Improvement in the Histopathology of Hearts from Cardiomyopathic BIO TO-2 Hamsters Following Long-Term Administration of Amlodipine and Cilnidipine

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The effect of long-term administration of amlodipine and cilnidipine was examined on the histopathology and 1,4-dihydropyridine (DHP) calcium channel antagonist receptors in the left ventricle of BIO TO-2 hamsters, a model of dilated cardiomyopathy (DCM). Oral administration of amlodipine (3 and 10 mg/kg/d, 19 weeks) in 7 week-old BIO TO-2 hamsters produced a significant reduction in calcium deposition and necrosis with little change in the cavity area and fibrosis. A reduction of calcium deposition and necrosis in the myocardium of BIO TO-2 hamsters was also seen following similar administration of cilnidipine (10 mg/kg/d). The long-term administration of amlodipine (3 and 10 mg/kg/d) caused a significant increase (36.6 and 21.7%, respectively) in the $B_{\max}$ for specific (+)-[³H]PN 200-110 binding in the myocardium from BIO TO-2 hamsters, compared with that in control hamsters. In conclusion, the present study has shown that long-term administration of amlodipine and cilnidipine improves calcium deposition and necrosis in the myocardium from BIO TO-2 hamsters. Thus, these data suggest that both agents may be effective pharmacological treatments of DCM.

Key words dilated cardiomyopathy (DCM); BIO TO-2 hamster; amlodipine; cilnidipine; histopathological change; myocardial calcium channel receptor

Systemic vasodilators are clinically used to treat heart failure and have been shown to reduce the mortality associated with this syndrome. The therapeutic effects of calcium channel antagonists on congestive heart failure are controversial. Nifedipine, 1,4-dihydropyridine (DHP) calcium channel antagonist, showed disappointing results in patients with heart failure, probably because of a negative inotropic effect or reflex neurohumoral activation.^{1,2)} However, a newer 1,4-DHP calcium channel antagonist, amlodipine, which has higher vasoselectivity and longer duration of action with slower onset when compared with nifedipine,^{3,4)} has been shown to improve the exercise capacity of patients with mild-to-moderate congestive heart failure. It also improved the survival of patients with heart failure due to nonischemic dilated cardiomyopathy in the PRAISE Trial.⁵⁾

Recently, Wang *et al.*⁶⁾ have reported that oral administration of amlodipine in a murine model of congestive heart failure induced by viral myocarditis, decreased significantly the histopathological grades of myocardial lesions and increased survival. In addition, amlodipine was effective in preventing cardiac remodeling in the early stage of cardiomyopathy in dilated cardiomyopathic (DCM) hamsters, BIO 53.58.⁷⁾ It is believed that a continuous elevation in circulating catecholamines in DCM hamsters may cause myocardial cell injury by inducing intracellular calcium overload and/or increasing peripheral vascular resistance. Although 1,4-DHP compounds are specific inhibitors of L-type voltage-dependent calcium channels, both amlodipine and cilnidipine, unlike other 1,4-DHP compounds, also inhibit N-type voltage-dependent calcium channels in nerves and secretory cells which are linked to both sympathetic nerve tone and catecholamine release.^{8–11)} Cilnidipine, like amlodipine, is a 1,4-DHP calcium channel antagonist with a slow onset of action and a long-lasting effect,¹²⁾ although little information

is available about its chronic effect on cardiac remodeling. Therefore, the purpose of this study was to examine the effect of long-term administration of amlodipine and cilnidipine on the histopathology and 1,4-DHP calcium channel antagonist receptors in DCM hamsters, BIO TO-2.

MATERIALS AND METHODS

Materials (+)-[³H]PN 200-110 (2.61 TBq/mmol) was purchased from DuPont-NEN Co., Ltd (Boston, Massachusetts, U.S.A.). Amlodipine besilate and cilnidipine were kindly donated by Pfizer Pharmaceutical Company (Tokyo, Japan) and Ajinomoto Company (Tokyo, Japan), respectively. Nifedipine hydrochloride was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). All other chemicals were obtained from commercial sources.

Animals Male golden Syrian BIO TO-2 hamsters, at 6 weeks of age, were obtained from Bio Breeders (Fitchburg, MA, U.S.A.). The hamsters were allowed free access to food and water. The hamsters at 7 weeks of age received repeated oral doses of amlodipine, 3 and 10 mg/kg/d in the drinking water, or cilnidipine, 10 mg/kg/d in the diet, for 19 weeks. During the initial and experimental period, the animal's intake of water and diet was determined, so as to calculate the drug concentration which would be sufficient to achieve dosages of 3 and 10 (amlodipine) or 10 (cilnidipine) mg/kg/d. Thus, the drug concentration in the drinking water and feeding diet was adjusted frequently, *i.e.* every 3 d. After animals were anesthetized with pentobarbital (40 mg/kg, *i.p.*), then, blood from the descending aorta was collected into tubes preloaded with EDTA and the hearts were perfused with 0.9% saline and excised. Plasma was separated by centrifugation, and plasma samples and portions of myocardial tissue were stored at –80 °C.

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Fig. 1. Effects of Oral Administration of Amlodipine on Cavity Area, Calcium (Ca) Deposition, Necrosis and Fibrosis in Left Ventricles of BIO TO-2 Hamsters

BIO TO-2 hamsters at 7 weeks of age received amlodipine (3 and 10 mg/kg/d) or vehicle (control) orally for 19 weeks; they were then sacrificed, and the cavity area, calcium (Ca) deposition, necrosis and fibrosis were determined using an imaging analyzer after staining the left ventricle sections with hematoxylin-eosin, Masson's trichrome and Von Kossa stain. Each column represents the mean $\pm$ S.E. of six to seven animals. *, significantly different from the control value at $p < 0.05$.

Histopathology of the Myocardium Hearts were processed for histopathological investigations and quantitatively assessed as previously described.¹³⁾ Briefly, after fixation by immersion in 10% phosphate-buffered formaldehyde for 24 h, the hearts were embedded in paraffin. The tissues were cut into serial sections 5 μ m thick with a sliding microtome (HN 400, Microm, Heidelberg). The serial sections were stained with hematoxylin and eosin, with Masson's trichrome for connective tissue and by the Von Kossa method for calcium deposits. The areas of necrosis, calcium deposits, left ventricular cavity and fibrosis were quantitatively determined using light microscope and an image analyzer (SP 500, Olympus, Tokyo).

Measurements of 1,4-Dihydropyridine Calcium Channel Antagonist Receptors Ventricular myocardium was homogenized in a Polytron homogenizer in 20 mM NaH_2PO_4 buffer. After centrifugation of the homogenate at $40000 \times g$ for 20 min at 4 $^\circ\text{C}$, the resulting pellet was resuspended in 20 mM NaH_2PO_4 buffer and recentrifuged. The final pellets were resuspended in assay buffer. The density of 1,4-dihydropyridine calcium channel antagonist receptors in myocardial homogenates from BIO TO-2 hamsters was measured using (+)-[^3H]PN 200-110, as previously described.¹⁴⁾ Briefly, the myocardial homogenate (approximately 200 μg protein) was incubated with different concentrations of (+)-[^3H]PN 200-110 (0.03–0.57 nM) in the dark under a sodium lamp for 60 min at 25 $^\circ\text{C}$ in 50 mM Tris-HCl buffer. Incubation was terminated by rapid filtration over Whatman GF/B filters. Filters were washed with 10 ml ice-cold buffer, and the tissue-bound radioactivity was extracted from the filters overnight with 5 ml scintillation fluid (2 l toluene, 1 l Triton X-100, 15 g 2,5-diphenyloxazole and 0.3 g 1,4-bis-[2-(5-phenyloxazolyl)]-benzene], and it was determined by a liquid scintillation counting. The specific binding was defined experimentally from the difference between counts in the absence and

presence of 1 μM nifedipine. All assays were conducted in duplicate. Every binding experiment was performed using the fresh tissue. The protein concentration was determined by the method of Lowry *et al.*¹⁵⁾ using bovine serum albumin as a standard.

Data Analysis Analysis of the binding data was performed as described previously.¹⁶⁾ The apparent dissociation constant (K_d) and maximal number of binding sites (B_{max}) for (+)-[^3H]PN 200-110 were estimated by Rosenthal analysis of the saturation data.¹⁷⁾ Statistical analysis of data was performed with one-way analysis of variance followed by Dunnett's test for single and multiple comparisons, respectively.

RESULTS

Pathological Observations The ratio of heart to body weight in BIO TO-2 hamsters was unchanged following oral administration of amlodipine and cilnidipine for 19 weeks (control = 3.52 ± 0.13 , amlodipine: 3 mg/kg/d = 3.69 ± 0.13 , 10 mg/kg/d = 3.93 ± 0.07 , cilnidipine 10 mg/kg/d = 3.83 ± 0.12 mg/g, $n = 9-10$).

Histopathological examination of the myocardium (left ventricle) from BIO TO-2 hamsters, given oral amlodipine (3 and 10 mg/kg/d) for 19 weeks, showed a significant reduction in calcium deposition and necrosis, compared with those in myocardium from vehicle-treated cardiomyopathic hamsters (Fig. 1). The reduction in the calcium deposition and necrosis at the dose of 3 mg/kg/d of amlodipine were 76.9 and 41.2%, respectively, and that at the dose of 10 mg/kg/d was 74.8 and 40.0%, respectively. There was no significant change in the cavity area and fibrosis of hearts of these amlodipine-treated BIO TO-2 hamsters.

Similarly, long-term administration of cilnidipine (10 mg/kg/d) for 19 weeks reduced markedly (88.4%) calcium deposition in the myocardium of BIO TO-2 hamsters, and

Fig. 2. Effects of Oral Administration of Cilnidipine on Cavity Area, Calcium (Ca) Deposition, Necrosis and Fibrosis in Left Ventricles of BIO TO-2 Hamsters

BIO TO-2 hamsters at 7 weeks of age received cilnidipine (10 mg/kg/d) or vehicle (control) orally for 19 weeks; they were then sacrificed, and the cavity area, calcium (Ca) deposition, necrosis and fibrosis were determined using an imaging analyzer after staining the left ventricle sections with hematoxylin-eosin, Masson's trichrome and Von Kossa stain. Each column represents the mean $\pm$ S.E. of six to seven animals. **, significantly different from the control value at $p < 0.01$.

Table 1. Effects of Oral Administration of Amlodipine and Cilnidipine on K_d and B_{max} of Myocardial (+)-[3 H]PN 200-110 Binding in BIO TO-2 Hamsters

Drugs	K_d (pM)	B_{max} (fmol/mg protein)
Control	72.1 $\pm$ 6.5	62.8 $\pm$ 5.0
Amlodipine (mg/kg/d)		
3	94.3 $\pm$ 6.6	85.8 $\pm$ 3.3**
10	75.1 $\pm$ 3.7	76.4 $\pm$ 3.8*
Cilnidipine (mg/kg/d)		
10	88.4 $\pm$ 8.6	67.7 $\pm$ 3.1

Values are the mean $\pm$ S.E. of ten animals. BIO TO-2 hamsters at 7 weeks of age were given orally amlodipine (3 and 10 mg/kg/d), cilnidipine (10 mg/kg/d) or vehicle (control) for 19 weeks; they were then sacrificed, and Rosenthal analysis of the specific binding of (+)-[3 H]PN 200-110 in myocardial homogenates of these hamsters was performed. * and **, significantly different from the control value at $p < 0.05$ and $p < 0.01$, respectively.

there was also a trend towards a reduction in necrosis with little effect on the cavity area and fibrosis (Fig. 2).

Effects on Myocardial 1,4-Dihydropyridine Calcium Channel Antagonist Receptors Long-term administration of amlodipine (3 and 10 mg/kg/d) in BIO TO-2 hamsters brought about a significant increase in the B_{max} value for specific (+)-[3 H]PN 200-110 binding in the myocardium of BIO TO-2 hamsters with little effect on the K_d value, compared with the values in vehicle-treated hamsters (Table 1). The increase produced by amlodipine at doses of 3 and 10 mg/kg/d was 36.6 and 21.7%, respectively.

DISCUSSION

The present study was undertaken to examine the effect of long-term oral administration of amlodipine and cilnidipine on the histology and 1,4-DHP calcium channel antagonist receptors in the myocardium of DCM hamsters, BIO TO-2.

Oral administration of amlodipine, 3 and 10 mg/kg/d for 19 weeks, reduced significantly calcium deposition and necrosis in the myocardium of BIO TO-2 hamsters, compared with vehicle-treated hamsters. These data are in reasonable agreement with previous observations in other models of heart failure.^{6,7} Wang *et al.*⁶ have reported that oral administration of amlodipine in a murine model of congestive heart failure induced by viral myocarditis, reduced significantly the histopathological grade of myocardial lesion and increased survival. In addition, amlodipine prevented cardiac remodeling in the early stage of cardiomyopathy and reduced cardiac dysfunction in BIO 53.58 hamsters.⁷ Inasmuch as a prolonged high level of intracellular calcium may lead to the death of myocytes in BIO 53.58 hamsters,^{18,19} it seems likely that amlodipine ameliorates calcium overload of myocytes by inhibiting calcium influx through voltage-dependent calcium channels. Taken together, our data support the idea that, in the myocardium of DCM, amlodipine may improve calcium handling, ameliorate calcium overload, and prevent calcium-mediated cell death.

Cilnidipine, like amlodipine, is another 1,4-DHP calcium channel antagonist having long-lasting pharmacological effects with slow onset of action¹² and concomitant blockade of N-type calcium channels.⁹⁻¹¹ In the present study, long-term administration of cilnidipine has been shown to cause a significant reduction in calcium deposition with a trend towards a reduction in necrosis in the myocardium of BIO TO-2 hamsters. To our knowledge, this is the first study to demonstrate a preventive effect by cilnidipine on calcium deposition and necrosis in the myocardium of the DCM model.

Although it is generally accepted that 1,4-DHP calcium channel antagonists are specific inhibitors of L-type calcium channel, it has been shown that amlodipine and cilnidipine may also block N-type calcium channels which are mainly distributed in sympathetic nerve terminals and secretory cells.⁸⁻¹¹ It is possible that chronic blockade of N-type cal-

cium channels in DCM patients may reduce the concentration of plasma catecholamine release.^{20,21)} Continuously elevated circulating catecholamines in BIO 53.58 hamsters may cause myocardial cell injury by inducing intracellular calcium overload and/or increasing peripheral vascular resistance, thereby resulting in exacerbation of congestive heart failure. The present study has revealed that long-term administration of amlodipine and cilnidipine causes a reduction in calcium deposition and necrosis in the myocardium of BIO TO-2 hamsters. Thus, it is conceivable that these beneficial effects are due to the inhibition of intracellular calcium influx in the myocardium which may result from the reduced catecholaminergic activity following the blockade of N-type calcium channel in addition to the blockade of L-type calcium channels.

Long-term administration of amlodipine at doses of 3 and 10 mg/kg/d caused a significant increase in the number of (+)-[³H]PN 200-110 binding sites in the myocardium of BIO TO-2 hamsters. This enhancement of (+)-[³H]PN 200-110 binding sites may reflect up-regulation of myocardial 1,4-DHP calcium channel antagonist receptors as a result of the prolonged blockade by amlodipine. The physiological significance of the increased density of 1,4-DHP calcium channel antagonist receptors in the myocardium of BIO TO-2 hamsters is at present uncertain. The voltage-dependent calcium channel in isolated cardiac cells may exist in interconvertible states, that is, active (open) and inactive (closed) states, and 1,4-DHP calcium channel antagonists bind predominantly to the inactive channels.^{22,23)} According to this assumption, it seems likely that the increased density of (+)-[³H]PN 200-110 binding sites in the BIO TO-2 myocardium reflects more inactive state of calcium channels, *i.e.* a relative decrease in the active state. Consequently, this phenomena may be the cause of the reduced calcium influx into myocardial cells of BIO TO-2 hamsters by long-term administration of amlodipine.

In conclusion, the present study has shown that long-term administration of amlodipine and cilnidipine has beneficial effects on calcium deposition and necrosis in the myocardium from BIO TO-2 hamsters. Thus, these data suggest that both agents may be effective drug treatment for DCM.

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