

**Comparison of the effects of cilnidipine and amlodipine on post-infarct cardiac remodeling
in spontaneously hypertensive rats**

(高血圧モデルラットにおける心筋梗塞後リモデリングに対するシルニジピンとアムロジピンの効果の比較)

Naseratun Nessa

Department of Clinical Pharmacology

Kyoto Pharmaceutical University

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Preface

Calcium channels are considered to be potential therapeutic targets for the treatment of hypertension. Hypertension increases the risk of myocardial infarction (MI). Calcium channel blockers (CCBs) is effective for the ischemic heart disease because they increase coronary blood flow and hypertension, resulting in an increased survival rate. In this study, we evaluated the effects of L/N-type and L-type calcium channel blockers on post-infarct cardiac remodeling in spontaneously hypertensive rats. We found that cilnidipine an L/N-type CCB attenuated the post-infarct remodeling by reducing the cardiac noradrenaline concentration and renin-angiotensin system enhancement more than amlodipine an L-type CCB, in hypertensive rats.

This study is divided into three chapters. The first chapter is titled the effects of CCBs on post-infarct cardiac function. The second chapter is titled effects of CCBs on post-infarct morphological remodeling and gene expression. The third chapter is titled the mechanism of differences between amlodipine and cilnidipine, which was published in Clinical and Experimental Pharmacology and Physiology.

This study will significantly aid in the treatment of hypertension with acute MI.

Abbreviations

ARB	Angiotensin receptor blocker
AMI	Acute myocardial infarction
ACE	Angiotensin-converting enzyme
ACEI	Angiotensin-converting enzyme inhibitor
ANOVA	One-way analysis of variance
BP	Blood pressure
BNP	Brain natriuretic peptide
CCBs	Calcium channel blockers
CMC	Carboxymethyl cellulose
cDNA	Complementary DNA
DBP	Diastolic blood pressure
DHP	Dihydropyridine
DAB	Diaminobenzidine
LV +dp/dt max	Maximum rates of LV pressure development
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
HE	Hematoxylin-eosin
HVA	High-voltage-activated
HW	Heart weight
HR	Heart rate
HF	Heart-frequency
HPLC	High-performance liquid chromatography
LVH	Left ventricular hypertrophy
LVFS	Left ventricular fractional shortening
LVEDP	Left ventricular end-diastolic pressure
LVEDd	Left ventricular end-diastolic diameter
LVESd	Left ventricular end-systolic diameter
LVW	Left ventricular weight
LW	Lung weight
LF	Low frequency
MI	Myocardial infarction
PLSD	Fisher protected least significant difference
PCR	Polymerization chain reaction

RAS	Renin angiotensin system
SBP	Systolic blood pressure
SHR	Spontaneously hypertensive rat
TGF	Transforming growth factor

Abstract

Hypertension is a major risk factor for cardiovascular diseases, such as myocardial infarction (MI) and stroke, and the number of patients with hypertension is increasing in aging societies. Calcium channel blockers (CCBs) are commonly used in the treatment of hypertension. The voltage-dependent Ca^{2+} channels are divided into the L, N, T, P/Q and R types based on their electrophysiological characteristics. Among anti-hypertensive drugs of CCBs, amlodipine blocks the L-type Ca^{2+} channels, and cilnidipine blocks L- and N-type Ca^{2+} channels therefore, cilnidipine suppresses the ischemia/reperfusion-induced cardiac arrhythmia and protects against renal diseases due to inhibition of vascular sympathetic neurotransmitters.

The mortality following MI is highly associated with progressive post-infarct cardiac remodeling and dysfunction. After MI, angiotensin-converting enzyme (ACE) inhibitors are generally used to control the blood pressure because these drugs suppress post-infarct cardiac remodeling and improve the survival rate. On the other hand, nifedipine, a short acting CCB, increases the mortality rate of patients with acute MI. Recently, long acting CCB amlodipine was reported to improve the prognosis of post-infarct cardiac remodeling in normotensive animals. However, the effects of long acting CCBs on post-infarct cardiac remodeling in hypertensive animals are still unclear. To clarify these effects, we investigated whether hypertension management with cilnidipine and amlodipine attenuates cardiac remodeling after MI in spontaneously hypertensive rats (SHRs).

Chapter 1: Effects of CCBs on post-infarct cardiac function.

To assess the effects of CCBs on post-infarct cardiac remodeling in SHRs, we administered 10 mg/kg/day of cilnidipine and amlodipine, orally by gavage 1 week before surgery and continued for 5 weeks. MI was introduced by 30 minutes of coronary artery occlusion followed by reperfusion, and MI rats were sacrificed 4 weeks after surgery. After MI surgery, both CCBs reduced systolic blood pressure to a similar extent. At the end of the experimental period, the ratios of heart weight to body weight and left ventricular (LV) weight to body weight significantly decreased in the cilnidipine-treated group but not in the amlodipine-treated group. In addition, rats in the cilnidipine-treated group had a slightly lower heart rate.

We assessed cardiac function by measuring LV fractional shortening (LVFS) and $+dP/dt_{\max}$ as LV systolic functions and tau as LV diastolic functions in all groups. Severity of the heart failure was assessed by LV end diastolic pressure (LVEDP). Cilnidipine but not amlodipine significantly attenuated the LVFS

reduction, the LVEDP increase, and tau prolongation after MI. Thus, treatment of hypertension after MI using cilnidipine improves post-infarct LV function in hypertensive animals.

Chapter 2: Effects of CCBs on post-infarct morphological remodeling and gene expression.

Morphological examinations after MI were carried out using hematoxylin-eosin (HE), Sirius red, and Masson's trichrome staining. The myocardial infarct size, which is one of the important factors affecting post-infarct cardiac function, was similar with or without CCB treatment. Myocyte hypertrophy and interstitial fibrosis in the non-infarct region are major histological changes in post-infarct cardiac remodeling. After MI, both CCBs exhibited comparable suppression of myocyte hypertrophy. Interstitial fibrosis was also inhibited by both CCBs, but cilnidipine reduced interstitial fibrosis more than amlodipine. The mRNA expression of brain natriuretic peptide (BNP), an indicator of myocyte hypertrophy, was increased in the untreated MI group, and was reduced by both CCBs to a similar extent. Increases in collagen III mRNA expression, a major component of cardiac fibrosis after MI was also suppressed by cilnidipine but not amlodipine.

Chapter 3: Mechanism of differences between amlodipine and cilnidipine.

The mRNA expression and activity of ACE, a key regulator of the renin–angiotensin system and potent activator of TGF β , in the non-infarct region increased in the untreated MI group. Cilnidipine but not amlodipine significantly suppressed the ACE increase. Consistent with post-infarct cardiac failure, the cardiac interstitial noradrenaline concentration, measured by micro-dialysis method, in the non-infarct region significantly increased in the untreated MI group. Cilnidipine also suppressed the increases in cardiac interstitial noradrenaline concentrations after MI by inhibiting N-type Ca^{2+} channels in sympathetic neuro-terminals, which may inhibit the renin angiotensin system.

Conclusion.

This study suggested that the long-acting CCBs, cilnidipine and amlodipine, attenuated LV remodeling after MI in SHRs. Cilnidipine reduced cardiac noradrenaline concentrations and inhibited the tissue renin–angiotensin system, which attenuated post-infarct remodeling more than amlodipine in hypertensive rats.

Introduction

Hypertension is defined as a resting blood pressure (BP) consistently above the normal level. According to the Japanese society of hypertension guidelines hypertension is diagnosed when the systolic blood pressure (SBP) in the office or clinic is ≥ 140 mmHg and/or the diastolic blood pressure (DBP) is ≥ 90 mmHg following repeated examinations [1–4]. Hypertension is a common lifestyle-related disease leading to cardiovascular diseases worldwide and is becoming more prevalent in aging societies [5–7]. The morbidity and mortality of cardiovascular diseases increase in accordance with increases in BP above 120/80 mmHg [8–12]. The annual number of cardiovascular deaths related to hypertension in Japan accounts for the largest portion of all cardiovascular deaths, which is estimated to be 100,000 [9, 10, 13]. Approximately 50% of cardiovascular disease deaths are due to a blood pressure $>120/80$ mmHg [6,7]. The number of hypertensive patients in Japan is estimated to be 43 million, including 31 million with poorly controlled hypertension. Among these 31 million hypertensive patients, 14 million are unaware of hypertension, 4.5 million are untreated despite disease awareness and 12.5 million have poorly controlled despite drug therapy [12].

The heart is one of the essential target organs under hypertension and left ventricular hypertrophy (LVH) develops in patients with hypertension due to maladaptive response to chronic pressure overload. LVH is generally considered as a morphological change of the heart, but it reflects and integrates the long-term cumulative effects of several risk factors for cardiovascular diseases [14–16]. Therefore, the development of LVH is considered a great risk factor for heart failure. Antihypertensive therapy leads to the regression of LVH, which reduces the risk of cardiac morbidity and mortality [17,18]. The goal of pharmacological therapy for hypertension is the reduction of the morbidity and mortality due to hypertension itself and its complications. Presently, many types of antihypertensive drugs are used. The 2019 guidelines for the management of hypertension from the Japanese Society of Hypertension recommend four classes of medication for the initial management of hypertension. Angiotensin-converting enzyme inhibitors (ACEI), angiotensin receptor blockers (ARB), diuretics and calcium (Ca^{2+}) channel blocker (CCB). According to the prescription analysis in Japan, ARBs and CCBs account for approximately 60% and 30%, respectively of the recent hypertension drug usage [19–21]. CCBs are preferred for their higher vascular selectivity, slower onset, and longer duration of hypotensive action [22–25]. Therefore, in the management of hypertension, CCBs are commonly selected for monotherapy and in combination with other medications.

Composition of Ca²⁺ channels

Voltage gated Ca²⁺ channels are classified into six subtypes, namely L, N, T, P/Q and R type based on electrophysiological and pharmacological characteristics [26]. Among them T type Ca²⁺ channels are low-voltage-activated Ca²⁺ channels which are activated and deactivated slowly, but inactivated rapidly. The other five types of Ca²⁺ channels are all high-voltage-activated (HVA) Ca²⁺ channels, which depolarize at approximately -40 mV. In excitable cells, such as smooth muscle cells, cardiac myocytes, and neurons, HVA Ca²⁺ channels play an important role in regulating cellular functions including muscle contraction, neuronal electrical activity, and release of neurotransmitters and hormones. The molecular biological examinations demonstrated that Ca²⁺ channels are composed of 5 subunits, $\alpha 1$, $\alpha 2$ - δ , β and γ , and each subunit is encoded by separate genes. Among them, the $\alpha 1$ subunit comprises the pore structure for Ca²⁺ and 10 different subclasses of $\alpha 1$ subunits have been reported. Subclasses of $\alpha 1$ subunits characterize the types of Ca²⁺ channels and regulate CCBs affinity, and the distribution of these subclasses is organ specific [26,27].

Classification of Ca²⁺ channel blockers

Calcium channel blocking agents are generally classified into three groups according to their chemical structure: benzothiazepines, phenylalkylamines, and dihydropyridines (DHPs) (Figure 1). DHPs have a greater selectivity for vascular smooth muscle than myocardium because they block smooth muscle calcium channels at concentrations below those required for significant cardiac effects, they have lower negative inotropic activity than benzothiazepines and phenylalkylamines [28]. Verapamil and diltiazem, classified in phenylalkylamines and benzothiazepines respectively, are less selective vasodilators than DHPs and have direct effects on myocardium causing depression of Sino-atrial and Atrio-ventricular nodal conduction. These drugs, especially verapamil, are used for the management of supraventricular tachyarrhythmia.

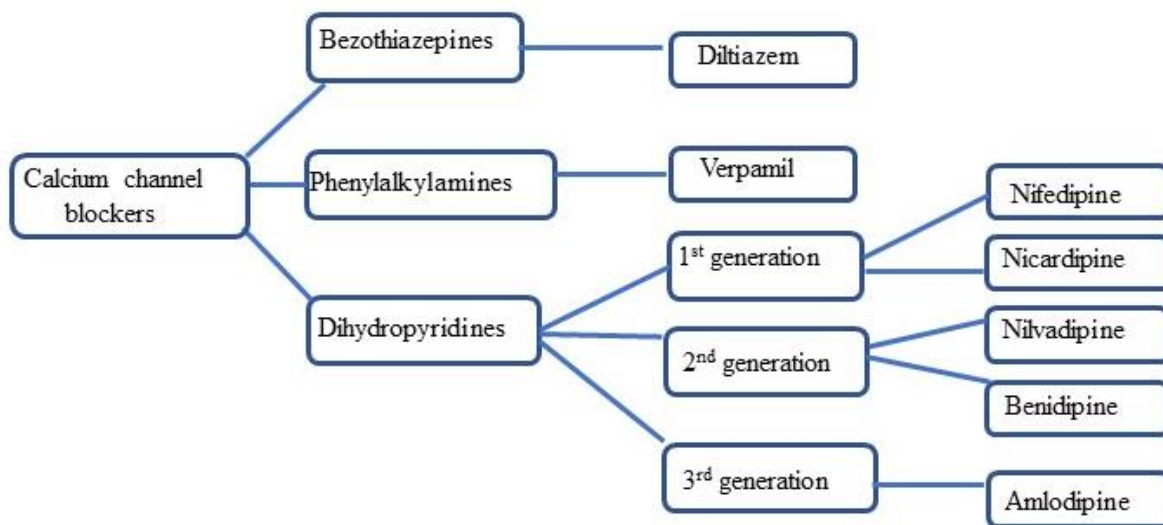


Figure 1. Classification of calcium channel blockers

DHPs are the most widely used clinical drugs for the management of hypertension. Based on the timing of their discovery and duration of their actions, DHPs are categorized into three generations. The first-generation DHP, nifedipine, was clinically introduced in the 1960s against hypertension, and exhibited showed powerful hypotensive action. However, it was also reported that nifedipine activates sympathetic neurotransmitters due to its short-acting vasodilating action and had negative inotropic effects by blocking L-type Ca^{2+} channels in myocytes. Clinical side effects by nifedipine on sympathetic activity are related not only to baroreflex responses, but also to stimulation of the renin-angiotensin system (RAS) [29]. Due to these adverse effects, clinical use of first generation nifedipine is limited. The second generation CCBs are nilvadipine and benidipine, which enabled better control of the therapeutic BP reducing effects and reduced tachycardia. The third generation DHP, amlodipine is more lipophilic with a longer action than second-generation agents, and more slowly induces of vasodilator action and is less cardio-selective; therefore, it is well tolerated by patients with heart failure [30,31]. However, several studies reported that long-term treatment using the slow release formula of amlodipine reduced the BP with an increased noradrenaline concentration via the sympathetic reflex [32].

Recent, fourth generation CCBs include the highly lipophilic dihydropyridine compound cilnidipine [33]. Cilnidipine provides therapeutic effects by reducing the BP and adverse effects, especially in those with ischemic arrhythmia and chronic kidney diseases due to its L/N type calcium channel blocking activity [31,34,35].

Distribution of L/N type calcium channels and characteristics of cilnidipine

Distributions of N-type Ca^{2+} channels are shown in Figure 2. N-type Ca^{2+} channels are abundantly expressed in the nervous system particularly in peripheral and central sympathetic nerve endings and mediate the fast release of noradrenaline and other neurotransmitters [33,36,37]. Noradrenaline released from sympathetic nerve endings constricts vessels, reflects positive inotropic and chronotropic effects in the heart, and constricts efferent arterioles of the glomerulus. Cilnidipine is a recently developed CCB and inhibits sympathetic N-type Ca^{2+} channel and vascular L-type Ca^{2+} channels activity. Cilnidipine was reported to suppress the excessive excitation of the sympathetic nervous system by blocking N-type Ca^{2+} channels and reducing the release of noradrenaline from sympathetic nerve endings, consequently suppressing the RAS. It was also reported to suppress renal sclerosis in hypertensive patients more than the L-type CCB amlodipine via the reduction of glomerular hypertension by vasodilation of efferent arterioles [35,38–41]. Moreover, cilnidipine suppresses ischemia/reperfusion-induced arrhythmia via the suppression of cardiac sympathetic activation [42].

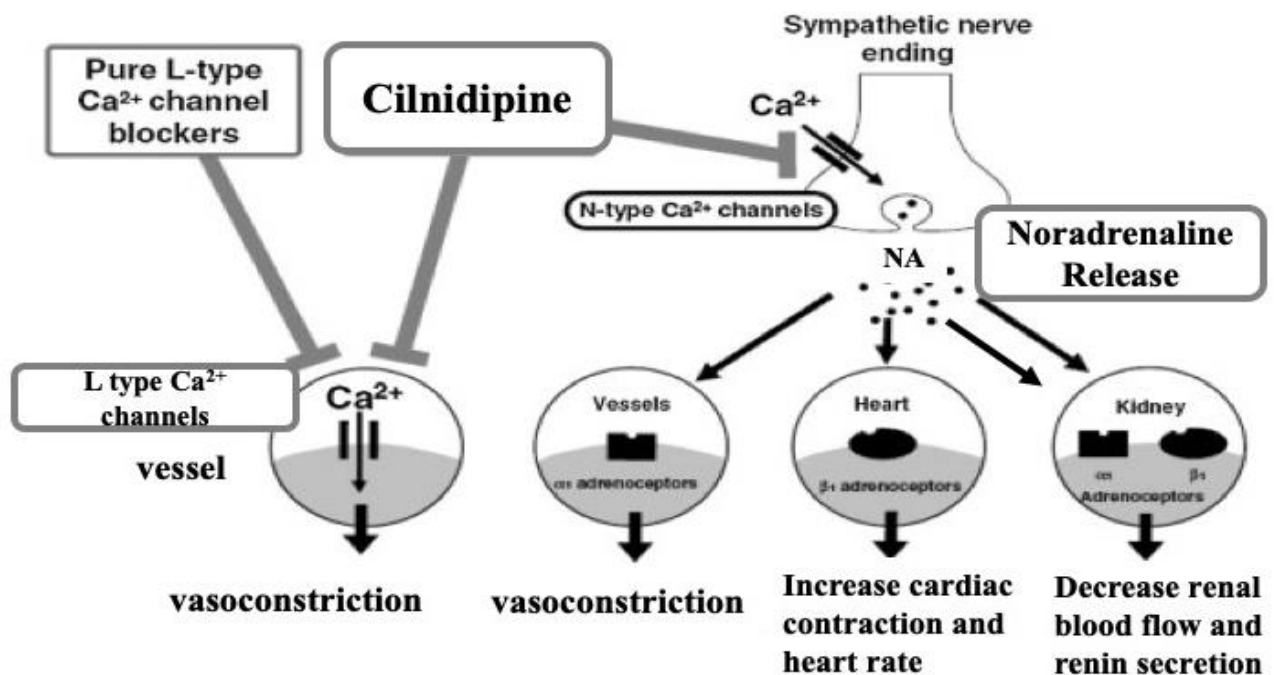


Figure 2. Distribution and roles of L- and N- type calcium channels. *Indian Heart J.* 2013; 65(6): 691–695, figure 1.

Pathophysiology of myocardial infarction (MI) and remodeling after MI

Myocardial infarction (MI) is the term used to describe irreversible myocardial necrosis resulting from the cessation of coronary blood flow to a specific area of the myocardium, and is the main cause of heart failure. Cardiovascular death is the second-leading cause of death in Japan and the risk of post-MI heart failure is higher in hypertensive patients than normotensive patients [12]. After MI, physiological and pathological changes occur, termed post-infarct LV remodeling. It is defined as a change in size, shape, and function of the heart after MI. This remodeling is influenced mainly by hemodynamic load and neurohormonal activation. After cessation of coronary blood flow, the process of cardiac remodeling begins within a few hours as myocyte necrosis. In the early phase of injury within several days, infarct wound healing by necrotic tissue resorption and granulation formation occurs in the infarct region. In the later phase within several weeks after MI, left ventricular dilatation by the expansion of the infarct area, leads to chronic heart failure with myocyte hypertrophy and interstitial fibrosis in non-infarcted myocardium [43–46] (Figure 3).

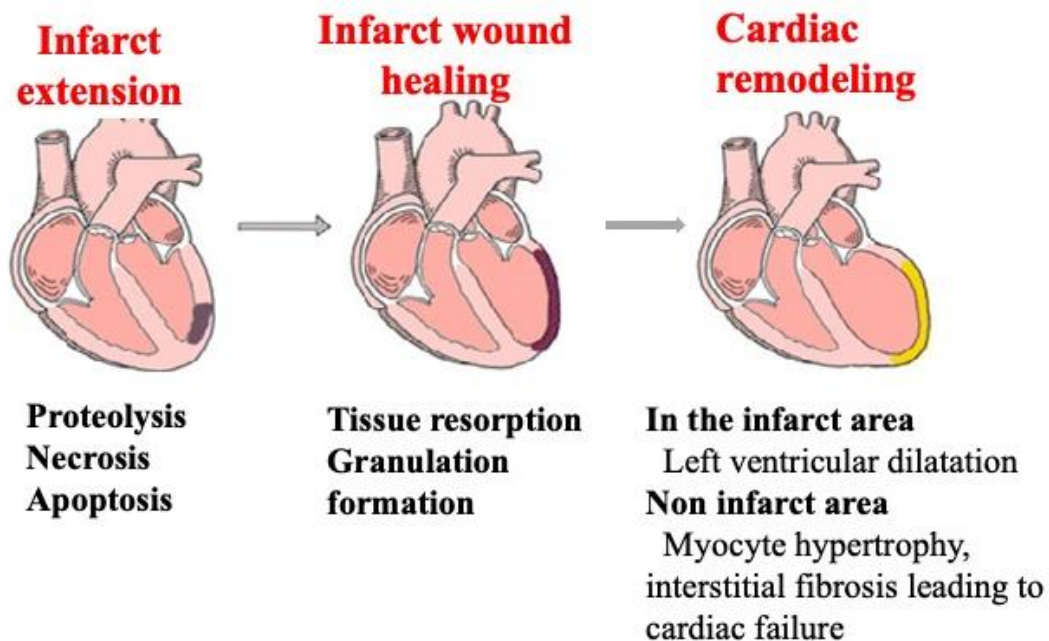


Figure 3. Schema of cardiac remodeling after MI. *J Biomed Sci.* 2017; 24: 13. Figure 1 a (partly modified)

MI and post-infarct LV remodeling are the major causes of death in Western countries and Japan [47]. In Japan, the prevalence of heart failure is rapidly increasing and cardiovascular diseases account for approximately 25% of deaths [4,7]. According to the Japanese Registry of All Cardiac and Vascular Diseases (JROAD), the annual number of patients hospitalized for with heart failure increased by 22% from 212,793 in 2012 to 260,157 in 2016 (acute myocardial infraction (AMI) increased by 6% during the same period) [48]. The number of patients with heart failure increases continuously, reaching 1.3 million by 2030, referred to as a pandemic of cardiac failure in Japan [49]. According to the guidelines for the management of acute MI by the Japanese Circulation Society, after MI ACEIs and ARBs are generally administrated for BP control because they suppress post-infarct LV remodeling and reduce mortality rates [50,51]. In addition, other clinical studies demonstrated that the short acting nifedipine increased the mortality rate in patients with ischemic heart diseases, particularly acute MI because of hypotension and reflex sympathetic activation with tachycardia [52]. According to these clinical studies, short-acting CCBs are defined as harmful drugs after MI. On the other hand, in recent animal studies on long acting CCBs, amlodipine and azelnidipine, attenuated post-infarct LV remodeling in normotensive animals [53–56]. However, the effects of long-acting CCBs on post-infarct LV remodeling in hypertensive animals remain unknown.

To clarify the effects of long acting CCBs on post-infarct LV remodeling, we investigated whether continuous hypertension treatment using the long-acting CCBs cilnidipine and amlodipine, attenuates LV remodeling after MI in spontaneously hypertensive rats (SHRs).

Chapter 1: Effects of Calcium Channel Blockers on Post-Infarct Cardiac Function

1. Background

Hypertension is a well-established independent risk factor for cardiovascular diseases [10]. In most developed and developing nations, approximately 20 to 30% of the adult population has a high BP and the prevalence increases with age [57]. Although hypertension increases the risk of cardiovascular diseases, pharmacological treatment is beneficial for achieving long-term reduction of the BP in association with the reduction of the risk of cardiovascular events [58]. Therefore, clinically, an important goal of antihypertensive therapy is to reduce the BP to the target level and prevent the cardiovascular events. The target BP is defined as a reduction of the systolic/diastolic BP of at least 20/10 mmHg and BP <130/90 mmHg in Japan. CCBs are potent vasodilators widely used in the management of hypertension which reduce the BP and prevent the hypertensive cardiac dysfunction [59,60]. In previous animal studies, the range of amlodipine and cilnidipine doses was approximately 1 to 10 mg/kg/day [39,61]. The BP reducing effects of amlodipine and cilnidipine at 1 mg/kg/day depended on the animal models, [62] and 5-10 mg/kg/day was sufficient to reduce the BP [63]. Based on these previous reports, we used amlodipine and cilnidipine 10 mg/kg/day in this study [64].

After MI, according to the progression of LV dysfunction, the chamber size of the LV dilates due to the Frank-Starling law. An increased LV diastolic diameter and left ventricular weight indicate worsening of post-MI remodeling [15,16,65]. The progression of cardiac dysfunction increases the incidence of sudden death and congestive heart failure [66]. LV failure is characterized by lung congestion and a high level of LV preload. The ratio of O₂ saturation is a non-invasive and useful indicator of lung congestion in patients with heart failure. On the other hand, in animal studies total lung weight is usually used as an index of lung congestion. The LV pre-load and severity of LV dysfunction reflect LV pressure at the end-diastolic phase (LVEDP). Therefore, LVEDP is usually measured as a useful indicator of the severity of heart failure in human and animal studies. Left ventricular function consists of systolic and diastolic functions. Pressure-overload, such as hypertension, increases LV wall thickness, which impairs diastolic function in the early phase [67]. On the other hand, acute MI rapidly reduces LV systolic function according to the necrosis of the ischemic LV region. In animal studies, the percentage of LV fractional shortening (LVFS (%)), LV ejection fraction, and maximum rate of LV pressure development ($+dP/dt_{max}$) are measured as an index of LV systolic function, and time constant of isovolumic pressure decay (τ) is measured as an index of LV diastolic function. This chapter includes the effects of cilnidipine and amlodipine on BP and post-infarct cardiac function.

2. Materials and methods

The procedures used conformed to the *Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health* and were approved by the Animal Care and Use Committee of Kyoto Pharmaceutical University, Kyoto, Japan.

2.1 Animals

Male SHRs aged 17 weeks and weighing 200 g (Japan SLC., Hamamatsu, japan) were acclimated for at least 1 week before the initiation of the experiment in a temperature ($23 \pm 1^{\circ}\text{C}$) and humidity-controlled room (65+5%) under a 12-hour light/dark cycle.

2.2 Surgical procedure

Experimental MI was performed by intraperitoneal injection of pentobarbital (30 mg/kg). After endotracheal tube intubation, controlled ventilation with room air using a rodent respirator was performed. Thoracotomy was performed at the level of the fourth intercostal space. The rat heart was exposed and a 6-0 silk suture with a needle was passed under the left coronary artery (Figure 4). Then, both ends of the suture were passed through a polyethylene tube (PE-50) and the coronary artery was occluded using this tube [68]

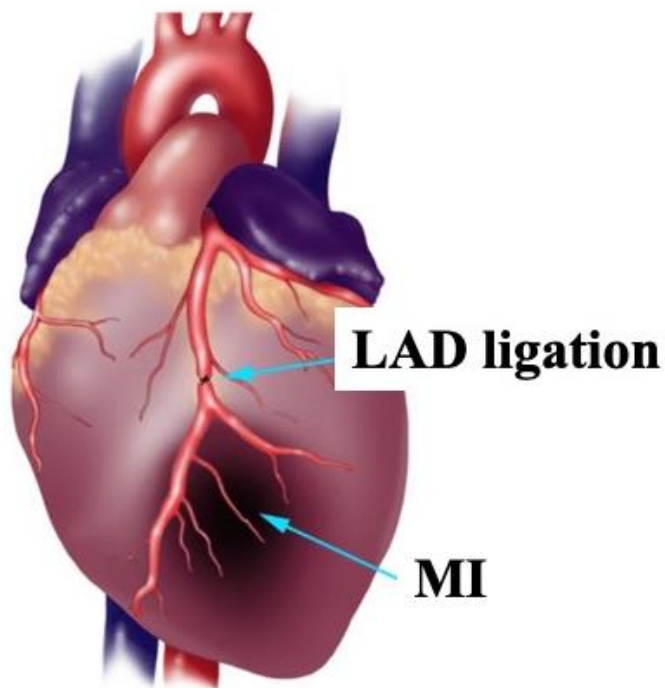


Figure 4. Ligation of the coronary artery in the experimental model *J Vis Exp.* 2011; 48: 2464. **Figure 1.**

2.3 Protocols

The experimental design of the study is shown in Figure 5. Rats were randomly divided into 4 groups: (1) Sham group - suture around the coronary artery was not tied, (2) MI group - MI without treatment, (3) cilnidipine-treated MI group, and (4) amlodipine-treated MI group. MI was performed by 30 minutes occlusion of the left coronary artery, followed by reperfusion. Cyanosis of the anterior left ventricle indicates the success of coronary occlusion. After 30 minutes of occlusion, the occluder was removed and the chest wall was immediately closed using sutures. In sham-operated animals, the coronary artery was not occluded. Cilnidipine (10 mg/kg/day; MI + Cil group) or amlodipine (10 mg/kg/day; MI + Aml group) was administered 1 week before surgery and continued for 5 weeks orally by gavage. Cilnidipine and amlodipine were both dispersed in 0.5 w/v (%) aqueous carboxymethyl cellulose (CMC), and a similar volume of CMC was administered by gavage as a placebo to sham-operated animals and untreated MI animals. Four weeks after surgery, rats were anaesthetized by intraperitoneal injection of urethane (1.2 mg/kg), and subjected to echocardiography and hemodynamic analyses. After euthanasia, the heart and lungs were excised for analyses. The BP was measured by the tail cuff method one week before the surgery and continued every week until the sacrifice. The BP was measured by the tail cuff method one week before the surgery and continued every week until the sacrifice.

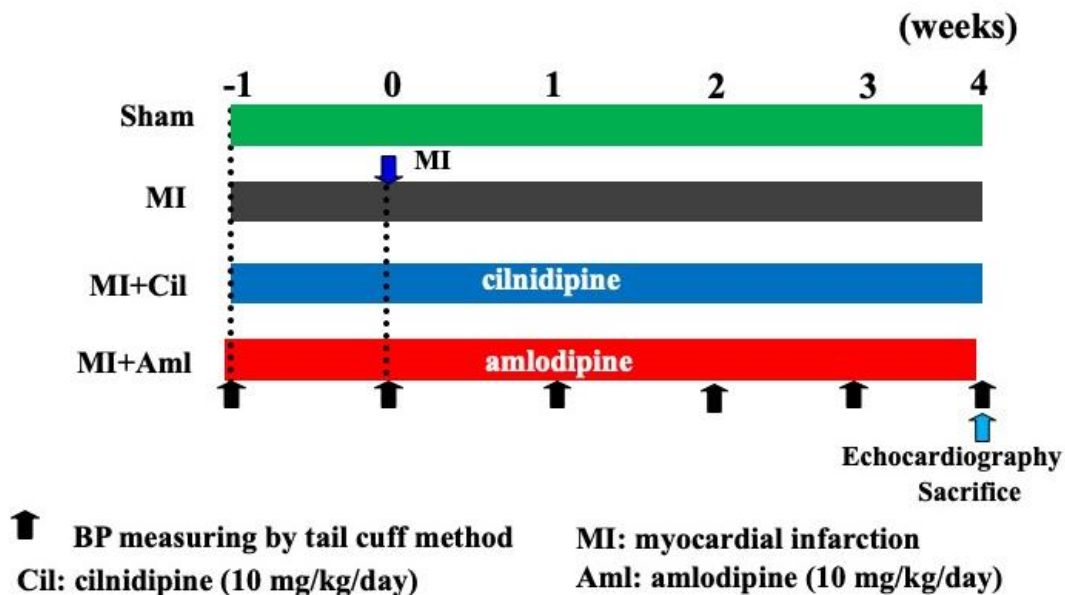


Figure 5. Experimental protocol

2.4 Echocardiographic studies and hemodynamic measurements.

Four weeks after surgery, transthoracic echocardiography was performed using a 15-MHz sector scan probe (SONOS 5500; Phillips Medical Systems Japan). This procedure was carried out under anesthesia with animals breathing spontaneously. The M-mode and B mode recordings were obtained from the short-axis view of the LV at the level of the papillary muscle (Figure 6, 8). The LV end diastolic diameter (LVEDd) and end-systolic diameter (LVESd) were measured on M mode tracing. LVFS was calculated as $LVFS (\%) = 100 \times ((LVEDd - LVESd) / LVEDd)$. Three beats were averaged for each measurement. Then, the LVEDP, LV +dP/dtmax, and tau were measured using a 1.6-F catheter-tip pressure manometer (Transonic Scisense Inc. Ontario, Canada). The catheter-tip was inserted through the right carotid artery.

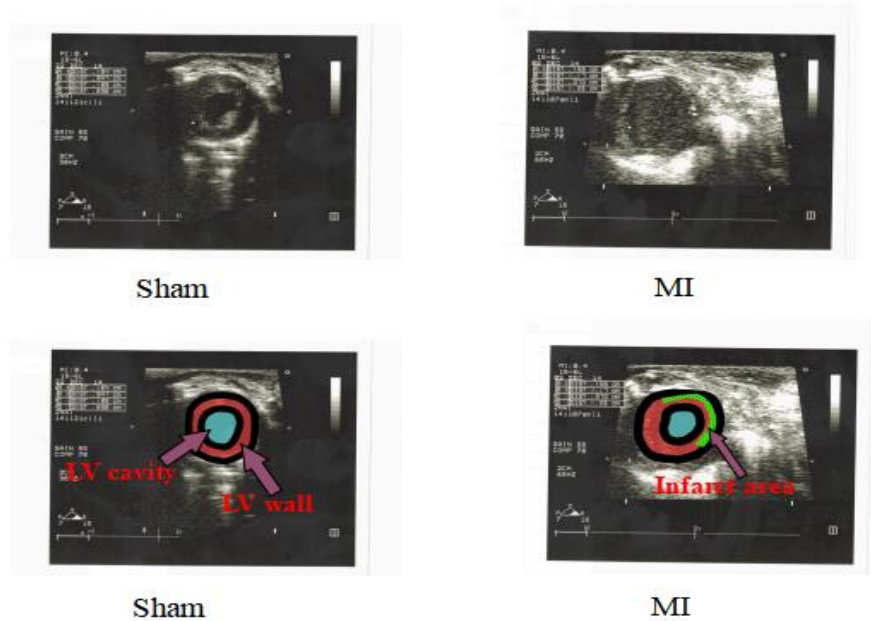


Figure 6. B mode echocardiography of the sham and MI

2.5 Statistical analysis

All data were expressed as the mean \pm SEM. The statistical analysis was performed using computer software (Stat View 5.0, SAS Institute Inc., North Carolina, USA). Inter-group comparisons (at the end of the study period) were performed using a one-way analysis of variance (ANOVA) for multiple comparisons, followed by Fisher's protected least significant difference (PLSD) test. The time course changes in BP were analyzed by a repeated-measure ANOVA with Fisher's PLSD test. P values < 0.05 were considered to be significant.

3 Results

3.1 Heart rate, body weight, and organ weights

The body and organ weights. of experimental animals 4 weeks after surgery are shown in Table 1. There were no significant differences in body weight among all groups. The ratios of heart weight to body weight and LV weight to body weight were significantly increased in all MI groups. Cilnidipine, but not amlodipine, significantly attenuated these increases in ratios. The ratio of lung weight to body weight was also increased in all MI groups and was slightly reduced by cilnidipine treatment. The heart rate slightly increased after MI in the untreated MI group in comparison with the sham group. Four weeks after surgery, the cilnidipine-treated MI group had a slightly lower the heart rate than the amlodipine-treated MI group.

Table 1. Body and organ weights, infarct size, and hemodynamic data of experimental groups

	Sham	MI	MI+Cil	MI+Aml
HR(/min)	328.9±8.5	336±15.8	307.1±10.2	330.1±10.2
BW(g)	373.8±6.4	368.5±3.5	369.1±5.5	359.4±2.7
HW/BW (mg/g)	3.7±0.1	5.6±0.2*	5.0±0.1*#	5.4±0.2*
LVW/BW (mg/g)	2.8±0.1	3.8±0.4*	3.3±0.2*#	3.5±0.1*
LW/BW (mg/g)	5.0±0.4	7.0±0.3*	6.6±0.1*	6.9±0.5*

* $p < 0.01$ versus. Sham, # $p < 0.01$ versus. MI, n=6-10. Sham, sham-operated group; MI, untreated MI group; MI+Cil, cilnidipine-treated MI group; MI+Aml, amlodipine-treated MI group. BW, body weight; HW, heart weight; LVW, left ventricular weight; LW, lung weight; HR, heart rate. **Clin Exp Pharmacol Physiol.** 2020; 00:1-9 table 1

3.2 Time course changes in SBP

The time course changes in SBP are shown in Figure 7. After surgery the SBP decreased in all MI groups irrespective of the treatments because of reduction of cardiac function. This BP decrease is temporary phenomenon and after the compensation of the cardiac function the BP remained high in the untreated MI group. The treatments with the CCBs, amlodipine and cilnidipine, both reduced the SBP to a similar extent in compare with the sham group and MI group.

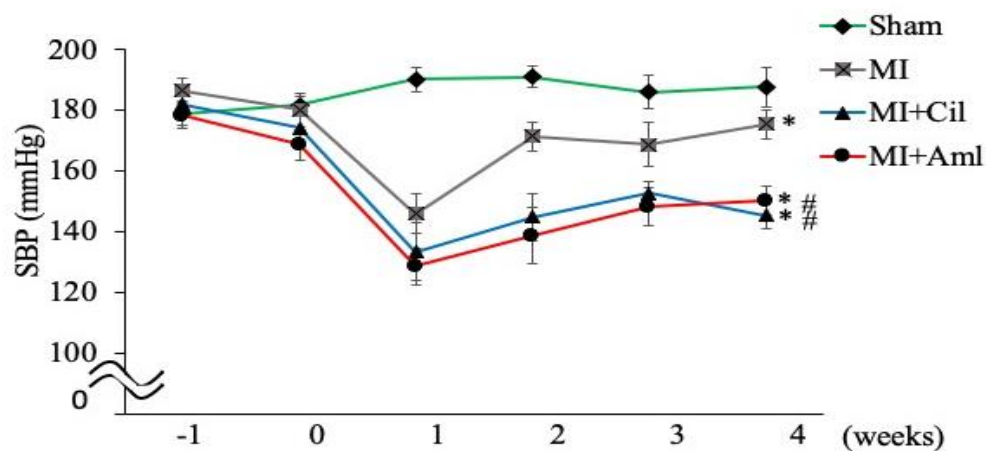


Figure 7. Time course changes in SBP during the experimental period. SBP was measured using tail-cuff method. Amlodipine or cilnidipine was orally administered during the experimental period. Rats were subjected to 30 minutes of coronary occlusion followed by reperfusion at 0 week in all MI groups (n=10–16). * $p < 0.05$ versus Sham, # $p < 0.05$ versus MI. **Clin Exp Pharmacol Physiol. 2020;00:1-9. Figure 1.**

3.3 Echocardiographic assessments

Echocardiographic assessments of LV geometry and function 4 weeks after surgery are shown in Figure 8. Representative M-mode echocardiography revealed marked LV dilatation and impaired of LV systolic function in MI rats (Figure 8 (A)). Cilnidipine, but not amlodipine, significantly attenuated the dilatation of LV end-diastolic diameter (LVEDd) and aggravation of LVFS (Figure 8 (B, C)).

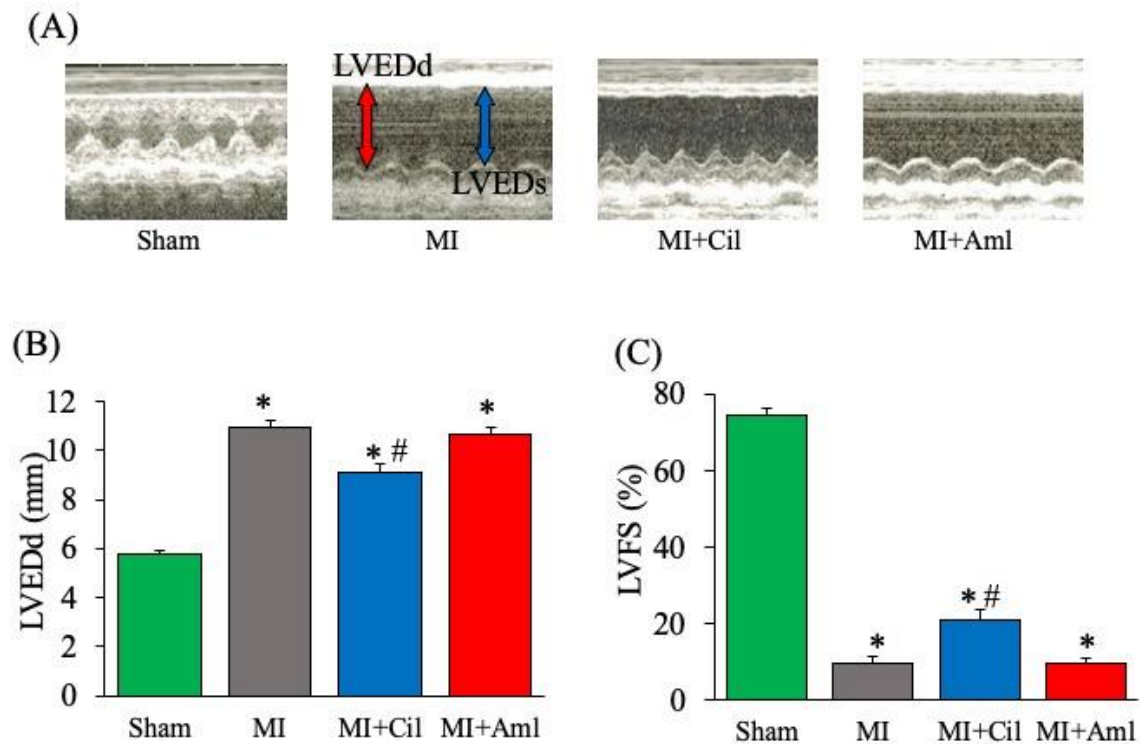


Figure 8. Effects of cilnidipine and amlodipine on echocardiographic measurements 4 weeks after MI. Representative M-mode echocardiography (A). Bar graphs indicate left ventricular end-diastolic diameter (LVEDd) (B) and left ventricular fractional shortening (LVFS) (C) (n=8-10). * $p < 0.05$ versus Sham, # $p < 0.05$ versus MI. *Clin Exp Pharmacol Physiol.* 2020;00:1-9. **Figure 2.**

3.4 Hemodynamic analysis

Hemodynamic data of LV 4 weeks after MI are shown in Figure 9. LV $+dP/dt_{\max}$ is the maximal rate of rise of LV pressure and another index of systolic function. LV $+dP/dt_{\max}$ significantly decreased in all MI groups. Among MI groups, only the cilnidipine-treated group had a slightly increased LV $+dP/dt_{\max}$. Tau, an indicator of diastolic function, increased in the untreated MI group, and was significantly improved only by cilnidipine. LVEDP, an index of LV preload and severity of heart failure, increased in the untreated MI group. Cilnidipine, but not amlodipine, significantly attenuated the increase in LVEDP.

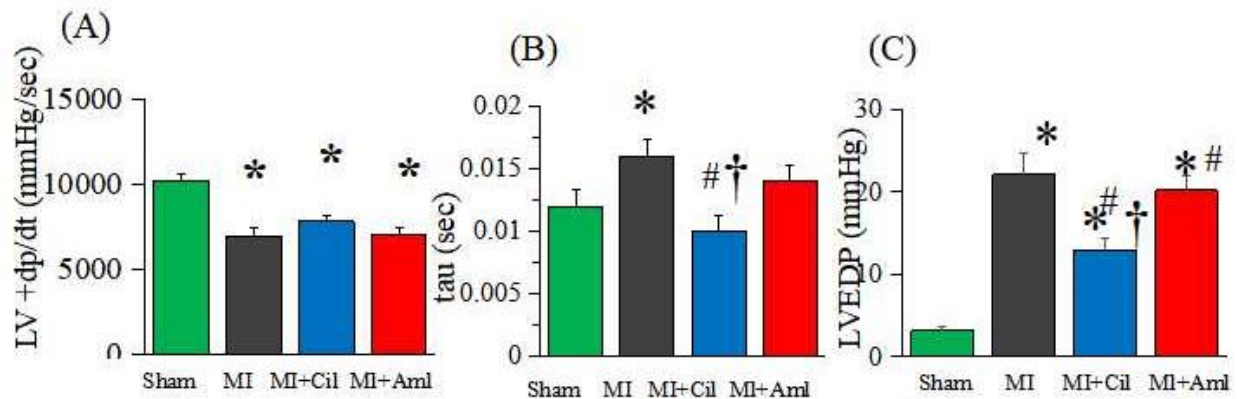


Figure 9. Effects of cilnidipine and amlodipine on hemodynamic measurements 4 weeks after MI. Bar graphs indicate maximum rates of LV pressure development (LV $+dP/dt_{\max}$) (A), tau (B) and left ventricular end diastolic pressure (LVEDP) (C). * $p < 0.05$ versus Sham, # $p < 0.05$ versus MI and † $p < 0.05$ versus MI+Aml. Clin Exp Pharmacol Physiol. 2020; 00:1-9. Table 1.

4. Discussion

Clinically, an important goal of antihypertensive therapy is to reduce the blood pressure at target level. Target BP' was defined as a reduction of systolic/diastolic BP of at least 20/10 mm Hg and BP <130/90 mm Hg in Japan. Recent large-scale clinical studies have also reported that CCBs achieved a higher rate of BP control than ACEIs (104) or ARBs (103). In the present study I selected cilnidipine and amlodipine at 10 mg/kg/day to clarify the effects of continuous treatment using a hypotensive dose of these two drugs. Administration of cilnidipine and amlodipine reduced SBP similarly by almost 20 mmHg and the reduction of BP by these CCBs was similar to that in clinical practice [69]. The pharmacokinetics of these two drugs, such as T_{max} and $T_{1/2}$, are differed, so the beneficial effects of cilnidipine may have been partially due to action of these pharmacokinetic differences [70]. On the other hand, cilnidipine reduces BP stably throughout the day similar to amlodipine under once-a-day administration despite its short T_{max} and $T_{1/2}$. this long-lasting hypotensive action is due to its high lipid solubility [71]. In the present model, the reduction of BP was similar between cilnidipine and amlodipine-treated MI groups. Therefore, the degree of inhibition of L-type Ca^{2+} channels, at least in vascular L-type Ca^{2+} channels, may be similar in both CCB-treated MI groups.

Left ventricular hypertrophy and remodeling are frequently seen in hypertensive subjects and result from a complex interaction of several hemodynamic and non-hemodynamic variables. Although increased blood pressure is considered the major determinant for the alteration of the LV mass and geometry. hypertension leads to concentric hypertrophy, as an adaptive response to normalize wall stress, which is then followed by chamber dilation and heart failure. In the present model, sham-operated rats showed remarkable LV wall thickening (data not shown) in echocardiography without dilatation of LV chamber and impairment of LVFS, indicating compensative phase of hypertensive heart disease. On the other hand, MI resulted in a markedly dilated LV chamber, and heart enlargement resulted in increased heart weight to body weight and LV weight to body weight. Cilnidipine but not amlodipine improved morphological remodeling after MI, suggesting attenuation of the severity of post-MI heart failure. On the other hand, the lung weight to body weight ratio significantly increased in the untreated MI group. Cilnidipine slightly reduced this ratio but not significantly. The ratio of lung weight to body weight is a useful index of lung congestion; however, extracted lungs were weighted after cardiac arrest, which may affect lung weight and reduce the sensitivity as an index of lung congestion.

I assessed cardiac function using echocardiography and cardiac catheterization, and measured LVFS and $LV +dP/dt_{max}$ as a LV systolic function, tau as diastolic function. Cilnidipine significantly improved the LVFS reduction, LVEDP increase, and tau prolongation after MI. High level of LVEDP increases the risk for developing clinical symptoms of heart failure. Notably, previous clinical studies have suggested that LVEDP at acute MI may have prognostic significance and LVEDP >18 mmHg has demonstrated to be a predictor of in-hospital mortality [72]. In the present study, cilnidipine suppressed LVEDP less than 18 mmHg, which may contribute to the symptom and mortality reduction in patients with post-MI heart failure. Among the indicators of LV systolic function, cilnidipine significantly improved the reduction of LVFS and led to the recovery of $LV +dP/dt_{max}$. In a previous report using a pacing dog model, the heart rate had a positive impact on $LV +dP/dt_{max}$ [102]. In the present study, the cilnidipine-treated MI group had a markedly slower heart rate, which may reduce the $LV +dP/dt_{max}$; therefore, the improvement of $LV +dP/dt_{max}$ by cilnidipine was not significant.

Chapter 2 Effects of CCBs on Post-infarct Morphological Remodeling and Gene Expression.

1. Background

Microscopic and morphological remodeling of LV progresses after MI. Within one hour after ischemic stimulation, accumulation of intercellular edema is found in the ischemic region. Then, infiltration of neutrophils with progressive coagulative necrosis of myocytes occurs in the ischemic region several hours later. The dead myocytes begin to disintegrate and are removed by macrophages, along with the proliferation of fibroblasts and formation of granulation tissue, which progressively replaces necrotic tissue (Figure 10) [73]. In addition to expansion of the infarct region due to the loss of necrotic myocytes and replacement with granulation tissue, remodeling in the non-infarct region progresses due to mechanical stress and neuro-humoral factors such as BP, noradrenaline, and angiotensin II. In this process in the non-infarct region, myocyte hypertrophy and interstitial fibrosis develop. Myocyte hypertrophy is known as a compensatory growth in the early phase of remodeling; however, continuous stimulation of growth signals induces oxidative stress and apoptosis in myocytes and further increases fibrosis, resulting in impaired cardiac function [74,75]. In addition to histological remodeling after MI, related gene expression is also altered [76,77]. Brain natriuretic peptide (BNP) is produced by left ventricular myocytes and its mRNA expression is usually used as an indicator of myocyte hypertrophy [78,79]. Collagen fibers are the major components in interstitial fibrosis in the non-infarct region. Therefore, mRNA expression of collagen III estimates fibrosis levels.

In this chapter, I describe the effects of CCBs on post-infarct histological remodeling and related gene expression in the non-infarct region.

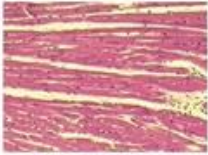
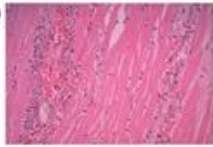
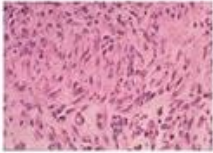

Time	Microscopic feature	
0-24 hr		Begins to undergo irreversible injury, release of necrotic cell.
1-3 days		Extensive coagulative necrosis. Tissue surrounding infarct shows acute inflammation with neutrophil.
3-14 days		Macrophage phagocytes necrotic tissue and replace with granulation tissue.
2 weeks to several months		Contracted scar complete.

Figure 10. Histological changes after MI. Nature Biotechnology. 2005; volume 23, 846, figure 1 (partly modified)

2. Materials and methods

2.1 Animals and experimental protocol

The procedures were performed in strict accordance with the *Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health* and were approved by the Animal Care and Use Committee of Kyoto Pharmaceutical University, Kyoto, Japan. Male SHR_s weighing 200 g and aged 17 weeks (Japan SLC., Hamamatsu, Japan) were acclimated to the standard laboratory conditioning with a 12-hour light/dark cycle in a temperature ($23 \pm 1^{\circ}\text{C}$) and humidity-controlled room (65+5%) at least 1 week before the initiation of the experiment. MI was performed following the same protocol and assessment as in Chapter 1.

2.2 Sample collection and histological analysis

Four weeks after surgery hearts were excised. The LV was separated from the right ventricle and weighted. The LV was cut transversely at the middle level. Then, it was fixed in 4% paraformaldehyde phosphate buffer for one day and embedded in paraffin wax. Tissue samples were sliced at a thickness of 4 μm and stained with hematoxylin-eosin (HE), Sirius Red, and Masson's Trichrome. In each MI animal, the percentage of the infarct size was assessed planimetrically using HE-stained specimens as the ratio of infarct tissue or scar tissue to the entire LV endocardial circumference [80]. Cardiac myocyte sizes were assessed using HE stained sections by measuring of the cross-sectional area of myocytes. To measure cross-sectional myocyte areas, a suitable area of the section was defined as one with circular capillary profiles and myofiber shapes (indicative of a true transverse section). Then, the circumferences of 300 myocytes were traced, and pixels inside were counted. Interstitial fibrosis was analyzed by assessing collagen deposition on Sirius Red-stained and Masson's Trichrome-stained sections. Pink-red pixels in Sirius Red-stained sections and blue pixels in Masson's Trichrome-stained sections were counted, as the interstitial fibrosis area, and the fibrosis ratio was calculated as the sum of the fibrosis area divided by the sum of all connective tissues and muscle areas in the field. Five independent fields of the myocardium from each rat were photographed using an optical microscope system (Olympus IX71; Olympus Japan).

2.3 RNA extraction and real-time PCR for BNP and collagen type III

The mRNA expression of BNP, collagen type III was assessed by real-time PCR, as described previously [74]. Total RNA was extracted from the non-infarct region using Isogen II reagent (NIPPON GENE, Tokyo, Japan) according to the manufacturer's instructions. Reverse transcription was performed to synthesize complementary DNA (cDNA) from 0.5 µg of total RNA using a reverse transcription kit (PrimeScript RT Master Mix, Takara Bio Inc., Shiga, Japan). The cDNA fragments were amplified by PCR using the SYBR Premix Ex Taq II kit (TaKaRa, Shiga, Japan). The following cycling parameters were used: initial denaturation at 95°C for 30 s, 45 cycles of denaturation at 95°C for 5 s, annealing at 58°C for 10 s, and extension at 72°C for 30 s. Expression levels were normalized to that of GAPDH (glyceraldehyde-3-phosphate dehydrogenase). The delta-delta Ct method was used to calculate all data and perform comparisons with the control group. Sequences for the relevant primers are shown in Table 2

Table 2. Primer sequences used in the real-time PC analysis

Biomarkers	Gene	Forward (5' – 3')	Reverse (5' – 3')
Reference gene	GAPDH	GGCACAGTCAAGGCTGAGAATG	TGCCCAAAGCAGCTTGAAC
Myocyte hypertrophy	BNP	TTCCTTAATCTGTCGCCGCT	AAGCAAACAGGGCCAATGTCCA
Interstitial fibrosis	Collagen-III	ACATGGATCAGGCCAATGGCAA	AAGCAAACAGGGCCAATGTCCA

PCR, polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; BNP, brain natriuretic peptide

2.4 Statistical analysis

Stat View software version 5.0 (SAS Institute) was used for statistical analyses. All data are expressed as the mean \pm SEM. Inter-group comparisons (at the end of the study period) were performed using a one-way analysis of variance (ANOVA) for multiple comparisons, followed by Fisher's protected least significant difference (PLSD) test. A P value <0.05 was considered to be significant.

3. Results

3.1 Effects of cilnidipine and amlodipine on infarct size

Four weeks after surgery, the myocardial infarct size was approximately 38% in the untreated MI group. Both CCBs did not affect infarct size (Figure 11).

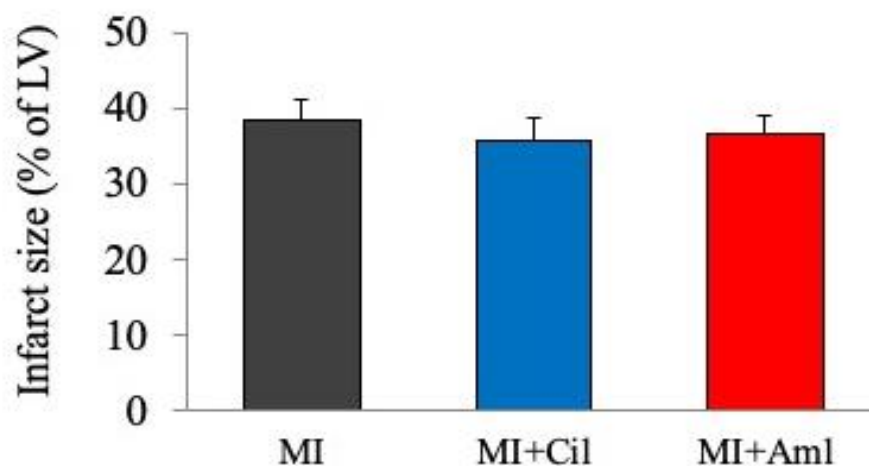


Figure 11. Effects of cilnidipine and amlodipine on the infarct size 4 weeks after MI (n=6–10). *Clin Exp Pharmacol physiol.* 2020; 00:1-9. Table 1.

3.2 Effects of cilnidipine and amlodipine on myocyte hypertrophy

The effects of CCB treatment on myocyte hypertrophy in the non-infarct region 4 weeks after surgery are shown in Figures 12. Representative HE-stained sections of the non-infarct region are shown in Figure 12 (A). The cross-sectional area of myocytes, an indicator of myocyte size, markedly increased in the untreated MI group. Cilnidipine and amlodipine suppressed myocyte hypertrophy similarly (Figure 12 (B)).

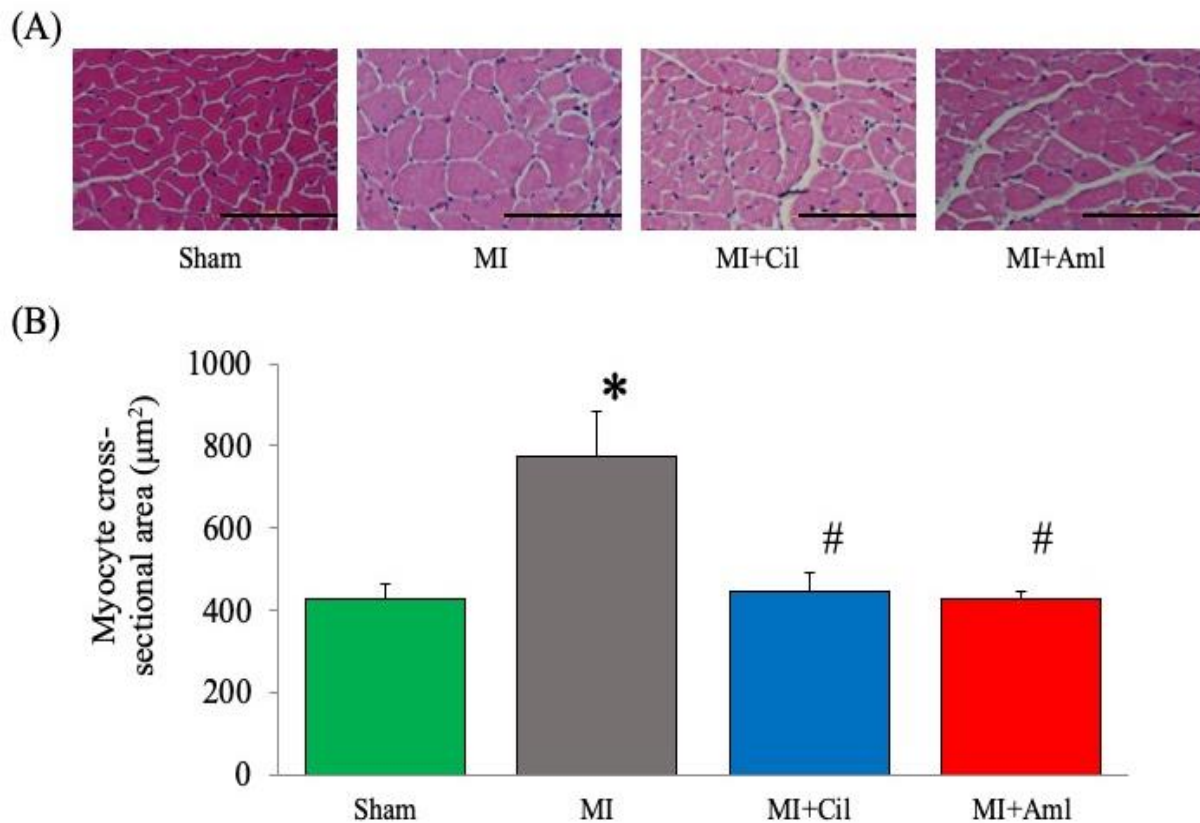


Figure 12. Effects of cilnidipine and amlodipine on myocyte hypertrophy in the non-infarct region 4 weeks after MI. Representative HE-stained sections in the non-infarct region (A). Bars indicate 100 µm. Cross-sectional area of myocytes in the non-infarct region (B) (n=7–8). * p<0.05 versus Sham, # p<0.05 versus MI. Clin Exp Pharmacol physiol. 2020;00:1-9. Figure 3.

3.3 Effects of cilnidipine and amlodipine on interstitial fibrosis

Interstitial fibrosis in the non-infarct region is shown in Figures 13 and 14. Representative Sirius Red-stained sections are shown in Figure 13 (A) and representative Masson's Trichrome-stained sections are shown in Figure 14 (A). After MI, interstitial fibrosis significantly increased in the untreated MI group. Cilnidipine and amlodipine both suppressed interstitial fibrosis, and cilnidipine reduced interstitial fibrosis more than amlodipine (Figures 13 (B), and 14 (B)).

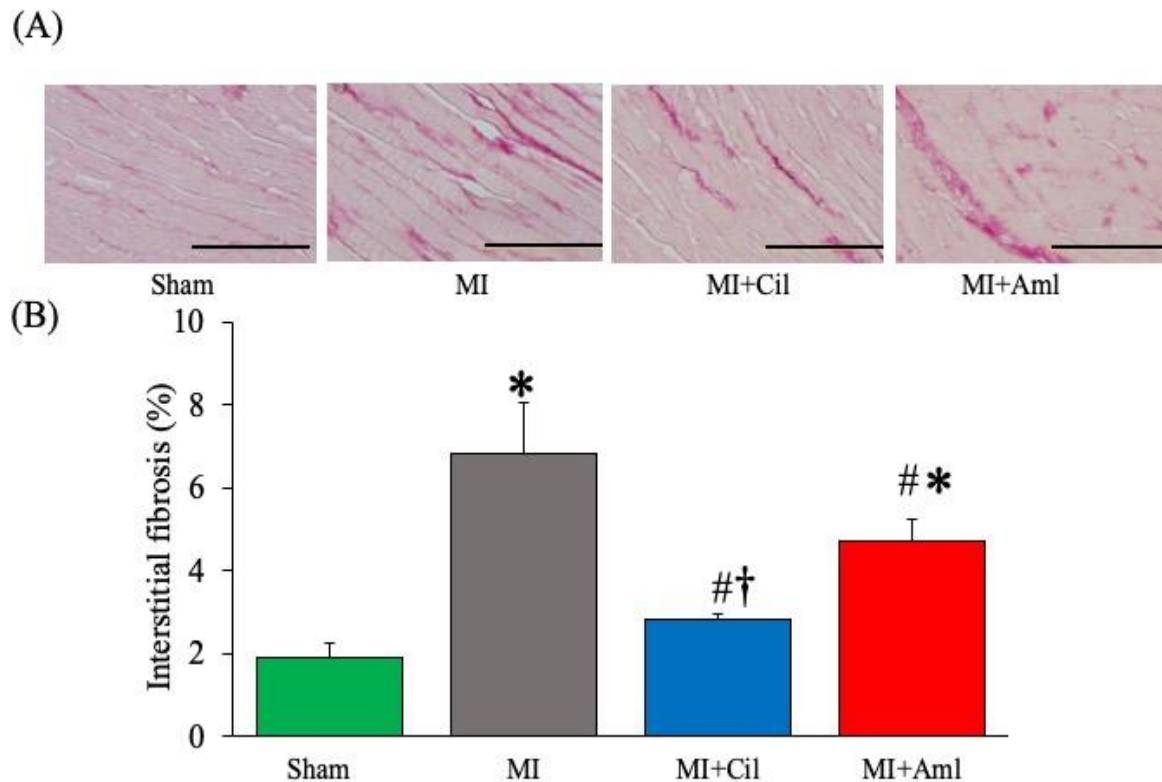


Figure 13. Effects of cilnidipine and amlodipine on myocardial fibrosis in the non-infarct region 4 weeks after MI. Representative Sirius Red-stained sections of the non-infarct region (A). Bars indicate 100 μ m. % of interstitial fibrosis in the non-infarct region (B) (n=7-8). * $p < 0.05$ versus Sham, # $p < 0.05$ versus MI, † $p < 0.05$ versus MI+Aml. *Clin Exp Pharmacol physiol.* 2020; 00:1-9. **Figure 4.**

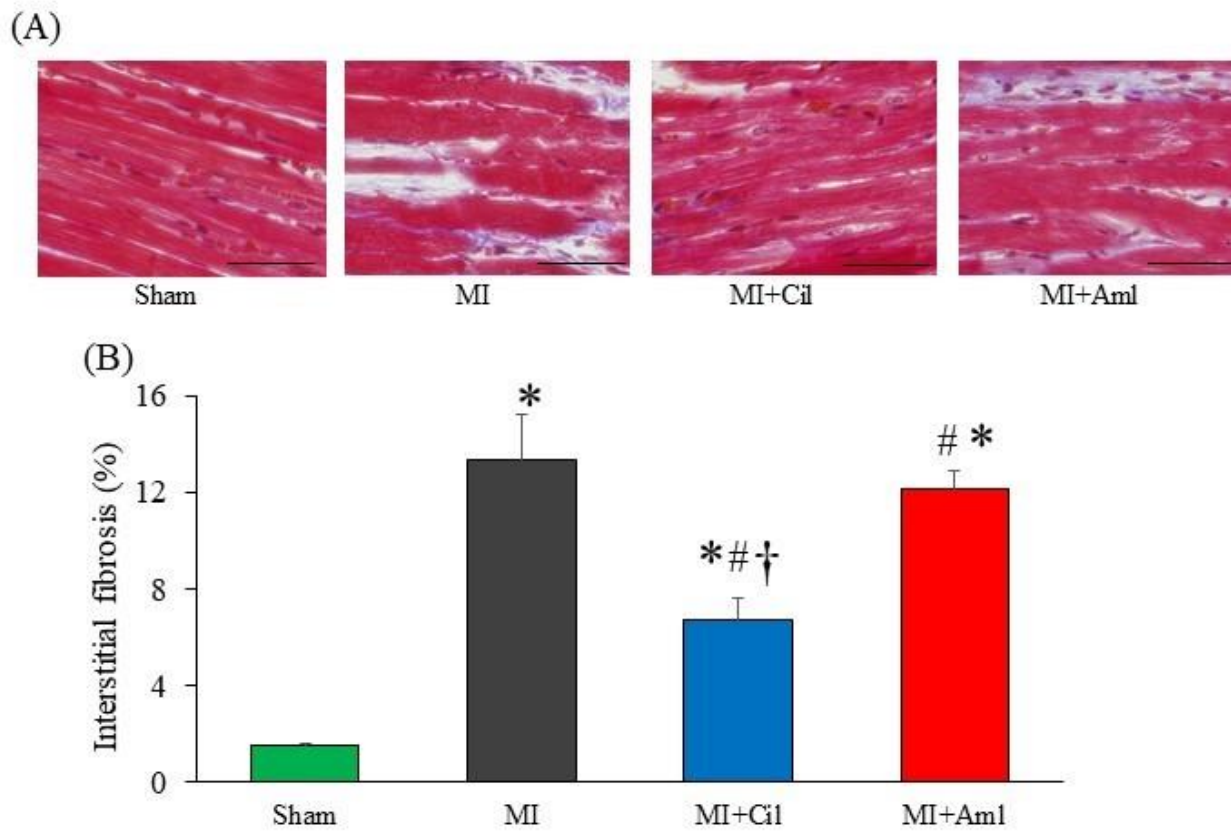


Figure 14. Effects of cilnidipine and amlodipine on myocardial fibrosis in the non-infarct region 4 weeks after MI. Representative Masson's trichrome stained sections of the non-infarct region (A). Bars indicate 100 μ m. % of interstitial fibrosis in the non-infarct region (B) (n=7-8). * $p<0.05$ versus Sham, # $p<0.05$ versus MI, † $p<0.05$ versus MI+Aml. *Clin Exp Pharmacol physiol.* 2020; 00:1-9. **Supplementary Figure.**

3.4 BNP and collagen III mRNA expression in the non-infarct region

BNP and collagen III mRNA expression in the non-infarct region 4 weeks after surgery is shown in Figure 15. The mRNA expression of BNP, an indicator of myocyte hypertrophy, increased in the untreated MI group. Both CCBs reduced BNP mRNA expression to a similar extent (Figure 15 (A)). Collagen III, a major component of fibrosis, was also increased in the untreated MI group, and cilnidipine, but not amlodipine, suppressed its expression (Figure 15 (B)).

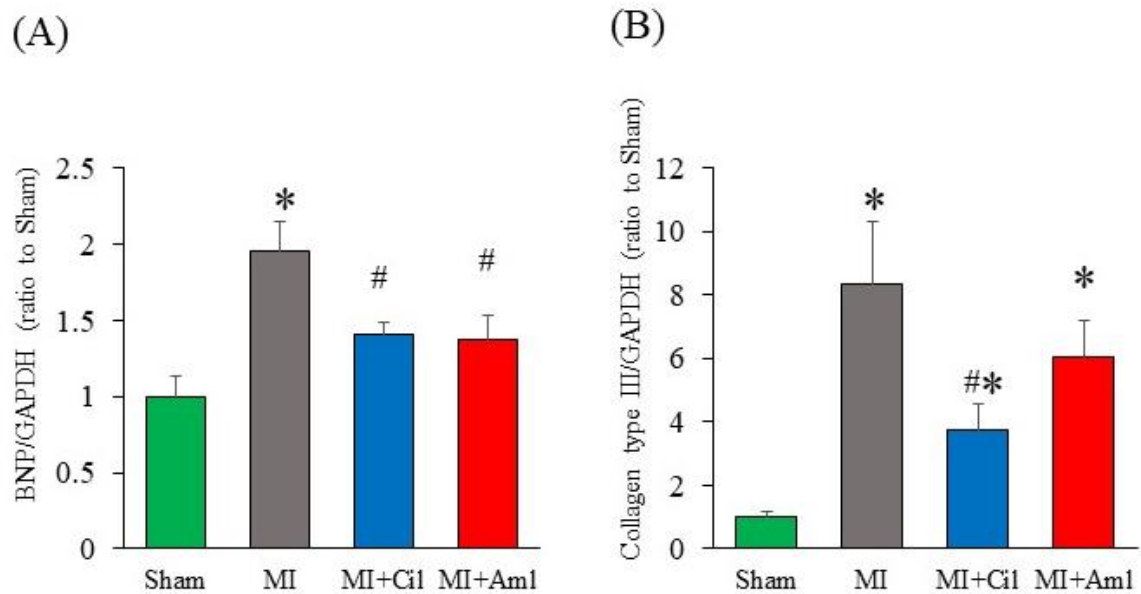


Figure 15. Effects of cilnidipine and amlodipine on the mRNA expression of BNP (A) and collagen III (B) in the non-infarct region 4 weeks after MI (n=7-13). * $p < 0.05$ versus Sham, # $p < 0.05$ versus MI. **Clin Exp Pharmacol physiol.** 2020;00:1-9. **Figure 5.**

4. Discussion

Myocardial infarct size is an important factor to assess post-infarct remodeling and cardiac function. In previous reports, amlodipine reduced the infarct size in normotensive animals [53]. In the present study, the infarct size after 4 weeks of MI was approximately 38% in untreated MI rats, and both CCBs did not affect infarct size. However, in our previous report, the infarct size was smaller by similar ischemia-reperfusion protocols using normotensive animals than in the present hypertensive animals [68]. It is difficult to completely account for the discrepant findings on the infarct size by CCBs among our and other studies, but a large infarct size by hypertension may attenuated the protective action of CCBs.

Myocyte hypertrophy and interstitial fibrosis in the non-infarct region are major histological changes in post-infarct LV remodeling [15,81]. In the present study, myocyte hypertrophy in the non-ischemic region was found in the untreated MI group. Cilnidipine and amlodipine attenuated myocyte hypertrophy similarly. In the post MI remodeling process, mechanical stress, such as mechanical stretching by BP, and humoral factors, such as the RAS, promote myocyte hypertrophy [21,74]. Among these factors, pressure overload is more important for myocyte hypertrophy than angiotensin II based on the report by Baba et.al. that pressure overload by aortic constriction induced myocyte hypertrophy and cardiac fibrosis, whereas an angiotensin II receptor blocker attenuated cardiac fibrosis, but not hypertrophy [82]. As expected, CCB attenuates LV hypertrophy due to BP reduction in stroke-prone hypertensive Rats [83]. In the present study, BP reducing effects by both CCBs were comparable as they both similarly ameliorated myocyte hypertrophy after MI. In addition, BNP is a well-known indicator of myocyte hypertrophy, and is released from ventricular myocytes due to their mechanical stretching and volume overload [78]. Therefore, the suppression of BNP mRNA expression in this study was also similar by both CCBs due to comparable reduction of pressure overload.

Interstitial fibrosis and collagen III mRNA expression were more suppressed in the cilnidipine-treated MI group than in the amlodipine-treated MI group. In the post-infarct remodeling process, major components in the fibrosis region are collagen I and III, and expression of both collagens is similarly regulated. Therefore, I assessed collagen III mRNA expression as an indicator of cardiac fibrosis. Takatsu et al. investigated the effects of cilnidipine and amlodipine on hypertensive cardiac remodeling using Dahl salt-sensitive rats fed a high salt diet. In this hypertensive model, salt-induced increases in SBP and associated myocyte hypertrophy were similarly attenuated by amlodipine and cilnidipine. On the other hand, cilnidipine reduced interstitial fibrosis more than amlodipine [84]. Although the present model was not

simple pressure overload-induced cardiac remodeling, but post-MI remodeling in hypertensive rats, our findings were consistent with this prior report.

I conclude that cilnidipine and amlodipine similarly attenuated myocyte hypertrophy and BNP. Cilnidipine also attenuated interstitial fibrosis and collagen III expression more than amlodipine.

Chapter 3: Mechanism of Differences Between Amlodipine and Cilnidipine

1. Background

In Chapter 1 and 2, I described that BP control with cilnidipine improves post-MI impairment of cardiac function and histological remodeling in SHR. In patients with MI, congestive heart failure due to MI stimulates the RAS and noradrenaline release. In animal experiments, noradrenaline is liberated as a result of the reflex-induced sympatho-neural stimulation, and the angiotensin II concentration in cardiac tissue increases after MI [75,85–87]. In addition, these neuro-humoral factors further exacerbate histological cardiac remodeling of myocyte hypertrophy and interstitial fibrosis [88,89]. Moreover, inhibition of these factors attenuates post-infarct cardiac remodeling [90]. Due to N and L-type Ca^{2+} channel inhibition, cilnidipine attenuated renal noradrenaline release in a hypertensive reno-sclerosis model, leading to suppression of glomerular hypertension [90]. Furthermore, sympathetic β stimulation increases angiotensin II concentrations in plasma after MI [91]. Therefore, we hypothesized that cilnidipine suppresses neuro-humoral factors after MI, leading to greater attenuation of interstitial fibrosis than amlodipine. We examined whether cilnidipine suppresses the cardiac interstitial noradrenaline concentration and production of angiotensin II in cardiac tissue.

2. Materials and methods

2.1 Animals

Animal were treated as described in chapters 1 and 2.

2.2 Experimental protocol

The experimental protocol was the same as in chapters 1 and 2.

2.3 Measurement of myocardial interstitial noradrenaline concentrations using the micro dialysis method.

After echocardiography, several rats were intubated and ventilated by mechanical ventilator. The fifth or sixth rib on the left side was partially removed to expose the heart to measure cardiac interstitial noradrenaline concentrations using the micro dialysis method [92]. A micro dialysis probe (EVAL, 5-mm length, 0.225 mm OD, 0.175 mm ID; Kawasumi Co.) was implanted in the non-infarct region of the LV. The micro dialysis probe was perfused with Ringer's solution at a rate of 1.5 $\mu\text{L}/\text{minute}$ by a microinjection pump. After 120 minutes of stabilization, the dialysate was sampled for 20 minutes (Figure 16). The dialysate samples were frozen at -80°C until analysis. Noradrenaline concentrations in collected dialysates were measured using high-performance liquid chromatography (HPLC) and an electrochemical detection system (ECD-300, Eicom, Kyoto, Japan).

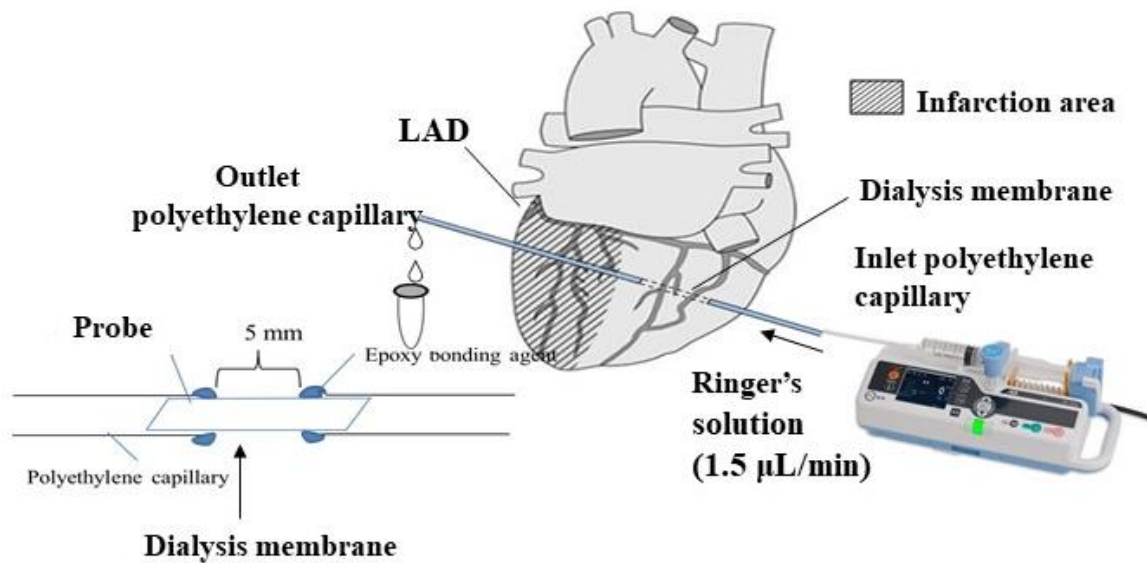


Figure 16. Measurement of the cardiac interstitial noradrenaline concentration using the micro dialysis method.

2.4 RNA extraction and real-time PCR for ACE and TGF β mRNA expression in the non-infarct region

Angiotensin II usually promotes cardiac fibrosis through TGF β production [93–95]. Therefore, I assessed mRNA expression of ACE and TGF β in the non-infarct region 4 weeks after MI using real-time PCR, as described above. After total RNA was extracted from the non-infarct region, reverse transcription was performed to synthesize cDNA from 0.5 μ g of total RNA. The cDNA fragments were amplified by real time PCR using the SYBR green method. Sequences for the relevant primers are shown in Table 3. Expression levels of target mRNA were normalized to that of GAPDH.

Table 3. Primer sequences used in the real-time PC analysis

Biomarkers	Gene	Forward (5' – 3')	Reverse (5' – 3')
Reference gene	GAPDH	GGCACAGTCAAGGCTGAGAATG	TGCCCAAAGCAGCTTGAAC
Profibrotic cytokine	TGF β	CGAGGTGACCTGGGCACCATCCATGAC	CTGCTCCACCTTGGGCTTGCGACCCAC
Renin-angiotensin system	ACE	TTGCAGCCGGGCAACTTTT	CGCATTCTCCTCCGTGATGT

GAPDH, glyceraldehyde-3-phosphate dehydrogenase; TGF β , transforming growth factor β ; ACE, angiotensin converting enzyme.

2.5 ACE activity

ACE activity was measured by the cleavage of hippuryl-L-histidyl-L-leucine (Hippuryl-His-Leu) as described by Hayashi, with modification (49). The frozen cardiac tissue (100 mg) of the non-infarct region was homogenized in 300 μ L of Tris-HCl buffer (10 mmol/L, pH 7.4) including 0.2 mol/L sucrose. After centrifugation, 50 μ L of the supernatant was added to 200 μ L of reaction buffer containing 0.11 mol/L boric acid, 0.9 mol/L NaCl, Na_2CO_3 , and 4.2 mmol/L Hippuryl-His-Leu as a reaction substrate at pH 8.2. After a 120-minutes incubation at 37°C, to stop the reaction, incubated aliquots (50 μ L) were transferred to 500 μ L of NaOH (1 mol/L), and samples were then incubated with 15 μ L 2% *o*-phthaldialdehyde at 37°C for 30 minutes. Thereafter, 500 μ L HCl (1 mol/L) was added and reaction samples were centrifuged at 1,000 g at 4°C for 10 minutes. Fluorescence in the supernatants was measured using a fluorometer (Perkin Elmer Japan, Osaka, Japan). Excitation and emission wavelengths were 360 and 500 nm, respectively. A standard curve was performed with His-Leu (Peptide Res Co., Osaka, Japan). Activity of ACE was expressed as nanomoles of His-Leu formed /minutes. The protein concentrations of the samples were measured using the Lowry method.

2.6 Immunostaining of TGF β in the non-infarct region

The protein expression of TGF β in the non-infarct region was examined using immune-histological staining 4 weeks after MI. Formalin fixed paraffin-embedded LVs were cut into 4- μ m-thick specimens. Deparaffinized sections were placed in an inhibition reagent containing 3% H_2O_2 for 5 minutes to quench endogenous peroxidase activity. After blocking with 10% normal goat serum, sections were incubated at 4°C overnight with an anti-TGF β polyclonal antibody (1:100 dilution, ab92486, Abcam Co., Ltd., MA, USA). Slides were then incubated with a biotinylated goat anti-rabbit IgG antibody for 1 hour, followed by the streptavidin-peroxidase complex for 20 minutes. Immunoreactivity to the primary antibodies was visualized by 3,3'-diaminobenzidine (DAB) and counterstaining was performed using hematoxylin. As a negative control, the primary antibody was omitted. Six different fields in the non-infarcted region from each rat were photographed using the optical microscope system (Olympus IX, Olympus Japan, Tokyo, Japan). We measured the density of TGF β expression using a computer-assisted image analysis program, and the mean density was calculated in each field.

2.7 Statistical analysis

Stat View software version 5.0 (SAS Institute) was used for statistical analyses. All data are expressed as the mean \pm SEM. A one-way analysis of variance (ANOVA) was used to assess the inter-group comparisons (at the end of the study period). For multiple comparisons, Fisher's protected least significant difference (PLSD) test was performed. A P value <0.05 was considered to be significant.

3. Results

3.1 Interstitial noradrenaline concentrations in the non-infarct region

Cardiac interstitial noradrenaline concentrations 4 weeks after surgery are shown in Figure 17. Interstitial noradrenaline concentrations increased in the untreated MI group and were significantly suppressed in the cilnidipine-treated MI group. However, amlodipine did not affect the interstitial noradrenaline concentration.

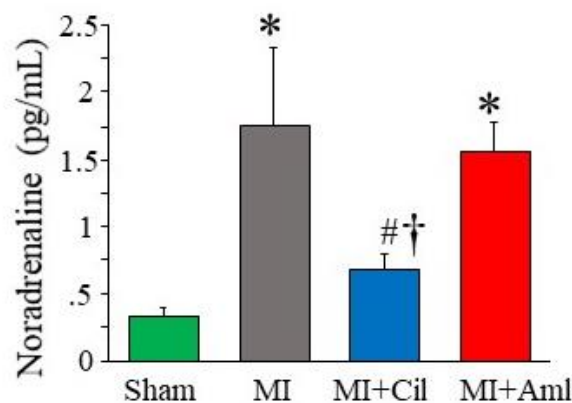


Figure 17. Effects of cilnidipine and amlodipine on interstitial noradrenaline concentrations in the non-infarct region 4 weeks after MI (n=6-12). * $p<0.05$ versus Sham, # $p<0.05$ versus MI, † $p<0.05$ versus MI+Aml. *Clin Exp Pharmacol physiol.* 2020; 00:1-9. Figure 7 C.

3.2 ACE mRNA expression and activity in the non-infarct region

The mRNA expression and activity of ACE, a key regulator of the RAS, in the non-infarct region 4 weeks after MI are shown in Figure 18 (A, B). ACE mRNA expression increased in the untreated and amlodipine-treated MI groups. Cilnidipine significantly reduced this increase to the level of that in sham-operated group. Furthermore, ACE activity in the non-infarct region was stronger in all MI groups than in the sham-operated group. Among MI groups, ACE activity was weaker in the cilnidipine-treated MI group than in the untreated MI group.

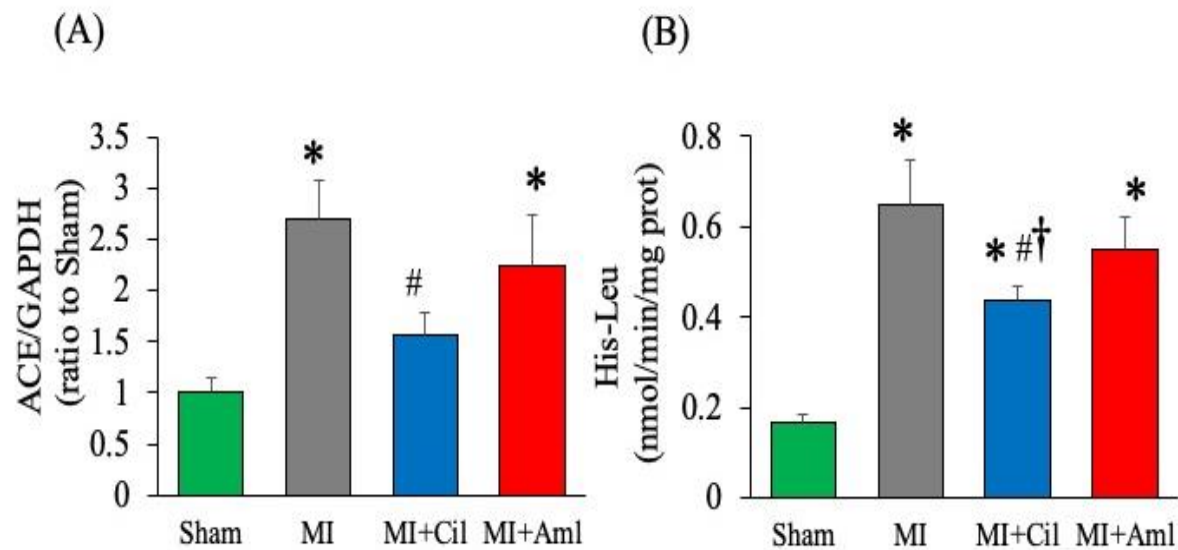


Figure 18. Effects of cilnidipine and amlodipine on ACE mRNA expression (n=13-16) (A) and activity (n=8-10) (B) in the non-infarct region 4 weeks after MI. * $p < 0.05$ versus Sham, # $p < 0.05$ versus MI, † $p < 0.05$ versus MI+Aml. *Clin Exp Pharmacol physiol.* 2020; 00:1-9. **Figure 7 A and B.**

3.3 mRNA and immunohistological protein expression of TGF β in the non-infarct region

The mRNA expression and immunostaining of TGF β , a well-known angiotensin II stimulating profibrotic cytokine, in the non-infarct region 4 weeks after surgery are shown in Figure 19. TGF β mRNA expression increased in the untreated MI group and amlodipine-treated MI group. Cilnidipine significantly attenuated the TGF β mRNA increase (Figure 19 (A)). Representative immunostaining of TGF β in the non-infarct region is shown in Figure 19 (B). The immuno-positive density of TGF β increased in all MI groups, and was suppressed by cilnidipine, but not amlodipine (Figure 19 (C)).

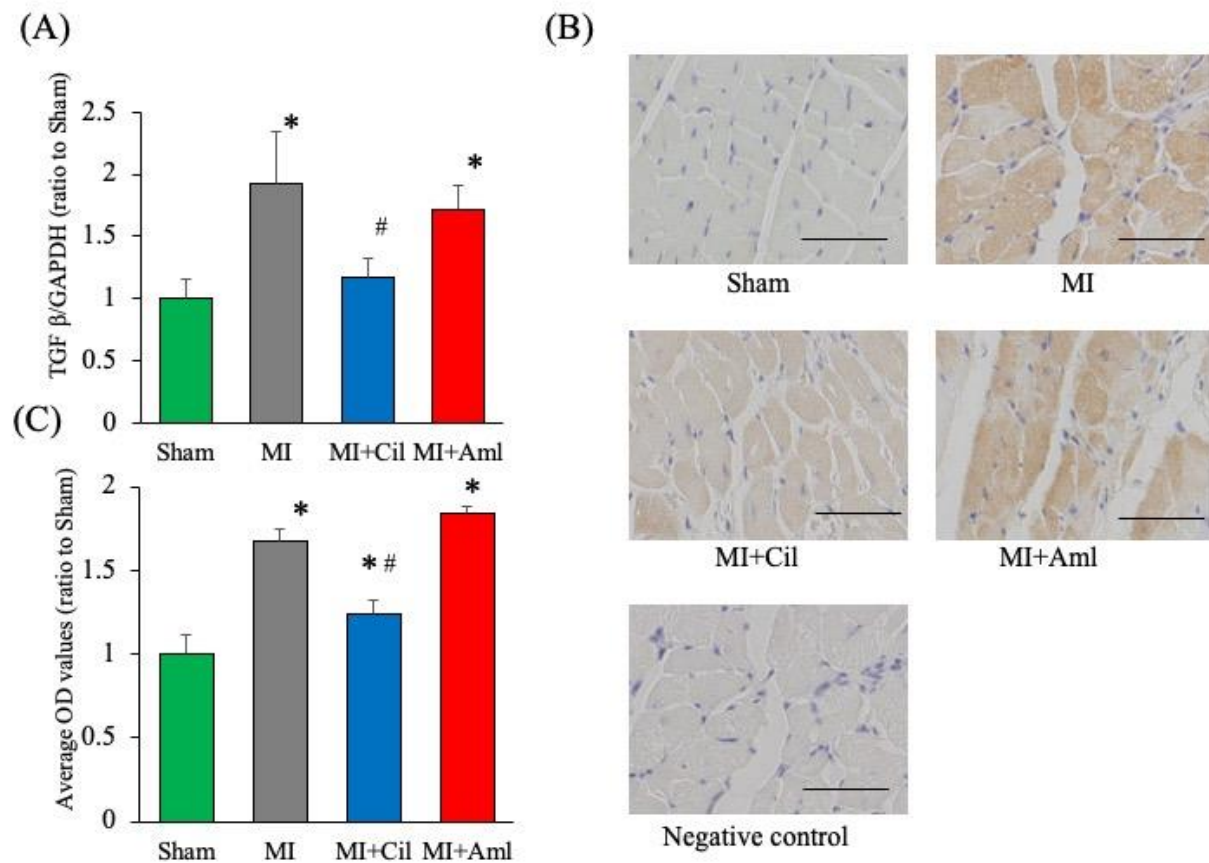


Figure 19. Effects of cilnidipine and amlodipine on TGF β in the non-infarct region 4 weeks after MI. TGF β mRNA expression (n=7-10) (A), representative immunohistochemical staining of TGF β (B), and the average OD value of TGF β (n=10-12) (C). Bars indicate 50 μ m. * p<0.05 versus Sham, # p<0.05 versus MI. Clin Exp Pharmacol physiol. 2020;00:1-9. Figure 6.

4. Discussion

In the present study, cilnidipine suppressed the noradrenaline concentration in the non-infarct region after MI. In renal tissue, reduction of the noradrenaline concentration by cilnidipine was previously reported in animal model of hypertensive renal diseases [87]. On the other hand, the reported effect of cilnidipine on the noradrenaline concentration in cardiac tissue are conflicting. Nagai et al. reported that cilnidipine treatment suppressed noradrenaline release from cardiac tissue using acute ischemia-reperfusion model rats [42]. On the other hand, Takatsu et al. noted comparable noradrenaline concentrations in cardiac tissue between cilnidipine and amlodipine using Dahl Salt-sensitive hypertensive rats with heart failure [82]. These discrepant results regarding the noradrenaline concentration by cilnidipine treatment may be due to different measurement methods. I and Nagai et al. measured the interstitial noradrenaline concentration using the micro dialysis method, whereas Takatsu et al. measured noradrenaline concentration using cardiac homogenates, which include noradrenaline in the sympathetic neuro-terminal and interstitial tissue. Noradrenaline is a well-known factor that induces myocyte hypertrophy and interstitial fibrosis [96]. Therefore, suppression of noradrenaline by cilnidipine attenuated of interstitial fibrosis more than amlodipine in the present model.

Activation of the sympathetic nerve increases RAS [97]. Hass et al. reported that sympathetic β stimulation by isoproterenol increases angiotensin II levels in plasma after MI [91]. In the present study, cilnidipine, but not amlodipine, also suppressed ACE mRNA expression and activity, which may have been due to the suppression of interstitial noradrenaline levels. Based on ACE, angiotensin II in the non-infarct region was suppressed in the cilnidipine-treated group. In previous animal studies, cilnidipine, but not amlodipine, prevented urinary protein excretion in association with the inhibition of the renal RAS in deoxycorticosterone acetate-salt hypertensive rats [62]. In addition, in a clinical trial of patients with hypertension, cilnidipine was superior to amlodipine at preventing the progression of proteinuria and RAS increase [40,98]. Therefore, the present results are consistent with these previous reports.

Recent evidence suggests that angiotensin II signaling plays an important role in mediating TGF β upregulation in the infarcted and remodeling myocardium [93–95]. In addition, TGF β is a well-known profibrotic cytokine and pharmacological blockade of TGF β potentially reduces fibrosis in the infarct and the non-infarct region [99]. In the present model, cilnidipine but not amlodipine reduced TGF β mRNA and protein expression. Therefore, cilnidipine likely suppressed the increase in noradrenaline enhancement after MI by inhibiting N-type Ca^{2+} channels in sympathetic neuro-terminals, which led to the inhibition of the RAS-TGF β pathway and attenuation of cardiac fibrosis after MI in this model (Figure 20).

In the present study, only cilnidipine was used as an N-type CCB because there is currently no other anti-hypertensive L- and N-type CCB in clinical use. Ω -conotoxin is a pharmacological selective inhibitor of N-type CCB but it is not suitable for use in *in vivo* experiments due to its toxicity. Moreover, cilnidipine was recently reported to protect mitochondria due to the regulation of mitochondrial dynamics in cardiac myocytes independent of L/N type Ca^{2+} channels inhibition[100]. Therefore, the present results do not exclude other potential mechanisms of cilnidipine on post-infarct cardiac remodeling, which is a limitation.

According to the treatment guidelines after MI, ACEIs and β blockers are essential drugs for the treatment of patients after MI. However, the antihypertensive effects of ACEIs and β blockers are not always sufficient. Therefore, in many cases, CCBs can be used as co-treatment with ACEIs and/or β blockers. In the present study, the superior beneficial effects of cilnidipine were likely due to noradrenaline and ACE suppression. Whether cotreatment with an ACEIs and/or β blockers and cilnidipine preserves the beneficial effects on post-MI remodeling is unclear. ACEIs are confidential drugs to prevent progression of post-infarct LV remodeling and heart failure. Hence, ACEIs are administrated just after MI at hypotensive dose. In patients with heart failure usefulness of cilnidipine in addition to ACEIs remain uncertain. However, in a previous randomized clinical trial of hypertensive patients with kidney disease, patients already receiving RAS inhibitors such as ACEIs and ARBs were treated using cilnidipine or amlodipine for one year. Although the anti-hypertensive effects in both CCB-treated groups were similar, the urinary protein to creatinine ratio was significantly reduced in the cilnidipine group compared with that in the amlodipine group [40]. Therefore, in the treatment of hypertensive patients with MI, cilnidipine in addition to ACEIs may promise additional prevention of heart failure.

β blockers are another essential drug used after MI. β blockers decrease the HR in association with reduction of workload in heart, leading to attenuation of heart failure. Therefore, β blockers without intrinsic sympathomimetic activity, such as carvedilol, bisoprolol, and metoprolol, are usually used after MI. On the other hand, systolic function of LV was remarkably decreased just after acute MI. To avoid exacerbation of heart failure by β blockers-induced negative inotropic action, safe dose of β blockers in initial use after MI are usually quarter or less dose of hypotensive dose. Therefore, additive β blocking effects would be expected by co-treatment with β blocker and cilnidipine. On the basis of these findings, additional treatment with cilnidipine to MI patients already receiving ACEIs and/or β blocker may further suppress post-infarct LV remodeling in hypertensive patients.

In a clinical study of heart rate variability, after treatment of hypertensive patients using CCBs for 6 months, the low-frequency (LF)-high frequency (HF) power ratio, an index of cardiac sympatho-vagal balance, was significantly lower with cilnidipine than with amlodipine [101]. Therefore, sympathetic nerve suppression by cilnidipine treatment after MI may be related to the prevention of post-infarct LV remodeling in hypertensive patients

Conclusion

In conclusion, the present results suggest that the long-acting CCBs, cilnidipine and amlodipine, attenuated LV remodeling after MI in SHRs despite negative inotropic effects. Cilnidipine reduced cardiac noradrenalin concentrations and suppressed the RAS, leading to further suppression of interstitial fibrosis more than amlodipine and attenuated post-infarct remodeling in hypertensive rats.

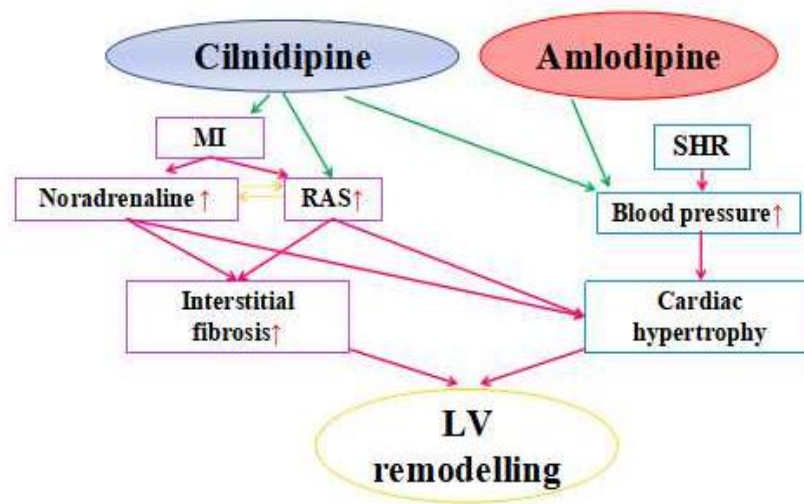


Figure 20. Proposed mechanism of treatment with CCBs in the development of post-infarct LV remodeling in hypertensive rats.

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