Beneficial Effects of Low-Dose Benidipine in Acute Autoimmune Myocarditis — Suppressive Effects on Inflammatory Cytokines and Inducible Nitric Oxide Synthase —

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Excessive production of nitric oxide (NO) by inducible NO synthase (iNOS) contributes to the progression of myocardial damage in myocarditis. Some dihydropyridine calcium channel blockers reportedly inhibit NO production and proinflammatory cytokines and the present study sought to clarify if a low dose of benidipine, a novel dihydropyridine calcium channel blocker, would ameliorate experimental autoimmune myocarditis (EAM). Rats with or without myocarditis were administered oral benidipine at a dose of $3 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$ for 3 weeks. Low-dose benidipine did not decrease blood pressure significantly compared with the untreated group, but markedly reduced the severity of myocarditis. Myocardial interleukin-1 (IL-1) expression and IL-1-positive cells were significantly less in rats with EAM that were treated with low-dose benidipine compared with untreated rats. Also, myocardial iNOS expression and iNOS-positive cells were markedly reduced in the treated rats compared with the untreated group. Furthermore, myocardial NO production and nitrotyrosine expression were suppressed by the treatment in rats with EAM. The cardioprotection of low-dose benidipine may be caused by suppression of inflammatory cytokines and inhibition of NO production. (*Circ J* 2003; 67: 545–550)

Key Words: Calcium channel blocker; Cytokine; Myocarditis; Nitric oxide

In humans, acute myocarditis is a potentially lethal disease, and precedes the development of dilated cardiomyopathy (DCM)! Two mechanisms to explain this progression have been proposed: (1) persistent viral infection, and (2) progressive autoimmune myocardial injury.²

Autoimmune giant cell myocarditis in rats mimics human fulminant myocarditis in the acute phase.³ Nitric oxide (NO), a gaseous free radical, is generated from L-arginine by enzymatic conversion in stimulated endothelial cells, activated macrophages and other cells,^{4–6} and there is increasing evidence that the NO pathway plays an important role in the pathogenesis of inflammatory and immunological diseases.⁷ Excessive production of NO by inducible NO synthase (iNOS) contributes to the progressive myocardial damage in myocarditis.⁸ Peroxynitrite, derived from NO, is a powerful oxidant that causes tissue damage. Its formation can be estimated immunohistochemically using anti-nitrotyrosine antibody because nitrotyrosine is a major product of peroxynitrite's reaction with proteins. Overexpression of iNOS by cardiomyocytes in mice results in peroxynitrite generation, heart block, and sudden death.⁹

Cytokines increase NO via induction of NOS in activated immune cells, which results in a direct negative inotropic effect and a modulation of inotropic responsiveness.¹⁰ Increased concentrations of circulating and intracardiac

inflammatory cytokines and iNOS have been reported not only in patients with heart failure and dilated cardiomyopathy, but also in mice with myocarditis!^{1,12} Recent reports have indicated that some dihydropyridine calcium channel blockers inhibit NO production in macrophages induced by lipopolysaccharide administration and the mechanism of action is thought to be inhibition of iNOS, possibly at the level of transcription!^{13,14} Dihydropyridine calcium channel blockers have been reported to also inhibit interleukin-1 (IL-1) production and the transcription of IL-1 mRNA!¹⁵ Those findings imply that benidipine, a novel dihydropyridine calcium channel blocker (Kyowa Co Ltd, Tokyo, Japan), may be an effective agent in countering myocardial inflammation by the removal of excess NO.

The purpose of the present study was to examine the effects of low-dose benidipine on an experimental autoimmune myocarditis (EAM) model, focusing on its inhibitory effects on inflammatory cytokines and NO production.

Methods

Immunization

Acute EAM was induced in 6-week-old Lewis rats by subcutaneous injection of 0.1 ml of porcine cardiac myosin (Sigma; 10 mg/ml), mixed with an equal volume of Freund's complete adjuvant (FCA) supplemented with Mycobacterium tuberculosis H37Ra (Difco), in the foot pads on days 1 and 8.¹⁶ Control rats were immunized with FCA alone. The day of infection was designated Day 1.

Medication

Experiment 1 To analyze the hemodynamics of benidipine in the rats with and without myocarditis, the control

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Table 1 Effects on HR and BP of Benidipine Treatment in Rats With and Without EAM in Experiment I

Group	HR (beats/min)				SBP (mmHg)				DBP (mmHg)			
	Day 1	Day 8	Day 15	Day 22	Day 1	Day 8	Day 15	Day 22	Day 1	Day 8	Day 15	Day 22
Con/V (n=3)	381±54	401±35	426±22	412±23	117±23	118±8	119±5	128±4	88±15	86±7	92±3	91±4
Con/Ben-3 (n=3)	398±47	375±25	391±13	375±25	118±2	124±4	123±2	124±4	88±4	92±3	89±9	92±3
Con/Ben-5 (n=3)	410±30	364±28	380±32	328±26**†	124±7	118±9	126±12	130±6	90±4	86±8	94±1	93±3
EAM/V (n=6)	401±44	362±26	400±28	489±26††	116±11	115±10	113±11	121±12	87±4	91±7	89±3	86±11
EAM/Ben-3 (n=3)	401±35	394±52	437±50	416±35**	120±9	128±6	122±8	122±18	90±7	92±9	91±3	91±14
EAM/Ben-5 (n=3)	416±22	389±30	421±74	464±17†	118±8	124±11	122±5	107±8*††	88±5	91±8	87±2	78±8*††

n, number of rats in each group; Con/V, normal rats treated with vehicle; Con/Ben-3, normal rats treated with low dose benidipine $3 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$; Con/Ben-5, normal rats treated with medium dose benidipine $5 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$; EAM/V, rats with EAM treated with vehicle; EAM/Ben-3, rats with EAM treated with low-dose benidipine $3 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$; EAM/Ben-5, rats with EAM treated with medium dose benidipine $5 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$. HR, heart rate; SBP, systolic blood pressure; DBP, diastolic blood pressure. * $p < 0.05$, ** $p < 0.01$ vs Con/V or EAM/V; † $p < 0.05$, †† $p < 0.01$ vs Day 1.

Fig 1. Histopathology and the effects of benidipine treatment in rats with EAM. (A,D) Histopathology of an intact heart (grade 0) immunized with FCA alone. (B, E) Representative histopathology of a rat with myocarditis treated with vehicle. Marked diffuse myocardial necrosis and cellular infiltration with multinuclear giant cells (arrows) can be seen in the inflammatory regions (grade 4). (C, F) Representative histopathology of a rat with myocarditis treated with low-dose benidipine ($3 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$; EAM/Ben-3). A small focus of cellular infiltration in the inflammatory regions (arrowheads) is shown (grade 1). Hematoxylin and eosin, original magnification, $\times 50$ (A and B, inset, $\times 100$), $\times 25$ (C), $\times 5$ (D, E, and F).

rats immunized with FCA alone were divided into 4 groups for 3-week oral administration of (1) vehicle (0.5% methylcellulose, Con/V, $n=3$), (2) low-dose benidipine $3 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$ (Con/Ben-3, $n=3$), (3) medium-dose benidipine $5 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$ (Con/Ben-5, $n=3$), and (4) high-dose benidipine $10 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$ (Con/Ben-10, $n=3$). The rats immunized with cardiac myosin were divided into another 3 groups and treated for 3 weeks with (5) vehicle (EAM/V, $n=6$), (6) low-dose benidipine $3 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$ (EAM/Ben-3, $n=3$), and (7) medium-dose benidipine $5 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$ (EAM/Ben-5, $n=3$). Vehicle or benidipine was administered for 3 weeks via the drinking water, starting simultaneously with the immunization with myosin on Day 1. The doses were chosen because benidipine does not change blood pressure (BP) at $3 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$, but does decrease it at $10 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$ in mice.¹⁷ BP and heart rate (HR) were determined by the tail-cuff method using a photoelectric tail cuff detection system (Softron BP-98A, Tokyo, Japan) on Days 1, 8, 15 and 22.

Experiment II From the results of experiment I (Table 1), we discontinued the preliminary studies using the medium and high doses of benidipine in the rats with EAM because of significant hypotension and aggravation of cardiac lesions. In experiment II, rats with and without myocarditis were divided into 4 groups: (1) Con/V, $n=4$, (2) Con/Ben-3, $n=4$, (3) EAM/V, $n=10$, and (4) EAM/Ben-3, $n=5$. The drug was administered for 3 weeks. All the rats were killed on Day 22 under ether anesthesia after the

hemodynamic measurements.

The investigations conformed with the 'Guide for the Care and Use of Laboratory Animals' published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Histopathology

At death, the macroscopic findings of the heart were graded on a scale of 0–4 and pericardial effusion was graded from 0 to 2. Microscopic findings of myocardial damage and cellular infiltration were graded on a scale of 0–4, as previously described.¹⁸

Western Blotting

The myocardial lysates underwent sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and were sequentially transferred to a membrane (Millipore) that was incubated with anti-iNOS (1:2,500, Calbiochem), anti-endothelial NO synthase (eNOS) (1:1,000, Calbiochem), and anti-IL-1 (Secotec) antibodies, then with a peroxidase-linked secondary antibody (Amersham). Chemiluminescence was detected and semiquantitatively analyzed using the NIH Image system.

Immunohistochemical Assay

We used an immunoperoxidase technique¹⁶ and the following primary antibodies [anti-IL-1 (1:250, Secotec), anti-iNOS (1:500, Calbiochem), and anti-nitrotyrosine

Table 2 Effects on HR, BP, Histopathology and Heart Weight/Body Weight Ratio by Low-Dose Benidipine ($3 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$) Treatment in Rats With EAM in Experiment II

Group	n	HW/BW (mg/g)	HR (beats/min)	SBP (mmHg)	DBP (mmHg)	Histopathological Score		
						Pericardial effusion	Macroscopic finding	Microscopic finding
Con/V	4	2.97 ± 0.07	410 ± 20	128.5 ± 3.7	93.8 ± 5.7	0	0	0
Con/Ben-3	4	3.02 ± 0.12	391 ± 31	130.3 ± 9.9	92.9 ± 11.1	0	0	0
EAM/V	10	5.75 ± 0.69	462 ± 51	131.3 ± 18.7	91.5 ± 16.9	1.8 ± 0.4	3.4 ± 0.8	3.5 ± 0.7
EAM/Ben-3	5	$4.49 \pm 1.12^{**}$	$412 \pm 39^{*}$	123.2 ± 13.3	88.8 ± 15.2	$1.2 \pm 0.8^{*}$	$2.2 \pm 0.8^{*}$	$2.3 \pm 0.3^{**}$

n, number of rats in each group; Con/V, normal rats treated with vehicle; Con/Ben-3, normal rats treated with low-dose benidipine $3 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$; EAM/V, rats with EAM treated with vehicle; EAM/Ben-3, rats with EAM treated with low-dose benidipine $3 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$. HW/BW, the ratio of heart weight to body weight; HR, heart rate; SBP, systolic blood pressure; DBP, diastolic blood pressure. $^{*}p < 0.05$, $^{**}p < 0.01$ vs EAM/V.

(1:100, Upstate Biotechnology) to detect IL-1, iNOS, and nitrotyrosine positive cells, respectively. The positive-staining cells in the heart tissue were counted blindly by 2 observers in 6 fields at $\times 400$ magnification (within a 1-mm^2 grid). The results were then averaged for each group.

NO Production

NO production was evaluated by measuring the nitrite/nitrate, the stable metabolite of NO.¹⁹ Frozen myocardium was homogenized and then centrifuged at 12,000G for 1 h at 4°C . After centrifugation, the supernatant was incubated for 15 min at 37°C in the presence of 5 mmol/L NADPH. Next, 100 μL of supernatant mixed with 100 μL of Griess reagent (1% sulfanilamide and 0.1% naphthylethylenediamide in 5% phosphoric acid), and the absorbency at 540 nm was then measured with microplate reader. Nitrite/nitrate contents were calculated by comparison with the standard solutions of sodium nitrite.

Statistical Analysis

All values are expressed as means $\pm$ standard deviation (SD). One-way analysis of variance (ANOVA), followed by Fisher's protected least significant difference test, was performed. A value of $p < 0.05$ was considered statistically significant.

Results

BP and HR of Rats With and Without EAM Undergoing Benidipine Treatment

In experiment I, rats with and without EAM were administered 3 dosages of benidipine. In general, in the control rats, high-dose benidipine ($10 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$) decreased BP and increased HR compared with the vehicle group (data not shown), but there were no significant differences between the low- ($3 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$) and medium- ($5 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$) dose benidipine groups compared with the vehicle group on Days 1, 8, and 15. None of the rats with EAM died during the course of the disease. The BP was decreased in the medium-dose benidipine group, but not in the low-dose group. We discontinued the preliminary studies using the medium and high doses of benidipine in the rats with EAM because of significant hypotension and aggravation of cardiac lesions.

Histopathology, Heart Weight/Body Weight (HW/BW), HR and BP in Rats With Acute EAM Undergoing Low-Dose Benidipine Treatment

In experiment II, low-dose benidipine ($3 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$) was given for 3 weeks to rats with EAM. None of the rats

Fig 2. Myocardial IL-1 protein expression. (A) Western blot analysis using 10 μg of each protein sample, loaded and electrophoresed on 15% SDS-PAGE. Vehicle, rat immunized with FCA alone treated with vehicle; Ben-3, rat immunized with FCA alone treated with benidipine $3 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$; EAM+Vehicle, rat with EAM treated with vehicle; EAM+Ben-3, rat with EAM treated with benidipine $3 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$. (B) Densitometric analysis of relative protein levels. In rats with EAM, the IL-1 protein expression was markedly increased and was decreased by benidipine treatment. Values are derived from 4 animals and are represented as percentage of controls. $^{*}p < 0.05$, $^{**}p < 0.01$ vs Vehicle; $^{\dagger}p < 0.01$ vs EAM+Vehicle.

died. At euthanasia on Day 22, the hearts showed severe, diffuse discolored myocarditis with massive pericardial effusion. Extensive injuries to myocytes with inflammatory changes and multinucleated giant cells (Fig 1B) were observed. Treatment with low-dose benidipine markedly reduced the severity of the disease, as assessed by HW/BW and histopathological scores (Table 2, Fig 1). HR were increased, and low-dose benidipine treatment suppressed the increment of HR in rats with EAM. There was no significant difference in BP compared with that of rats with EAM treated with vehicle.

Myocardial Expression of IL-1

Western blotting showed that myocardial IL-1 was upregulated 2.2-fold in rats with EAM and treated with vehicle compared with the intact heart (Fig 2). Benidipine treatment decreased the upregulated IL-1 expression in EAM. Immunohistochemistry showed that IL-1-positive cells were localized mainly in infiltrating inflammatory cells (Fig 3A). Low-dose benidipine treatment markedly reduced the number of IL-1-positive cells in the inflam-

Fig3. Immunohistochemical assay for IL-1, iNOS and nitrotyrosine in the heart. (A) Immunohistochemical staining. Serial paraffin sections of heart tissue from rats with EAM were stained with antibodies to IL-1 (a), iNOS (b) and nitrotyrosine (c). IL-1 -positive cells were localized mainly in infiltrating inflammatory cells (arrows). iNOS-positive cells were found predominantly in inflammatory infiltrating cells (arrowheads) and endothelium-like cells. Nitrotyrosine-positive cells were mainly in the damaged myocytes (arrowheads) and in infiltrating cells (arrows). Counterstained with hematoxylin (a) and methyl green (b, c, and negative), original magnification, $\times 200$. (B) Immunohistochemical assay on the effects of benidipine. The number of IL-1, iNOS and nitrotyrosine-positive cells ($/\text{mm}^2$) were markedly decreased by benidipine treatment compared with in rats with EAM treated with vehicle. $*p < 0.01$ vs EAM + Vehicle.

matory lesions compared with rats with EAM treated with vehicle (Fig 3B).

Myocardial Expression of iNOS

Western blotting showed that myocardial iNOS was upregulated 2.9-fold in rats with EAM treated with vehicle compared with the intact heart (Fig 4). Low-dose benidipine treatment decreased the upregulated iNOS expression in rats with EAM. There was no significant difference in the eNOS expression among the 4 groups. Immunohistochemical staining showed that iNOS-positive cells were found predominantly in infiltrating inflammatory cells and endothelium-like cells (Fig 3A). Low-dose benidipine treatment markedly reduced the number of iNOS-positive cells in the inflammatory lesions compared with rats with EAM treated with vehicle (Fig 3B). iNOS-positive cells were not found in the intact heart (data not shown).

Myocardial Production of NO and Peroxynitrite

The results showed that nitrotyrosine-positive cells were localized mainly in infiltrating cells and in the damaged myocytes (Fig 3A). Low-dose benidipine reduced the number of nitrotyrosine-positive cells in the inflammatory

Fig4. Myocardial expression of iNOS and eNOS protein. (A) Western blot analysis using $30\mu\text{g}$ of each protein sample, loaded and electrophoresed on 7.5% SDS-PAGE. Vehicle, rat immunized with FCA alone treated with vehicle; Ben-3, rat immunized with FCA alone treated with benidipine $3\text{mg}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$; EAM + Vehicle, rat with EAM treated with vehicle; EAM + Ben-3, rat with EAM treated with benidipine $3\text{mg}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$. (B) Densitometric analysis of relative protein levels. In rats with EAM, iNOS protein expression was induced by inflammation and partially abrogated by benidipine treatment. The eNOS expression was not significantly changed by myocardial inflammation or by benidipine treatment. Values are derived from 4 animals and represented as percentage of controls. $*p < 0.01$ vs Vehicle; $\dagger p < 0.01$ vs EAM + Vehicle.

Fig5. Myocardial NO production. To determine whether benidipine treatment attenuates NO production in EAM, we measured the myocardial nitrite/nitrate contents using the Griess reaction. Vehicle, rat immunized with FCA alone and treated with vehicle; Ben-3, rat immunized with FCA alone treated with benidipine $3\text{mg}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$; EAM + Vehicle, rat with EAM treated with vehicle; EAM + Ben-3, rat with EAM treated with benidipine $3\text{mg}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$. In rats with EAM, the myocardial nitrite/nitrate contents were significantly increased, and were reduced by 42% by benidipine treatment. Values are derived from 4 animals and represented as nmol/mg of heart. $*p < 0.01$ vs Vehicle; $\dagger p < 0.01$ vs EAM + Vehicle.

lesions (Fig 3B). Rarely were nitrotyrosine-positive cells found in the normal heart (data not shown).

To determine whether low-dose benidipine treatment attenuates NO production in rats with EAM, we measured the myocardial nitrite/nitrate content using the Griess reaction. NO production was significantly increased in rats with EAM treated with vehicle, and was decreased by 42% ($p < 0.01$) by benidipine treatment (Fig 5).

Discussion

The participation of proinflammatory cytokines in the pathogenesis of cardiac diseases has been described.²⁰ The concentrations of circulating proinflammatory cytokines, such as tumor necrosis factor (TNF)- α , IL-1 and IL-6, are elevated in patients with myocarditis,²¹ and in a murine model of viral myocarditis, the intracardiac expression of TNF- α , IL-1, interferon (INF)- γ , and IL-2 genes was increased.²² The degree of their expression correlated with the severity of the disease; namely, the overproduction of proinflammatory cytokines may aggravate the disease. This scenario is supported by the recent findings that the overexpression of TNF- α in the heart causes severe myocarditis in transgenic mice²³ and that IL-1 as well as TNF- α promoted coxsackievirus B3-induced myocarditis in the virus-resistant mice.²⁴ It has been previously demonstrated that the suppression of inflammatory cytokines can ameliorate acute myocarditis.^{11,16,25,26}

Calcium channel blockers are reported to suppress both cytokine production and transcription of cytokine genes in vitro and in vivo.^{27–29} In the present study, low-dose benidipine treatment modified the increased IL-1 expression in acute EAM, and the immunohistochemical study showed that the number of IL-1-positive inflammatory cells was markedly reduced by the treatment. Therefore, the beneficial effects of low-dose benidipine in EAM may be partly caused by suppression of inflammatory cytokines. The inhibition of signal transduction pathways, such as nuclear factor kappa B (NF- κ B) by calcium channel blockers, has been postulated.²⁸

Another finding of the present study is the concomitant inhibition of NO production and expression of iNOS by low-dose benidipine treatment. iNOS-positive cells were found predominantly in inflammatory cells and endothelium-like cells, and the numbers of those were decreased by the treatment. Increased production of NO (nitrite/nitrate) and nitrotyrosine in the lesions of EAM was also decreased by this treatment. These biochemical and histopathological findings indicate that excessive NO might aggravate myocardial injury in rats with EAM, and that low-dose benidipine treatment reduces myocardial inflammation by removing the excess NO. This hypothesis is supported by studies in which the inhibition of NO synthesis by specific iNOS inhibitor decreased myocardial inflammation and necrosis in rats with EAM.^{8,30} Although it is not clearly understood how benidipine interferes with the induction of iNOS, dihydropyridine calcium channel blockers have been reported to inhibit IL-1 production and transcription of IL-1 mRNA in monocytes.¹⁵ Indeed, low-dose benidipine administration inhibited cytokine production associated with the suppression of iNOS in acute EAM in the current study.

The rationale for prescribing calcium channel blockers in patients with heart failure is based on their vasodilator action, anti-ischemic effects, and the capacity to improve left ventricular function. Despite initial studies with nifedipine that reported improvement of the hemodynamic profiles, later evaluation revealed controversial results, including deterioration in the hemodynamics, increased incidence of cardiac events with progression of heart failure, and death.³¹ Other calcium channel blockers, such as felodipine, nisoldipine, and nicardipine, have resulted in no effects or deterioration of clinical status.^{32,33} Amlodipine, another calcium channel blocker, had favorable effects³⁴ and the clinical trial, PRAISE, disclosed that long-term treatment with

amlodipine improved the morbidity and mortality rates in patients with non-ischemic dilated cardiomyopathy.³⁵ In a murine model of viral myocarditis, amlodipine reduced myocardial inflammation and necrosis, and improved survival.³⁶ Verapamil has not produced significant improvement in survival in mice with myocarditis despite histopathological improvement.³⁷ Benidipine has been reported to inhibit IL-1 production and the transcription of IL-1 mRNA¹⁵ and thus may be an effective agent for suppressing myocardial inflammation through the removal of excess NO. In the present study, low-dose, but not medium- or high-dose, benidipine treatment for 3 weeks ameliorated myocarditis on the basis of HW/BW ratio and histopathological scores. Although the actual effect might differ between drugs, dosages, and experimental models, low-dose benidipine for the treatment of patients with myocarditis appears to have clinical significance.

Study Limitation

Although we aimed to clarify the hypothesis that benidipine ameliorates EAM independent of hemodynamic modifications, benidipine treatment resulted in decreased HR on Day 22 in control rats (Table 1). Accordingly, further studies are necessary to clarify the precise effects of benidipine in acute EAM.

Conclusions

The present findings clearly demonstrated that low-dose benidipine markedly reduced the severity of acute EAM in rats and that the cardioprotective effect of benidipine may be suppression of inflammatory cytokines and inhibition of NO production.

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