micrographs representing typical glomerular lesion from the kidney of vehicle-treated Dahl S rats, cilnidipine-treated Dahl S rats and Dahl R rats. In normotensive Dahl R rats, glomeruli and tubule were normal or almost normal. In contrast, vehicle-treated Dahl S rats showed severe matrix expansion in glomeruli and tubular dilation.

Glomerular Sclerosis and Tubular Injury: Treatment of cilnidipine reduced the glomerular sclerosis and tubular injury compared with vehicle-treated Dahl S rats in appearance (Fig 4).

**Renal Cortical NE and Ang II Level**  The NE and angiotensin II (Ang II) levels in renal cortical tissue are shown in Fig. 5. In the vehicle-treated Dahl S rats, the renal tissue level of NE (65±10 pg/g tissue) and Ang II (441±95 pg/g tissue) were not different from the level observed in Dahl R rats (NE: 73±6 pg/g tissue, Ang II: 293±15 pg/g tissue). Both NE and Ang II levels tended to decrease in cilnidipine-treated Dahl S rats (NE: 55±7 pg/g tissue, Ang II: 246±26 pg/g tissue).

**DISCUSSION**

N-Type (neuronal) calcium channels, predominantly distributed on the sympathetic nerves, have been widely recognized to control the neurotransmitter releases. In the kidney, renal sympathetic nerve activity contributes to the hypertension and progressive renal disease through the regulation of renal blood flow, glomerular filtration rate, electrolyte transport, and hormonal release.6,22)

Cilnidipine is a dihydropyridine (DHP)-type calcium chan-
nel blocker, and it has been demonstrated to inhibit both N-type and L-type (long acting) calcium channels in various types of neurons. In anesthetized dogs, the increase in heart rate and plasma norepinephrine (NE) level induced by bilateral carotid artery occlusion was blocked by cilnidipine through an inhibitory effect on sympathetic nerve overactivity. Cilnidipine has also been shown to reduce NE secretion in response to renal nerve stimulation in anesthetized dogs. This result was not observed by nifedipine treatment, a selective L-type calcium channel blocker. From the N-type calcium channel blocking action of cilnidipine, there are some possibilities to suppress progressive renal injury. Since anti-sympathetic profile of cilnidipine has not ever been shown in animal model with renal injury, the present study was designed to clarify the renal protective effects of anti-sympathetic agents, cilnidipine in salt-sensitive Dahl (Dahl S) rats.

The Dahl S rats develop hypertension and renal injuries when challenged with a high-salt diet. It has been demonstrated that environmental stress increases renal sympathetic nerve activity more in Dahl S rats on a high-salt diet than on low-salt diet. McCarty et al. showed that stress-induced increase in plasma catecholamine level in Dahl S rats on a high-salt diet is higher than Dahl R rats on a low-salt diet regardless of the same basal plasma catecholamine levels in both treatment group. Furthermore, Wu et al. reported that high-salt induced changes in intrarenal vessel structure and renal haemodynamic function in Dahl S rats are dependent on the activity of the sympathetic nervous system. Thus increased sympathetic nerve activity is considered to be one of incentive factor of progressive renal disease in Dahl S rats. In this study, cilnidipine at a dose of 30 mg/kg/d effectively reduced increase in creatinine clearance and progression of glomerular sclerosis. Plasma norepinephrine level was decreased by the treatment of cilnidipine suggesting that cilnidipine suppressed the renal sympathetic nerve activity in Dahl S rats. These data suggest that inhibition of sympathetic nerve activity by cilnidipine led to suppression of renal failure in Dahl S rats. The present result on plasma norepinephrine level was consistent with the clinical and experimental evidences that cilnidipine suppressed the cardiac sympathetic overactivity and cold stress-induced pressor response.

Renal sympathetic nerve is a one of the main regulator of renin secretion from juxtaglomerular apparatus. Holmer et al. suggested that activation of beta-adrenergic receptors is a powerful stimulus of renin secretion and renin gene expression in juxtaglomerular cells in vivo, albeit the kinetics of up-regulation of renin secretion and renin expression are markedly different. Therefore, the sympathetic tone might be a major factor determining the activity of the renin system in vivo. In this experiments, cilnidipine significantly lowered plasma rennin activity in Dahl S rats compared with vehicle-treated group. In some experiments, selective L-type calcium channel blockers failed to reduce the plasma rennin activity in Dahl S rats with high salt diet in spite of enough blood pressure control. One of the reasons for these differences may be considered to be consequence of suppression in the renal sympathetic nerve activity through the blocking action of N-type calcium channels.

In agreement with recent studies, the present study showed that PRA was markedly reduced by a high-salt diet in Dahl S rats. On the other hand, there is much evidence that Ang II is formed locally in the kidney, and acts as a mediator of renal injury in various forms of hypertension. Previous studies suggest that treatment with angiotensin converting enzyme inhibitors or angiotensin receptor blocker reduces cardiac or renal dysfunction in Dahl S rats with high-salt diet. Furthermore, Nakaya et al. reported that an angiotensin receptor blocker causes partial attenuation of hypertension and ameliorates the renal damage in adult Dahl S rats fed high salt diet. These data suggest that the local renin-angiotensin system is regulated in a different manner from the circulating renin-angiotensin system and contribute to development of renal dysfunction in Dahl S rats. Interestingly, Nishiyama et al. suggests that increase in angiotensinogen production in the kidney could help maintain Ang II level in the presence of low plasma rennin activity. In this study, kidney tissue Ang II level in Dahl S rats was not statistically increased by high-salt diet, but an upward trend was noted. Same results were reported by Kobori et al. using Dahl S rats. Of note, cilnidipine treatment tended to decrease the kidney tissue Ang II levels in Dahl S rats. This result may come from the suppressive effects of cilnidipine on local renin-angiotensin system including angiotensinogen production. Further studies were needed to elucidate the effects of cilnidipine on the renin-angiotensin system.

Endothelial dysfunction plays an important role in the development of atherosclerotic vascular disease in hypertensive patients. It has been shown that plasma von Willebrand factor (vWF) level, a maker of endothelial injury is higher in patients with chronic renal disease. In patients with type 2 diabetes, microalbuminuric patients have increased plasma vWF level than normoalbuminuric patients. These results suggest that plasma vWF level is thought to be one of the predictor of renal injury. Recently, Fu et al. suggest that, NE induces apoptosis in neonatal rat endothelial cells mainly through down-regulation of Bcl-2 protein and activation of the beta-adrenergic (beta2>beta1) and caspase-2 pathways. Furthermore, Szabo et al. demonstrated that Ang II, via AT1 receptor subtype 1 activation triggers DNA breakage, which activates nuclear enzyme poly(adenosine diphosphate-ribose) polymerase in the vascular endothelium, leading to the development of endothelial dysfunction in hypertension. Thus sympathetic nerve activity and renin-angiotensin system are responsible to endothelial dysfunction. Recent studies suggest that, Dahl S rats fed high salt diet have endothelial dysfunction in renal artery that led to vascular hypertrophy and glomerulosclerosis. In this study, plasma vWF level was increased in Dahl S rats with high salt diet and cilnidipine inhibited the increase. Cilnidipine showed suppressive effects against progressive renal injury in Dahl S rats, which is due at least in part to the suppressing effect on sympathetic nerve activity and renin-angiotensin system in addition to hypotensive effects.

Despite the improvement of creatinin clearance and glomelular sclerosis, cilnidipine showed no effects on the urinary protein excretion. Although this discrepancy has been also demonstrated with other calcium channel antagonists, the exact mechanism is still unknown. In this study, antihypertensive effects of cilnidipine were sustained during the treatment period, but the blood pressure level may have not sufficient to reduce the urinary protein excretion in a se-
vere renal disease model. High dose treatment studies are needed to confirm the effect of cilnidipine on proteinuria in Dahl rats.

L-Type calcium channel blocker has been reported to protect renal injury in an animal model with hypertension. These protective effects are mainly explained by the L-type calcium channel blocking action that improves systemic hypertension and glomerular hypertension. As same as these L-type calcium channel blocker, cilnidipine is thought to suppress renal injury mainly by improving systemic hypertension and glomerular hypertension through the blocking action of L-type calcium channel. But from the results of reduced plasma NE level and PRA, the mechanism is not only arising from a systemic antihypertensive effect.

In conclusion, L/N-type calcium channel blocker, cilnidipine provided suppression against renal failure in Dahl S rats with high-salt diet. The mechanism cannot be attributed only to a systemic antihypertensive effect because plasma NE level and PRA were suppressed, which may be partly explained by actions on the renal sympathetic nerve activity through the N-type calcium channel blocking action. Cilnidipine may provide beneficial effects for treating chronic renal disease in patients with hypertension.

REFERENCES