Regular Article

Effects of the L/N-Type Ca\(^{2+}\) Channel Blocker Cilnidipine on the Cardiac Histological Remodelling and Inducibility of Atrial Fibrillation in High-Salt-Fed Rats

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High salt intake has been shown to induce hypertrophy and fibrosis in the atria and ventricles, which could result in the development of atrial fibrillation (AF). Whereas the development of AF is suggested to be prevented by renin–angiotensin system (RAS) inhibitors, recent findings have indicated that this prevention is closely associated with their antihypertensive effects. In this study, we investigated whether the L/N-type Ca\(^{2+}\) channel blocker cilnidipine counteracts salt-induced atrial and ventricular remodelling and the inducibility of AF. Cilnidipine was orally administered to Dahl salt-sensitive rats fed with an 8% NaCl diet at 10 mg/kg for 5 weeks, and then electrophysiological evaluation and histological analyses were performed. The effects were compared with those of the L-type Ca\(^{2+}\) channel blocker amlodipine at 3 mg/kg. Following the intake of the 8% NaCl diet, the blood pressure (BP) increased, and fibrosis was induced in the atria and ventricles. Cilnidipine decreased BP, and the extent of the decrease in the cilnidipine group was similar to those in the amlodipine group. Cilnidipine produced a greater decrease in the fibrotic area in the atria and ventricles than amlodipine. The cilnidipine group shortened the AF duration from 7.43 ± 3.16 to 2.95 ± 1.73 s, which had been increased by NaCl intake. Plasma noradrenaline levels in the cilnidipine group were lower than those in the amlodipine group. Thus, the suppressive effects of cilnidipine on the salt-induced atrial and ventricular remodelling, fibrosis, and AF sustainability might be closely associated with its N-type Ca\(^{2+}\) channel-blocking actions.

Key words cilnidipine; atrial fibrillation; L/N-type Ca\(^{2+}\) channel blocker; salt-sensitive hypertension; cardiac fibrosis

INTRODUCTION

Calcium channel blockers (CCBs) are a family of drugs commonly used for patients with hypertension.\(^1\) Among them, cilnidipine has a unique characteristic of inhibiting not only L-type calcium channels but also N-type channels, leading to beneficial actions, such as the suppression of the hyperactivation of the sympathetic nervous system.\(^2\) We have shown that cilnidipine also decreases plasma renin activity, plasma angiotensin II and aldosterone levels \(via\) the suppression of N-type calcium channels, leading to the suppression of fibrosis and remodelling in the kidney and heart.\(^3\) Thus, cilnidipine can exhibit organ-protective activity in the kidney and heart \(via\) N-type Ca channel suppression.

Atrial fibrillation (AF) is the most frequent arrhythmia in the clinic.\(^4\) When AF occurs, thrombus tends to be formed in the atrium, which may travel to the brain and can cause cerebral infarction. Thus, in the treatment of AF, the prevention of this cerebral infarction is important, and anticoagulants are used; however, medication over a lifetime is necessary, and it is symptomatic therapy rather than fundamental treatment. Catheter ablation is performed as a fundamental therapy, but recurrence of AF is still high.\(^5\) Therefore, as a treatment for AF, it is important to prevent the onset of AF. Hypertension is an important risk factor for AF, and the prevention of atrial and pulmonary vein remodelling caused by high blood pressure is thought to be an important strategy as an upstream therapy for AF.\(^6\) Although angiotensin II receptor blockers (ARBs) are recommended among antihypertensive drugs, there is also a theory that blood pressure reduction itself is important for the treatment of AF, and no conclusion has been obtained.\(^7\) Therefore, it is unknown what effect CCBs exhibit in the treatment or prevention of AF.

Dahl salt-sensitive rats are a commonly used animal model of hypertension in humans. When a high-salt diet is fed to Dahl rats, salt-sensitive hypertension develops, and pressure loading occurs as the blood circulation volume increases, causing remodelling in the kidney and heart.\(^6\) It has been reported that this myocardial remodelling occurs not only in the ventricle but also in the atrium and is related to the onset of AF. Therefore, in this study, we investigated and compared the effects of cilnidipine as an L/N-type CCB and amlodipine as an L-type CCB on the remodelling of atria and ventricles using histological evaluation and electrophysiological experiments.

MATERIALS AND METHODS

All procedures involving the care and use of animals were approved by the Institutional Animal Care and Use Committee of the Pharmaceutical Research Laboratories of Ajinomoto Pharmaceuticals Co., Ltd. (currently known as EA Pharma Co., Ltd.), before they were performed. Male Dahl salt-sensitive (DS) rats (5 weeks of age) were purchased from Japan SLC, Inc. (Shizuoka, Japan) and were fed with standard laboratory chow (CRF-1, Charles River, Kanagawa, Japan) until 6 weeks of age. Tap water was provided \(ad\) \(libitum\) throughout the experiment.

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Experimental Procedure A high-salt diet (F2Dahl-8.0 containing 8% NaCl; Oriental Yeast Co., Ltd., Tokyo, Japan) was provided to 32 DS rats from 7 weeks of age. Six weeks later, the rats were divided into 3 treatment groups, which were assigned by stratified randomization based on their blood pressure and body weight. Then, cilnidipine (10 mg/kg/d, cilnidipine group, n = 11), amlodipine (3 mg/kg/d, amlodipine group, n = 12) or vehicle containing 0.5% hydroxypropyl methylcellulose (2 mL/kg/d, vehicle group, n = 9) was orally administered every morning from 13 to 18 weeks of age. The human clinical doses of cilnidipine and amlodipine are 5–20 and 2.5–10 mg/d, respectively. The dosages of this study used were selected based on the blood pressure in our preliminary study. Five DS rats fed a normal-salt diet (F2Dahl-0.3 containing 0.3% NaCl; Oriental Yeast Co., Ltd.) served as a normal control group.

Measurement of Systolic Blood Pressure Systolic blood pressure was measured weekly by tail-cuff plethysmography (BP-98A, Softron Co., Ltd., Tokyo, Japan). The rats were introduced into a plastic wire holder and placed in a thermally warmed tube, which was maintained at 34–36°C during the measurements. After environmental acclimatization, 3 measurements were performed for each animal, and the mean values were recorded.3)

Electrophysiological Study The rats were anesthetized with intraperitoneal administration of pentobarbital 3–4 h after final oral administration of cilnidipine, amlodipine or vehicle and subjected to electrophysiological study. The surface lead II electrocardiogram (ECG) was obtained from the limb electrodes. The atrial electrogram recording/pacing combination catheter (3 F, inter-electrode distance 2 mm, SMC-304; Physio-Tech, Tokyo, Japan) was positioned at the atrial septum via the right jugular vein. Electrograms were amplified with a bioelectric amplifier (AB-621G, Nihon Kohden, Tokyo, Japan) and fed into a computer-based data acquisition system (Pow-erLab, ADInstruments, Castle Hill, Australia). The atrial effective refractory period (AERP) was assessed by programmed electrical stimulation using a cardiac stimulator (SEN-7203, Nihon Kohden). The 2 V stimulation pulses (approximately twice the threshold voltage) were rectangular in shape and had a duration of 3 ms. The pacing protocol consisted of 8 beats in a cycle length of 150 ms followed by an additional stimulus at various coupling intervals. Starting in the atrial diastole, the coupling interval was shortened by consecutive reductions until the additional stimulus could no longer elicit a response. The AERP was defined as the shortest coupling interval that still produced an electrical response. AF was induced by pacing at the septum of the atrium with burst pacing (2 V output; 3-ms pulse width; 40-ms cycle length for 30 s) 10 times using a stimulator (SEN-7203, Nihon Kohden). In this study, AF was defined as a period of rapid irregular atrial rhythm resulting in an irregular baseline of the ECG. The duration of AF was measured after its induction, and the cycle length was determined using the atrial electrogram.

Measurements of Biochemical Parameters Before the electrophysiological study, blood samples were collected under pentobarbital anaesthesia from the jugular vein for the measurement of plasma noradrenaline and adrenaline. Blood samples were centrifuged (3000 × g) for 15 min and stored at −80°C until analysis. Plasma noradrenaline and adrenaline concentrations were measured using HPLC, as described previously.2) For the measurement of plasma brain natriuretic peptide (BNP) levels, blood samples were collected from the postcaval vein after the electrophysiological study, which were centrifuged and stored as described previously. Its concentration was measured using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (Assay Max Rat BNP-32, AssayPro, St. Charles, MO, U.S.A.).

Histological Studies Immediately following sacrifice, the hearts were removed, weighed and fixed in 10% formalin. Sections of the myocardium were stained with Masson’s trichrome stain to quantify the myocyte cross-sectional dimensions and interstitial fibrosis. The analysis of cardiac sections was performed in a blinded fashion with a light microscope (BX50, Olympus Corporation, Tokyo, Japan). The relative volume occupied by each tissue element of the atrium and the ventricle (myocardial fibres and fibrous tissue) was quantified using image processing software (WinROOF version 3.5, Mi-tani Corporation, Fukui, Japan).

Materials Cilnidipine and amlodipine were purchased from Ajinomoto Co., Inc. (Tokyo, Japan) and Lek Co., Inc. (Kolodvorska, Slovenia), respectively. The drugs were suspended in 0.5% hydroxypropyl methylcellulose (Sigma-Aldrich Co., St. Louis, MO, U.S.A.) immediately prior to oral administration to animals.

Statistical Analysis Data are expressed as the means ± the standard error of the means (S.E.Ms.). Parameters in the vehicle group and normal control group were compared using unpaired t-test to evaluate the pathophysiology due to high salt intake. Dunnett’s test was used to assess the differences between the vehicle group and the cilnidipine group or the amlodipine group when evaluating the effect of drug administration. Parameters in the cilnidipine group and the amlodipine group were compared by the unpaired t-test. The analysis of AF duration was performed after converting the numerical value to a logarithmic value because the value of AF duration was

Table 1. Systolic Blood Pressure in Dahl Salt-Sensitive Rats

| Diet          | n   | Pre            | 2 weeks after administration | 4 weeks after administration |
|--------------|-----|----------------|-----------------------------|-----------------------------|
| Normal diet  | 5   | 124.5 ± 2.5    | 129.9 ± 4.8                | 134.7 ± 2.2                 |
| High Na⁺ diet| 9   | 175.5 ± 4.1     | 201.3 ± 5.3                | 205.4 ± 7.0***             |
| Cilnidipine  | 11  | 173.4 ± 5.0    | 148.0 ± 3.7***             | 152.6 ± 2.8                |
| Amlodipine   | 12  | 177.7 ± 5.0*   | 153.5 ± 2.5                | 154.2 ± 2.6                |

Data are expressed as mean ± standard error of the mean (S.E.M.) Cilnidipine (10 mg/kg, n = 11), amlodipine (3 mg/kg, n = 12) or their vehicle (n = 9) was administered for 5 weeks to Dahl salt-sensitive rats fed with high Na⁺ diets. Vehicle was also administered to Dahl salt-sensitive rats fed with a normal diet for 5 weeks (n = 5). *p < 0.05, ***p < 0.001, compared with Vehicle group.
considered to be log-normally distributed. Differences were considered statistically significant with \( p \) values less than 0.05.

RESULTS

**Systolic Blood Pressure**  Systolic blood pressure was significantly higher in the vehicle group than in the normal control group (\( p < 0.001 \) for each comparison; Table 1). The blood pressure decreased after the oral administration of cilnidipine or amlodipine, and the extent of the decrease in the cilnidipine group was similar to that in the amloidipine group (Table 1).

**Electrophysiological Effects** Figure 1A shows a typical tracing of the RA electrogram, surface ECG and blood pressure from a Dahl salt-sensitive rat fed a normal diet or a high-salt diet. After burst pacing, AF was induced in Dahl salt-sensitive rats fed a high-salt diet, and the duration of

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Fig. 1. Burst Pacing-Induced Atrial Fibrillation (AF) in Dahl Salt-Sensitive Rats

[A] Representative electrograms of AF induced by burst pacing in Dahl salt-sensitive rats treated with vehicle, which were fed a normal diet (upper panel) and a high Na\(^{+}\) diet (lower panel). [B] Effects of cilnidipine and amlodipine on the duration of AF induced by burst pacing in Dahl salt-sensitive rats fed with a high Na\(^{+}\) diet. Cilnidipine (10 mg/kg, \( n = 11 \)), amlodipine (3 mg/kg, \( n = 12 \)) or their vehicle (\( n = 9 \)) was administered for 5 weeks. Vehicle was also administered to Dahl salt-sensitive rats fed a normal diet for 5 weeks (\( n = 5 \)). There was no significant difference in AF cycle length in each group (Normal: 38.8 ± 3.1 ms; Vehicle: 45.8 ± 2.2 ms; Cilnidipine: 39.6 ± 1.3 ms; Amlodipine: 44.2 ± 2.4 ms). Values are mean ± S.E.M. *\( p < 0.05 \) compared with normal group. *\( p < 0.05 \) compared with Vehicle group.
rats fed a normal diet for 5 weeks (n = 11), amloidpine (3 mg/kg, n = 12) or their vehicle (n = 9) was administered for 5 weeks to Dahl salt-sensitive rats fed with high Na⁺ diets. Vehicle was also administered to Dahl salt-sensitive rats fed with a normal diet for 5 weeks (n = 5). Values are mean ± S.E.M. *p < 0.05 compared with normal group. **p < 0.01 compared with Vehicle group.

burst pacing induced AF in the vehicle group (7.43 ± 3.16 s) was significantly longer than that in the normal control group (0.45 ± 0.17 s, p < 0.05, Fig. 1B). The AF duration in the cilnidipine group was significantly shorter than that in the vehicle group (2.95 ± 1.73 s, p < 0.05), while that in the amloidpine group was shorter than that in the vehicle group, which was statistically insignificant (3.94 ± 2.66 s, Fig. 1B).

Table 2 summarizes the electrocardiographic parameters in Dahl salt-sensitive rats. Longer QRS width and QT intervals and shorter AERPs in the vehicle group than in the normal control group were detected. QT intervals were shorter and the R amplitude was less in the cilnidipine and amloidpine groups than in the vehicle group. The AERP in the cilnidipine group was longer than that in the vehicle group, and the difference was statistically insignificant.

**Plasma Catecholamine and BNP Levels** Figure 2 summarizes neurohumoral parameters in Dahl salt-sensitive rats. Plasma levels of noradrenaline, adrenaline and BNP in the vehicle group were greater than those in the normal control group. Among the 3 groups, plasma levels of the neurohumoral factors were lowest in the Dahl salt-sensitive rats receiving cilnidipine.

**Histological Assessment** Typical photomicrographs of longitudinal sections of the atrial tissues obtained from a DS rat in each of the 4 animal groups are shown in Fig. 3A, where interstitial fibrosis was observed. As shown in Figs. 3B and C, the areas of fibrosis in the vehicle group in the atria and ventricles were significantly greater than those in the normal control group. The areas of atrial and ventricular fibrosis in the cilnidipine and amloidpine groups were significantly less than those in the vehicle group.

**Organ Weight** Groups with high salt intake showed increased heart and kidney weights compared to the normal control group. Liver and heart weights in the amloidpine group were significantly lower than those in the vehicle group (Table 3). There was no significant difference between the cilnidipine and amloidpine groups in organ weights.

**DISCUSSION**

In this study, we assessed the effects of the L/N-type CCB, cilnidipine, and the L-type CCB, amloidpine, on atrial and ventricular remodelling and the induction of AF in Dahl salt-sensitive rats using electrophysiological, blood biochemical and histological methods. The administration of cilnidipine and amloidpine lowered blood pressure, and the degree of...
Fig. 3. Effects of Cilnidipine and Amlodipine on Cardiac Fibrosis in Dahl Salt-Sensitive Rats Fed with a High Na\(^+\) Diet

Masson’s trichrome staining of atrium [A], measurement of fibrosis area in atria [B] and ventricle [C]. Cilnidipine (Cil; 10 mg/kg, n = 11), amlodipine (Aml; 3 mg/kg, n = 12) or their vehicle (Veh; n = 9) was administered for 5 weeks. Vehicle was also administered to Dahl salt-sensitive rats fed a normal diet for 5 weeks (Normal; n = 5). Values are mean ± S.E.M.\(^{##}p<0.01,^{###}p<0.001\) compared with normal group. *\(p<0.05,^{***}p<0.001\) compared with Vehicle group. (Color figure can be accessed in the online version.)

Table 3. Effects of Cilnidipine and Amlodipine on Organ Weights in Dahl Salt-Sensitive Rats

|                        | Normal diet vehicle (n = 5) | High Na\(^+\) diet |
|------------------------|-----------------------------|-------------------|
|                        | Vehicle (n = 9)             | Cilnidipine 10 mg/kg (n = 11) | Amlodipine 3 mg/kg (n = 12) |
| Liver (mg/100 mg BW)   | 3.64 ± 0.03                 | 3.82 ± 0.06       | 3.66 ± 0.09       | 3.60 ± 0.04* |
| Heart (mg/100 mg BW)   | 0.30 ± 0.00                 | 0.41 ± 0.01\(^{**}\) | 0.39 ± 0.01       | 0.39 ± 0.01* |
| Kidney (mg/100 mg BW)  | 0.69 ± 0.01                 | 0.89 ± 0.02\(^{**}\) | 0.88 ± 0.01       | 0.88 ± 0.01  |
| Adrenal glands (mg/100 mg BW) | 0.013 ± 0.000               | 0.014 ± 0.001    | 0.013 ± 0.000    | 0.013 ± 0.000 |

Data are expressed as mean ± S.E.M. Cilnidipine (10 mg/kg, n = 11), amlodipine (3 mg/kg, n = 12) or their vehicle (n = 9) was administered for 5 weeks to Dahl salt-sensitive rats fed with high Na\(^+\) diets. Vehicle was also administered to Dahl salt-sensitive rats fed a normal diet for 5 weeks (n = 5). \(^{**}\)p<0.001, compared with normal group. *\(p<0.05\), compared with Vehicle group.
blood pressure reduction was similar. With the increase in blood pressure, cardiac disorder occurred. The administration of a CCB suppressed the elevation of blood BNP concentration. Histological evaluation of the atria and ventricles showed fibrosis in rats fed a high-salt diet, and the administration of cilnidipine and amlodipine suppressed fibrosis in the atria and ventricles. In electrophysiological evaluations, burst pacing induced AF in rats fed with high-salt diets, and the administration of cilnidipine and amlodipine decreased the duration of AF. Both cilnidipine and amlodipine decreased AF duration and cardiac fibrosis; on the other hand, a shorter AF duration was observed in the cilnidipine group.

**Effects on Electrical Remodelling** AF is caused by an abnormality in stimulation generation and an abnormality in excitatory drive. One of the mechanisms by which AF persists is explained by electrophysiological changes (electrical remodelling). When the atrial effective refractory period (AERP) is shortened, the excitatory conduction velocity is decreased, atrium enlargement occurs, reentry tends to be formed and AF occurs.20 In this experiment, AF was induced by burst pacing; however, in a healthy heart, AF did not continue even if burst pacing was performed, and it stopped immediately. In the vehicle group, high salt intake caused the shortening of the AERP, and heart weight was increased (Tables 2, 3); therefore, reentry was considered to have formed, AF easily occurred, and, therefore, AF duration was increased (Fig. 1).

Both the cilnidipine and amlodipine groups had reduced AF duration compared to the vehicle group (Fig. 1). Since cardiac weight was lower in the amlodipine group than in the vehicle group (Table 3), it was suggested that antihypertensive treatment suppressed cardiac hypertrophy. The AERP of the cilnidipine group was longer than that of the vehicle group (not significant) and closer to that of the normal-diet group, which may prevent the formation of reentry. On the other hand, the AERP of the amlodipine group was similar to that of the vehicle group. Therefore, cilnidipine and amlodipine may have different effects on remodelling, suggesting that only cilnidipine suppressed electrical remodelling.

**Effects on Histological Remodelling** Another factor involved in the incidence and duration of AF is histological remodelling. Histological remodelling includes changes such as myocardial hypertrophy, cell death, atrial enlargement, and fibrosis.20 Fibrosis is the most important histological change in the maintenance of AF and forms the basis for reentry due to the reduction and heterogeneity of conduction velocity.21 Stretching and expansion of the atria due to volume and pressure loading causes mechanical stimulation to increase angiotensin II and causes atrial fibrosis via extracellular signal-regulated kinase (ERK)1/2 activation.22 It has been reported that mice overexpressing myocardium-specific angiotensin II converting enzyme (ACE) show remarkable enlargement and fibrosis in the atrium and are prone to AF.23 In this experiment, the increase in sympathetic nerve activity, the increase in blood BNP, and the increase in fibrosis (Fig. 3) might be caused by the high salt feeding, resulting in the decrease in conduction velocity in the atria and ventricles. This fibrosis was suppressed in the cilnidipine and amlodipine groups, which was more strongly suppressed in the cilnidipine group. It is speculated that amlodipine reduced mechanical stress by depression and suppressed the continuation of AF, but sympathetic activation was recognized as a reaction (Fig. 2). Cilnidipine has been reported to suppress fibrosis in the Dahl S rat kidney and in the myocardium of Sprague–Dawley (SD) rats treated with Ang II and to suppress cardiac remodelling in the Dahl S rat.8,9,14,24 As a mechanism, cilnidipine suppresses fibrosis by changing the balance of angiotensin I/II and VII. In this model, cilnidipine may also be directed to suppress the duration of AF by suppressing sympathetic nerve activity and suppressing fibrogenesis via angiotensin (1–7) in addition to reducing mechanical stress due to blood pressure reduction.25 Cilnidipine showed greater effects on fibrosis suppression and the reduction of AF duration at a dose showing lower plasma catecholamine levels via N-type Ca2+ channel-blocking actions. However, further study is needed to clarify whether effects of cilnidipine on cardiac fibrosis, AERP and AF duration are due to N-type Ca2+ channel-blocking action, for example, using knockout animals.

Another mechanism to be considered is the effect of cilnidipine on mitochondria. Recently, it has been reported that cilnidipine suppressed mitochondrial hyperfission-associated myocardial senescence and heart failure after myocardial infarction (MI) by disrupting Drp1-FLNa complex.26 Cilnidipine also suppressed hypoxia/reoxygenation-induced cardiomyocyte senescence and reactive oxygen species (ROS) production. Since other drugs, including dihydropyridine-derived L-type Ca2+ channel blockers and other antihypertensive drugs, did not affect hypoxia-induced mitochondrial hyperfission, this pharmacological effect of cilnidipine is suggested to be independent of blockage of voltage-dependent L/N-type Ca2+ channels.26 Therefore, one of the mechanisms of inhibitory effect of cilnidipine on AERP, AF duration, and cardiac fibrosis observed in this study may be due to its effects on mitochondrial division during cardiac remodelling.

**Consideration of the Application of CCBs to the Treatment of AF** In this study, we investigated the AF persistence mechanism during stimulation by burst pacing when salt-sensitive hypertension existed as a basic disease. Antihypertensive treatment with a CCB reduced mechanical stress due to volume and pressure load, and it is possible to prevent reentry and the duration of AF by suppressing fibrosis. Electrical and structural remodelling of the atria formed by persistent atrial fibrillation leads to refractoriness of the atrial fibrillation itself.20,27,28 When hypertension exists as the underlying disease, antihypertensive treatment with a CCB could be an effective treatment as one of the upstream treatments. Moreover, it was suggested that the effect on sympathetic nerve activity may differ depending on the type of CCB. It has been reported that cilnidipine has an inhibitory effect on AF induced by repeated burst pacing stimulation in animals that do not exhibit hypertension.29 Therefore, CCBs may be effective in treating AF even in the absence of hypertension, but in any case, further verification in humans is necessary.

**CONCLUSION**

In salt-sensitive hypertensive rats, administration of cilnidipine and amlodipine lowered BP, suppressed atrial and ventricular fibrosis and suppressed sustained AF after burst pacing. Cilnidipine showed greater effects on fibrosis suppression and the reduction of AF duration at a dose showing lower plasma catecholamine levels via N-type Ca2+ channel-
blocking actions.

Conflict of Interest  
Eri Harada and Kazumi Sugino are employees of Ajinomoto Co., Inc. and were seconded to Ajinomoto Pharmaceuticals Co., Ltd., which is currently known as EA Pharma Co., Ltd. Akira Takahara received a research funding from Ajinomoto Pharmaceuticals Co., Ltd.

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