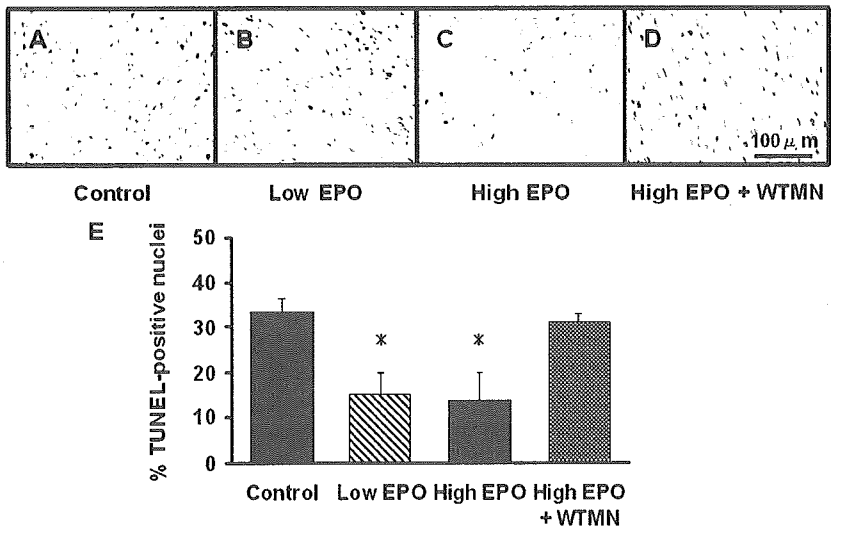

*Fig. 6. TUNEL staining in canine hearts after 90 min ischemia followed by 6 h of reperfusion. Representative examples of TUNEL-staining from canine hearts in the control (A), low EPO (B), high EPO (C), and high EPO + WTMN groups (D). (E) Quantitative data of the percentage of TUNEL-positive nuclei to total cell nuclei. *p < 0.05 vs. control group.*

increased the incidence of thrombotic events such as deep venous thrombosis or pulmonary embolisms in patients with breast cancer [18]. Furthermore, there are some reports that rhEPO increases the number of platelets in normal rats [19] and chronically hemodialyzed patients [20]. In the present study, we didn't find that either low or high dose of rhEPO, as a single injection, changed hematometric parameters. Although these findings suggest that a single administration of 1,000 IU/kg of rhEPO, that induced marked reduction of myocardial infarct size, could be used safely, we must be careful for the use of a high dose of rhEPO for the treatment of myocardial infarction.

Previous reports have shown that both phosphorylation of Akt and inhibition of apoptosis are associated with infarct size-limiting effects due to rhEPO [4,6–8]. Recently, it was reported that PI3 kinase activity is required for rhEPO to recover contractile dysfunction and to block apoptosis induced by myocardial ischemia-reperfusion in isolated hearts (*ex vivo*) [10]. Although the recovery of contractile function could be related to the reduction of infarct size, no evidence was presented that rhEPO reduced infarct size via the PI3 kinase-dependent pathway. In the present study we have demonstrated that the infarct size-limiting effect of rhEPO was blunted by the intracoronary administration of wortmannin in dogs. This is the first evidence showing that the infarct size-limiting effect of rhEPO is dependent on the PI3 kinase pathway in *in vivo* hearts.

In the present study, low and high doses of rhEPO equally increased phosphorylation of Akt and decreased equivalent number of TUNEL-positive cells in the ischemic myocardium of dogs. Either Akt phosphorylation or a decrease in the number of TUNEL-positive cells was prevented by the PI3 kinase inhibitor, wortmannin. This finding suggests that rhEPO prevents apoptotic cell death through PI3 kinase/Akt-dependent pathway in canine hearts. However, since the TUNEL method also detects single strand breaks occurring in the course of necrotic cell death [21], it is likely that rhEPO attenuates apoptotic and necrotic cell death. Indeed, if rhEPO only inhibits the apoptotic cell death, it may be difficult to explain the marked reduction of infarct size by rhEPO. Interestingly, the previous studies reported that the PI3 kinase activates not only Akt but also protein kinase C or mitogen-activated protein kinase in ischemia/reperfusion models [22–24], either of which mediates the cellular protection against necrotic process [25,26]. Furthermore, recent reports suggest that rhEPO can inhibit the release of free radicals from neutrophils [27] and act as a radical scavenger [28], both of which may reduce cardiac cell death after ischemia/reperfusion. Although further investigation will be needed, these characteristics of rhEPO may contribute to the reduction of necrotic as well as apoptotic cell death in ischemia/reperfused myocardium. In addition, since wortmannin inhibits not only PI3 kinase but also PI4 kinase and PI kinase related protein kinase, there is a limitation in using wortmannin as a specific inhibitor of PI3 kinase [29].

In clinical settings, ventricular arrhythmias are often observed in patients following reperfusion therapy and they can be life-threatening [30]. Importantly, the present study demonstrated that a high, but not a low dose of rhEPO prevented VF during reperfusion via the PI3 kinase-dependent pathway. Since low and high doses of rhEPO equally increased phosphorylation of Akt, it is unlikely that Akt is responsible for

the rhEPO-induced anti-arrhythmic effect. There are several possible mechanisms by which rhEPO exerts anti-arrhythmic effects via the PI3 kinase-dependent, but Akt-independent, pathway. First, under conditions of reperfusion, production of inositol-1,4,5-trisphospate (IP3) increases when phospholipase C (PLC) is activated through α-adrenoreceptors on the myocardial cell membrane [11]. This increase in IP3 activates IP3 receptors on the sarcoplasmic reticulum causing the release of Ca^{2+}. The increases in the intracellular Ca^{2+} levels caused by IP3 have been reported to initiate slow Ca^{2+} oscillations, which underlies the delayed afterdepolarizations that trigger many arrhythmias including VF [11,31]. PLC hydrolyzes phosphatidylinositol-4,5-bisphospate (PIP2) to produce IP3. Since PI3 kinase and PLC can act upon the common substrate, PIP2 [32], rhEPO may prevent lethal arrhythmia by activating the PI3 kinase pathway that results in the decrease in PIP2 levels, which will lead to prevent Ca^{2+} overload by IP3. Second, since oxygen-derived free radicals are involved in the generation of reperfusion arrhythmia [30,33,34], rhEPO may decrease reperfusion arrhythmia through the prevention of free radicals release from neutrophils or acting as a radical scavenger [27,28]. Finally, we need to consider that rhEPO exerts anti-arrhythmic effects by the reduction of myocardial infarct size.

In conclusion, our findings, when translated into clinical practice, may support the use of rhEPO as a cardioprotective agent in the treatment of patients with myocardial infarction.

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Amlodipine ameliorates myocardial hypertrophy by inhibiting EGFR phosphorylation

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Abstract

The effects of long-acting calcium channel blockers on pressure overload-induced cardiac hypertrophy have been little studied in experimental animals and the underlying mechanisms are not fully understood. We previously reported that cardiomyocyte hypertrophy could be induced via phosphorylation of the epidermal growth factor receptor (EGFR). In this study, we investigated whether amlodipine attenuates cardiac hypertrophy by inhibiting EGFR phosphorylation. We found that amlodipine dose-dependently inhibited epinephrine-induced protein synthesis and EGFR phosphorylation in cultured neonatal rat cardiomyocytes. Our in vivo study revealed that amlodipine could ameliorate myocardial hypertrophy induced by transverse aortic constriction (TAC) in C57/B6 mice. One week after TAC, amlodipine treatment (3 mg/kg/day) significantly reduced the heart-to-body weight ratio (6.04 ± 0.16 mg/g vs. 6.90 ± 0.45 mg/g in untreated TAC mice, $P < 0.01$). These results indicate that amlodipine ameliorates cardiomyocyte hypertrophy via inhibition of EGFR phosphorylation.
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Keywords: Calcium channel blocker; Cardiomyocyte; Hypertrophy; Epidermal growth factor; Phosphorylation; Mouse

Calcium channel blockers (CCBs) are widely used for the treatment of hypertension. Amlodipine is a long-acting dihydropyridine CCB that is effective for lowering the blood pressure, amelioration of cardiac remodeling, and reduction of mortality and morbidity [1]. However, the mechanisms underlying the beneficial effects of CCBs on cardiac remodeling are not fully understood. We have reported that stimulation of the G protein-coupled receptor (GPCR) in cardiomyocytes causes the release of heparin-binding epidermal growth factor (HB-EGF), which subsequently binds to the epidermal growth factor receptor (EGFR) and produces cardiac hypertrophy [2]. There is evidence that calcium channels play an important role in activation of the EGFR [3]. Calcium channels were reported to be involved in endothelin-1-induced activation of the EGFR [3], and calcium channels also induce tyrosine phosphorylation of this receptor to levels that can activate the mitogen-activated protein kinase signaling pathway [4]. In addition, blockade of calcium uptake and mobilization by mammary gland epithelial cells suppress EGF-induced cell proliferation [5]. Considering these findings, we hypothesized that amlodipine may ameliorate cardiomyocyte hypertrophy by inhibiting EGFR phosphorylation. In the present study, we evaluated the effect of amlodipine on EGFR phosphorylation induced by a GPCR agonist in vitro and

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on cardiomyocyte hypertrophy induced by left ventricular pressure overload in vivo.

Materials and methods

Cell culture. Rat neonatal ventricular myocytes were isolated as described previously [2], and were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma) supplemented with 10% FBS (Equitech-Bio). The medium was changed to serum-free medium after 72 h and cells were cultured under serum-free conditions for 48 h before addition of agents. Protein synthesis by the cultured cells was evaluated through analysis of [^{3}H]leucine incorporation [2,6]. Cardiomyocytes were exposed to either epinephrine (Epi: 10^{-5} M) or HB-EGF (10^{-8} M) for 24 h in the presence or absence of amlodipine (kindly provided by Sumitomo Pharmaceuticals, Japan), and the increase of [^{3}H]leucine incorporation was examined.

EGFR phosphorylation. Cultured cardiomyocytes were exposed to 10^{-5} M Epi or 10^{-8} M HB-EGF with or without pretreatment by amlodipine (10^{-6} or 10^{-9} M) or HB-EGF neutralizing antibody #19 for 30 min. Cells were lysed by incubation for 20 min at 4 °C in a buffer (50 mM Tris–HCl, pH 7.3; 150 mM NaCl; 2 mM EDTA; 0.5% sodium fluoride; 10 mM sodium pyrophosphate; 0.5 mM Na_3VO_4; 100 μg/ml phenylmethylsulfonyl fluoride; 2 μg/ml aprotinin; protease inhibitor cocktail; and 1% Nonidet P-40). Immunoprecipitation with an antibody directed against the EGFR and immunoblotting using phosphorylation antibody (Anti-pY) were performed as described elsewhere [7].

Animal model. All procedures were performed in accordance with the institutional guidelines for animal research. Male C57BL/6 mice (8–9 weeks-old, wt 19–25 g) were anesthetized with a mixture of xylazine (5 mg/kg) and ketamine (100 mg/kg intraperitoneally). The animal model of pressure overload was created as described previously [8]. Briefly, transverse aortic constriction (TAC) was produced by tying a 7-0 suture tied twice around the aorta and a 27-gauge needle, after which the needle was gently removed to yield 60–80% constriction of the aortic arch.

To determine whether amlodipine could attenuate cardiac hypertrophy induced by TAC, we treated the mice with saline (TAC group) or oral amlodipine 3 mg/kg/day. To confirm that the extent of pressure overload was similar between the amlodipine-treated and untreated groups, we measured the pressure in the ascending aorta of 2–3 mice from each group using a 1.4 F Millar catheter on the 2nd day after TAC. The tail-cuff blood pressure and heart rate (BP-98A, Softron, Tokyo, Japan) were examined before sacrifice. One week after the induction of pressure overload, mice were killed to determine organ weights and perform morphometric analysis. The cross-sectional surface area of cardiomyocytes was measured using three hearts in each group with the method described previously [6].

Statistical analysis. Multiple comparisons were performed by one-way ANOVA with the Tukey–Kramer exact probability test. Results are reported as means ± SEM. For all analyses, $P < 0.05$ was considered statistically significant.

Results and discussion

Amlodipine attenuates the induction of cardiomyoctyte protein synthesis by epinephrine

As shown in Fig. 1A, amlodipine markedly inhibited epinephrine-induced neonatal rat cardiomyocyte protein synthesis over a concentration range of 10^{-7}–10^{-5} M. Epinephrine is one of the GPCR agonists and is well known to induce cardiomyocyte hypertrophy. Pignier et al. [9] reported that hypertrophy induced by long-term stimulation of α_1-adrenoceptors is accompanied by an increase in the expression of functional calcium channels in neonatal rat cardiomyocytes, indicating the existence of a novel α_1-mediated pathway for positive regulation of the L-type calcium current. This agrees with our finding that blockade of L-type calcium channels inhibits cardiomyocyte hypertrophy. There is substantial evidence to support the notion that calcium signaling pathways contribute to the progression of cardiac hypertrophy [10,11], so it is likely that blockade of calcium signaling would lead to the regression of hypertrophy.

Amlodipine causes concentration-dependent inhibition of EGFR phosphorylation induced by epinephrine

Based on our earlier demonstration that EGFR activation by GPCR agonists led to the development of cardiac hypertrophy [2] and the present in vitro finding that

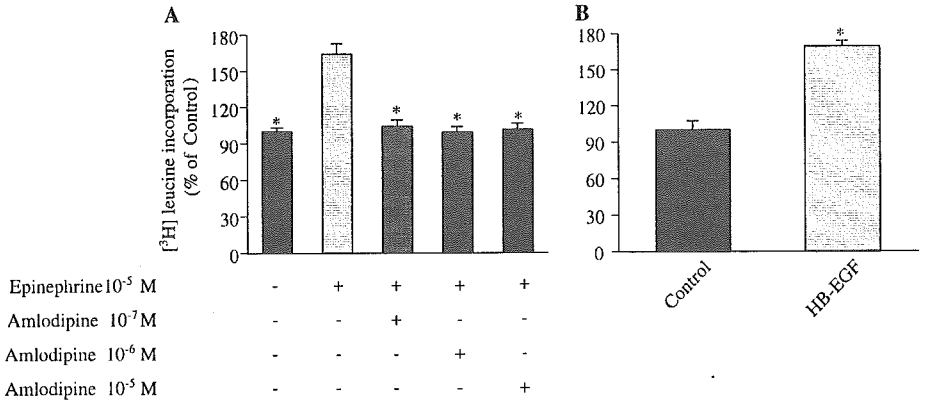

Fig. 1. Effect of amlodipine and HB-EGF on protein synthesis in rat cardiomyocytes. (A) Protein synthesis stimulated by epinephrine (10^{-5} M) was inhibited by amlodipine at concentrations ranging from 10^{-7} to 10^{-5} M. *$P < 0.01$ vs. epinephrine alone. (B) HB-EGF (10^{-8} M) significantly increased myocyte protein synthesis. *$P < 0.01$ vs. Control.

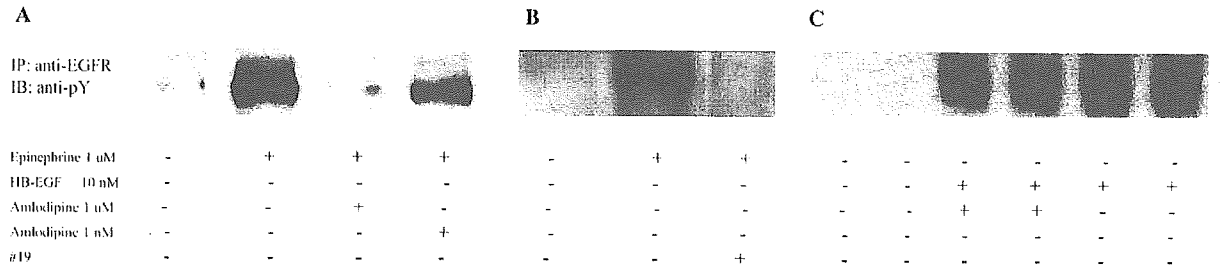

Fig. 2. EGFR phosphorylation and release of HB-EGF. (A) EGFR phosphorylation showed concentration-dependent inhibition by amlodipine. (B) HB-EGF neutralizing antibody #19 blocked epinephrine-induced EGFR phosphorylation. (C) Amlodipine did not influence EGFR phosphorylation induced by HB-EGF. Each experiment was repeated at least three times.

amlodipine inhibits cardiomyocyte protein synthesis stimulated by a GPCR agonist (epinephrine), we hypothesized that amlodipine may also inhibit cardiomyocyte hypertrophy by preventing tyrosine phosphorylation of the EGFR. In the present study, HB-EGF significantly increased protein synthesis by neonatal rat cardiomyocytes (Fig. 1B), a finding that agreed with our previous report [2]. Interestingly, we also demonstrated that amlodipine inhibits EGFR phosphorylation in cardiomyocytes in a concentration-dependent manner (Fig. 2A). In recent years, information about the mechanisms related to Ca^{2+} influx has accumulated. Zwick et al. [12] reported that calcium-dependent EGFR activation led to subsequent activation of the Ras/mitogen-activated protein pathway in neurons. In addition, Kawanabe et al. [3] have shown that Ca^{2+} influx plays an important role in endothelin-1-induced EGFR activation, and endothelin-1 is well known to stimulate cardiomyocyte growth.

Amlodipine inhibits epinephrine-induced release of HB-EGF

We previously reported that phenylephrine induces EGFR activation by increasing the release of the HB-EGF ectodomain [2]. Here we found that amlodipine could inhibit EGFR activation by reducing the epinephrine-induced release of HB-EGF. Since the extracellular level of the ectodomain of HB-EGF (soluble HB-EGF) was generally too low to be detected by Western blotting, we assessed it by an indirect method. If epinephrine induces release of the HB-EGF ectodomain, its depletion was assumed to block epinephrine-induced EGFR activation. As expected, we found that an HB-EGF neutralizing antibody #19 almost completely prevented epinephrine-induced phosphorylation of the EGFR (Fig. 2B), suggesting that epinephrine-induced EGFR activation is mediated by the release of HB-EGF, at least in newborn rat cardiac myocytes. When we investigated whether amlodipine prevents HB-EGF-induced activation of the EGFR, we found that this drug did not have any influence on HB-EGF-mediated EGFR phosphorylation (Fig. 2C), suggesting that it acts upstream of HB-EGF. Finally, we revealed that amlodipine caused marked inhibition of epinephrine-induced phosphorylation of the EGFR (Fig. 2A), a result that supported an inhibitory effect of the drug on EGFR activation by preventing the release of HB-EGF. Further studies are needed to elucidate the exact mechanism by which CCBs inhibit EGFR phosphorylation. Src kinase is reported to contribute to EGFR activation by GPCR agonists [13,14], while a link between calcium release through L-type calcium channels and Src has also been demonstrated [4,15–18], and the release of calcium seems to be necessary for activation of Src [4,18]. Thus, it is likely that amlodipine blocks the signal transduction pathway upstream of Src.

Amlodipine inhibits myocardial hypertrophy in vivo

We used a well-established mouse model of left ventricular pressure overload to further confirm the preventive effect of amlodipine on cardiac hypertrophy. An increase of GPCR agonists, such as catecholamines [6], angiotensin II, and endothelin-1, is known to occur in the myocardium of these mice. Since EGFR activation leads to cardiomyocyte hypertrophy [2] and amlodipine inhibits epinephrine-induced EGFR phosphorylation in cardiomyocytes in vitro, as shown in the present study, it would seem plausible that amlodipine also attenuates cardiac hypertrophy induced by TAC. Indeed, consistent with our in vitro results, we found that oral administration of amlodipine (3 mg/kg/day) for 1 week markedly ameliorated cardiac hypertrophy. Histological examination confirmed that myocyte hypertrophy was less severe (Figs. 3A and B) in mice treated with amlodipine. Compared with sham mice, the heart-to-body weight ratio (HW/BW) increased by about 43% in TAC mice, while the amlodipine-treated mice only showed an increase of about 25% (Fig. 3C). Cardiomyocytes cross-surface area was also significantly decreased in amlodipine-treated mice (Fig. 3D). Hemodynamic parameters are summarized in Table 1; amlodipine did not significantly affect either the tail-cuff systolic blood pressure or the heart rate. Ascending aortic pressure was similar in the TAC and amlodipine-treated TAC

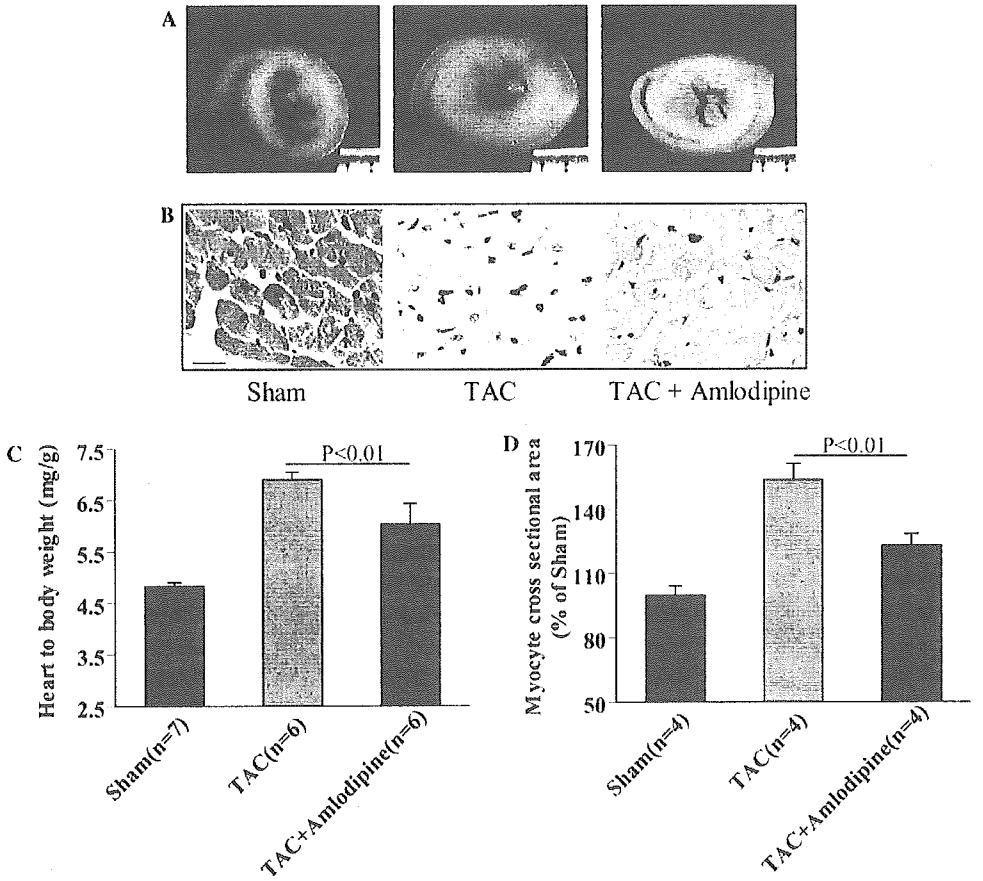

Fig. 3. Effects of amlodipine on cardiac hypertrophy induced by pressure overload in mice. (A) Representative cross-sections of hearts from the three groups. (B) Histological examination showed that cardiomyocyte hypertrophy was less severe in hearts of amlodipine-treated mice (Bar, 20 μm, HE stain). The heart-to-body weight ratio (HW/BW) (C) and cardiomyocyte cross-sectional surface area (D) were significantly lower in TAC mice treated with amlodipine (3 mg/kg/day) in comparison with untreated TAC mice.

Table 1
General characteristics in three experimental groups

Group	AASBP (mmHg)[a]	BW (g)	Tail SBP (mmHg)	HR (bpm)
Sham ($n = 7$)	101 ± 5	23 ± 0.2	112 ± 4	644 ± 26
TAC ($n = 6$)	157 ± 9[b]	22.4 ± 0.3	100 ± 5[b]	670 ± 24
TAC+amlodipine ($n = 6$)	161 ± 8[b]	20.1 ± 0.8[b,c]	93 ± 3[b]	675 ± 19

TAC, transverse aortic constriction; AASBP, ascending aortic systolic blood pressure (SBP); AASBP was measured in three mice in each group at 2nd day after TAC, while those mice were randomly selected and did not receive amlodipine treatment, because we just wanted to confirm that the pressure overload was similar between TAC and amlodipine-treated groups. BW, body weight; HR, heart rate. BW, tail SBP, and HR were measured before sacrifice.

[a] $n = 2$ in each group.
[b] $P < 0.05$ vs. Sham.
[c] $P < 0.05$ vs. TAC.

groups, indicating that there was no significant difference of the pressure load on the left ventricle.

Our data suggested that amlodipine was effective for ameliorating cardiomyocyte hypertrophy independently of any decrease in the blood pressure. This antihypertrophic effect was attributable, at least partly, to the inhibition of EGFR phosphorylation by amlodipine and this drug is also likely to exert an antihypertrophic effect through the nitric oxide signaling pathway, as indicated by previous studies [19].

Although various clinical trials have demonstrated that amlodipine is effective and safe for the treatment of hypertension and reducing cardiac events [20–22], the underlying mechanisms remain poorly understood. The present study is the first to show that amlodipine ameliorates cardiac hypertrophy by inhibiting EGFR

activation. This suggests the possibility of using the regulation of Ca^{2+} influx as a therapeutic approach for controlling cell growth and proliferation.

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Benidipine, a long-acting calcium channel blocker, inhibits cardiac remodeling in pressure-overloaded mice

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Abstract

Objective: The effects of long-acting calcium channel blockers (CCBs) on pressure overload-induced cardiac remodeling are seldom studied in animals. We evaluated the effects of benidipine, a long-acting CCB, on cardiac remodeling.
Methods: Rat neonatal cardiac myocytes were used to examine the influence of benidipine on protein synthesis. Cardiac remodeling was induced in C57 B6/J mice by transverse aortic constriction (TAC). Then the effects of benidipine (10 mg/kg/d) were assessed on myocardial hypertrophy and heart failure, cardiac histology, and gene expression.
Results: Benidipine significantly inhibited protein synthesis by cardiac myocytes stimulated with phenylephrine (PE), and this effect was partially abolished by cotreatment with a nitric oxide synthase (NOS) inhibitor [N(G)-nitro-L-arginine methylester (L-NAME)]. Four weeks after the onset of pressure overload, benidipine therapy potently inhibited cardiac hypertrophy and prevented heart failure. The heart to body weight ratio was 6.89±0.48 mg/g in treated mice vs. 8.76±0.33 mg/g in untreated mice ($P<0.01$), and the lung to body weight ratio was 7.39±0.93 mg/g vs. 10.53±0.99 mg/g, respectively ($P<0.05$). Left ventricular fractional shortening (LVFS) was improved on echocardiography. Plasma NO levels were increased, while B type natriuretic peptide, protein inhibitor of neuronal NOS, and procollagen IV alpha were down-regulated in benidipine-treated mice.
Conclusion: These results indicate that benidipine inhibits cardiac remodeling due to pressure overload at least partly by acting on the nitric oxide signaling pathway.
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Keywords: Calcium channel blocker; Heart failure; Hypertrophy; Gene expression

1. Introduction

Calcium channel blockers (CCBs) are one of the most frequently used classes of drugs for the treatment of hypertension. Although early clinical studies showed a disappointing outcome when short-acting dihydropyridine CCBs were used to reduce cardiovascular risk [1,2], well-designed prospective randomized controlled clinical trials have demonstrated that long-acting dihydropyridine CCBs are effective for reduction of the blood pressure (BP), inhibition of cardiac remodeling, and decreasing the risk of cardiovascular endpoints [3]. However, the underlying mechanism of the beneficial effect of CCBs on cardiac remodeling is not fully understood. An earlier study performed by our laboratory showed that the vasodilator hydralazine significantly lowered the systemic blood pressure but did not exert any effect on cardiac hypertrophy induced in rats by N(G)-nitro-L-arginine methylester (L-NAME), a nitric oxide (NO) synthase inhibitor [4], suggesting that blood pressure reduction alone was not sufficient to inhibit cardiac remodeling. We also

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reported that a long-acting CCB, benidipine, could increase coronary flow and reduce myocardial ischemia by promoting the release of NO [5,6]. NO is also known to lessen the severity of cardiac hypertrophy and heart failure [7,8]. Furthermore, benidipine has been demonstrated to inhibit myocardial fibrosis in diabetic rats [9]. Based on these lines of evidence, we hypothesized that benidipine may inhibit cardiac remodeling via the NO signaling pathway.

Because the occurrence of cardiac remodeling has been shown to be associated with subsequent cardiovascular events, therapeutic approaches that inhibit cardiac remodeling are likely to improve the prognosis. Chronic left ventricular pressure overload induced by transverse aortic constriction (TAC) is a well established animal model for investigation of cardiac remodeling [10–12], but few experimental studies have attempted to clarify the effects of long-acting CCBs on cardiac remodeling using this model. Therefore, we evaluated the effects of benidipine on cardiac hypertrophy and heart failure in a murine model of pressure overload due to TAC and explored the mechanisms involved.

2. Methods

2.1. Cell culture

Rat neonatal cardiac myocytes were isolated, as described previously [13]. The myocytes were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma) supplemented with 10% FBS (Equitech-Bio), which was changed to serum-free medium after 72 h. Cells were cultured under serum-free conditions for 48 h before agents were added. Protein synthesis by cultured cells was evaluated from [^{3}H] leucine incorporation, as described elsewhere [11,13]. Cardiac myocytes were exposed to 10^{-4} M phenylephrine (PE) for 24 h in the presence or absence of benidipine (kindly provided by the Pharmaceutical Research Laboratories of Kyowa Hakko Kogyo Sunto, Shizuoka, Japan), and the increase of [^{3}H] leucine uptake was examined. To determine whether the NO signaling pathway was involved in the inhibition of protein synthesis by cardiac myocytes, we examined whether the in vitro effect of benidipine could be blocked by the NO synthase (NOS) inhibitor L-NAME (10^{-5} M).

2.2. Animal model

All procedures were performed in accordance 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). Male C57BL/6J mice aged 8–9 weeks and weighing 19–23 g were anesthetized with a mixture of xylazine (5 mg/kg) and ketamine (100 mg/kg) injected intraperitoneally. Then pressure overload was created, as described preciously [10]. Briefly, endotracheal intubation was performed, and the cannula was connected to a volume-cycled rodent ventilator with a tidal volume of 0.5 ml (room air) and a respiration rate of 100/min. The chest was entered via the second intercostal space at the upper left sternal border. After the arch of the aorta was isolated, TAC was created using a 7–0 suture tied twice around a 27-gauge needle and the aortic arch between the innominate and left common carotid arteries. After the suture was tied, the needle was gently removed, yielding 60–80% constriction of the aorta. More than 1000 murine TAC models have been created at our laboratory, and cardiac hypertrophy occurs in 100% of these animals. The dispersion of heart weight to body weight ratio evaluated with statistical parameter coefficient variance at 4 weeks following TAC is about 20%, as we reported previously [11,14].

To test whether benidipine could inhibit the cardiac hypertrophy due to TAC, we treated the mice with either saline (TAC group) or benidipine at 10 mg/kg/d (po, mixed with 0.3% carboxymethyl cellulose sodium and suspended in water) from the 2nd day after surgery. The benidipine dose was based on previous reports from our [4] and another [9] laboratory as well as a preliminary study. To confirm that the extent of the pressure overload was similar between benidipine-treated and untreated animals, three mice were randomly selected from each group to measure the pressure in ascending aorta, using a 1.4 F Millar Pressure Catheter on the 2nd day after TAC. Four weeks after the creation of pressure overload, both the tail cuff blood pressure (BP) and the heart rate (HR; BP-98A, Softron, Tokyo, Japan) were measured 1 day before sacrifice. LV hemodynamic studies were performed by cannulation of the right carotid artery with a Millar Pressure Catheter that was carefully advanced to the LV. Then the mice were killed to measure organ weights and to perform histological analysis.

2.3. Histological examination

The cross-sectional area of cardiac myocytes and the extent of myocardial fibrosis were measured, as described elsewhere [4,15]. Briefly, the cardiac myocyte area and myocardial fibrosis area were analyzed quantitatively by morphometry of either HE-stained or Azan/Mallory-stained sections. The original images were digitized and transformed into binary images, after which the cardiac myocyte area or fibrosis area was calculated with an automatic area quantification program (NIH Image). One hundred myocytes per heart were counted, and the average value was determined. The total myocardial fibrosis index was defined as the sum of the total area of fibrosis in the entire microscopic field divided by the sum of total connective tissue area plus the myocardial area in the entire field.

2.4. Echocardiography

Transthoracic echocardiography was performed with a Sonos 4500 and a 15–6 L MHz transducer (Philips, the Netherlands). Mice were weighed, lightly anesthetized with

2.5% avertin (0.06 ml/10 g), and set in the left lateral decubitus position or the supine position. After the mouse recovered to complete consciousness (about 10 min), two-dimensional short-axis views of the left ventricle were obtained for guided M-mode measurement of the left ventricular diastolic posterior wall thickness (LVPWd), left ventricular end-diastolic dimension (LVEDd), and left ventricular end-systolic dimension (LVESd). Left ventricular fractional shortening (LVFS) was calculated as follows: LVFS=(LVEDd−LVESd)/LVEDd*100.

2.5. Microarray analysis

To determine the gene expression profile during cardiac remodeling, we performed microarray studies of murine hearts after pressure overload for 1 or 4 weeks. Data about the time course of the induction of NO synthase and fibrosis-related genes were needed to investigate their roles in cardiac hypertrophy and heart failure. Total RNA was prepared from murine hearts using Triazol (Gibco-BRL), according to the manufacturer's instructions. Microarray hybridization was performed in duplicate using Affymetrix Murine Genome U74v2A gene chips and RNA from hearts of animals in the TAC or sham operation groups at 1 or 4 weeks after surgery. Data were analyzed using Genespring 6 software [16].

2.6. Measurement of plasma nitric oxide

Blood was obtained from the right ventricle with a 23-gauge needle at the time of sacrificing the mice. The plasma concentrations of NOx (NO_2+NO_3) was measured with an autoanalyzer (ENO-10, Eicom Kyoto, Japan), as described elsewhere [5,6,17]. Samples were applied to an analytical column that was connected to a copperized cadmium reduction column to reduce NO_2 to NO_3, which was then reacted with Griess reagent, and the absorbance of the product was measured at 540 nm.

2.7. Quantitative PCR

Based on the results of microarray analysis, we chose three genes that were consistently up-regulated at both 1 and 4 weeks after the onset of LV pressure overload and were closely related to cardiac hypertrophy or heart failure. We further investigated the effects of benidipine on these genes by real-time PCR. The three genes were the natriuretic peptide precursor type B (BNP) gene, protein inhibitor of neuronal nitric oxide synthase (PIN) gene, and procollagen IV alpha gene. Primers were designed using Gene Express software. Using 50 ng/μl of total RNA as the template, quantitative measurement was performed with an ABI Prism 7700 sequencing system. Amplification was done by the one-step method using a Quantitect SYBR Green RT-PCR kit (QIAGEN). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was amplified as an endogenous control, and quantitation of target gene levels was performed relative to this gene.

2.8. Statistical analysis

For all statistical tests, multiple comparison was performed by one-way ANOVA with the Tukey–Kramer exact probability test. The least-squares method was used for linear correlation between selected variables. Results are reported as the mean±S.E.M., and $P<0.05$ was considered statistically significant.

3. Results

3.1. Benidipine reduces cardiac myocyte protein synthesis stimulated by PE

Benidipine (10^{-4} M) did not affect basal [^{3}H] leucine uptake by cardiac myocytes, but it inhibited PE-induced

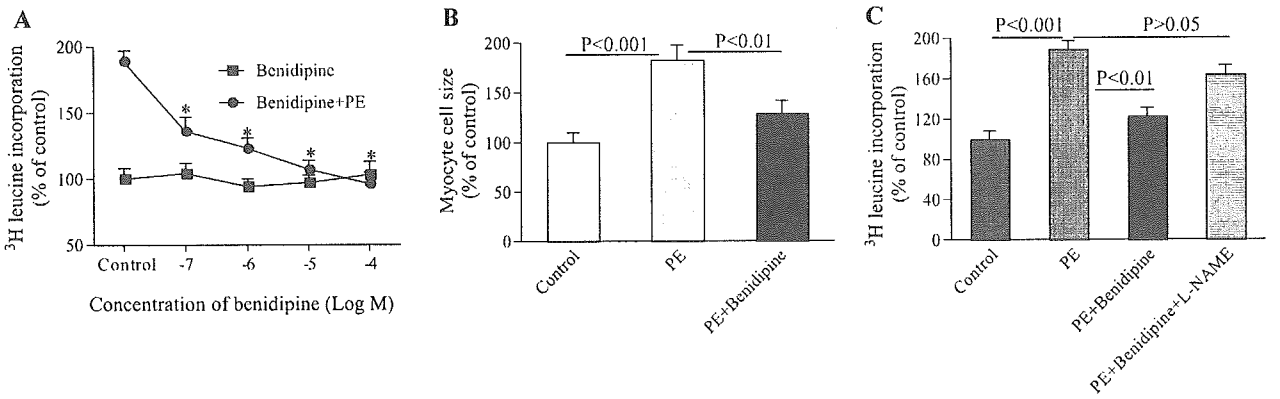

Fig. 1. Effect of benidipine on protein synthesis and the size of neonatal rat cardiac myocytes. (A) Protein synthesis stimulated by 10^{-4} M phenylephrine (PE) was inhibited by benidipine at concentrations ranging from 10^{-7}–10^{-4} M in a dose-independent fashion, and this concentration range did not affect normal cardiac myocytes. *$P<0.01$ vs. control. (B) The cell size was calculated from 200 cells in every group. The increase of cell size caused by PE (10^{-4} M) was inhibited by treatment with benidipine (10^{-5} M). (C) The inhibitory effect of benidipine (10^{-5} M) on protein synthesis induced by PE was partially blocked by cotreatment with L-NAME (10^{-5} M).

Table 1
Hemodynamic and echocardiographic data obtained at 4 weeks

Group	BW (g)	HR (bpm)	SBP (mm Hg)	LVPWd (mm)	LVEDd (mm)	LVESd (mm)
Sham	25.2±0.4**	651±11	114±3	0.65±0.02***	3.07±0.06	1.64±0.04**
TAC	22.64±0.41	686±26	101±5	0.98±0.04	3.38±0.12	2.29±0.04
TAC+Beni	23.1±0.4	652±26	105±2	0.77±0.03***	3.04±0.06*	1.69±0.12**

Beni—benidipine (10 mg/kg/d po); BW—body weight; HR—heart rate; SBP—Tail cuff systolic blood pressure; LVPWd—LV diastolic posterior wall thickness; LVEDd—LV end-diastolic dimension; LVESd—left ventricular end-systolic dimension. The number of mice in the sham, TAC, and TAC+benidipine groups was 10, 17, and 11, respectively, for BW, LVPWd, LVEDd, and LVESd; and 10, 9, and 7 for HR and SBP.

* $P<0.05$.

** $P<0.01$.

*** $P<0.001$ vs. TAC (transverse aortic constriction).

protein synthesis in a concentration-dependent fashion (Fig. 1A). The enlargement of cells induced by PE was also inhibited by benidipine (Fig. 1B). The inhibitory effect of benidipine on PE-induced protein synthesis was partially blocked by L-NAME (Fig. 1C).

3.2. Benidipine inhibits pathological cardiac hypertrophy

The hemodynamic and echocardiographic data obtained just before sacrifice are shown in Table 1. Benidipine (10 mg/kg/d) did not significantly affect the tail cuff systolic blood pressure, but the LV wall was thinner, and LV dimensions were smaller in benidipine-treated mice than in TAC mice (Table 1).

Echocardiography and hemodynamics showed no differences among the three groups of mice before surgery (data not shown). The ascending aortic systolic blood pressure was measured on the 2nd day after TAC or sham operation without drug treatment in order to evaluate the extent of pressure overload (in three mice per group), no significant difference was noted between the TAC and benidipine groups (98±5 mm Hg in the sham group, 163±4 mm Hg in the TAC group, and 161±3 mm Hg in the benidipine group).

LV hemodynamics were similar between TAC mice with or without benidipine treatment (Fig. 2), suggesting that an oral dose of 10 mg/kg did not significantly affect LV function.

Consistent with the in vitro results, benidipine markedly inhibited cardiac hypertrophy at 4 weeks after TAC (Fig. 3). Histological examination showed that the extent of myocyte hypertrophy (Fig. 4A,B) was reduced and that myocardial fibrosis was less severe in benidipine-treated mice (Fig. 4C,D).

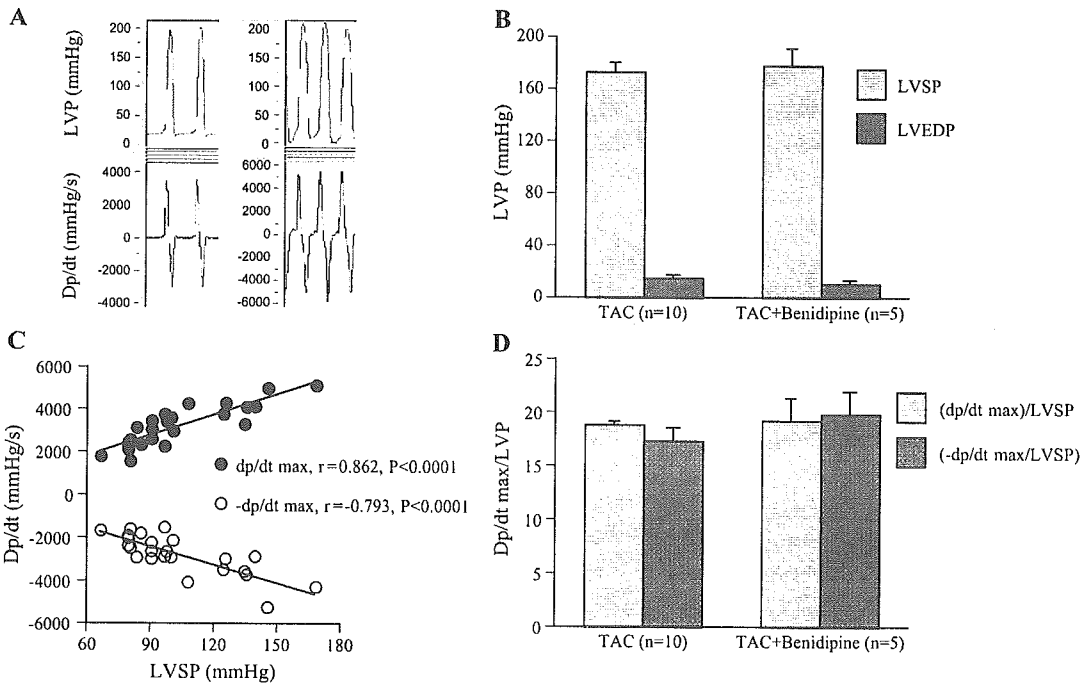

Fig. 2. Left ventricular (LV) hemodynamics measured with a Millar Catheter at 4 weeks after TAC. (A) LV pressure and dp/dt in the TAC and benidipine groups. (B) No significant differences of LV systolic pressure (LVSP) and LV end-diastolic pressure (LVEDP) were noted between TAC mice with or without benidipine. (C) ±Dp/dt max was closely correlated with LVSP in untreated mice. (D) ±Dp/dt max/LVSP was not significantly increased in benidipine-treated mice.

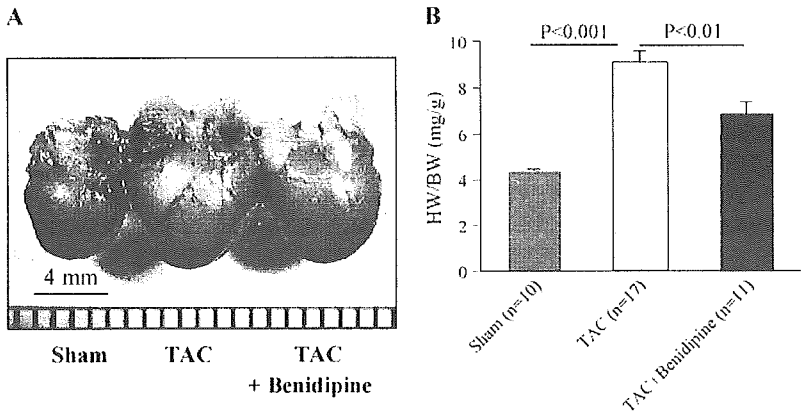

Fig. 3. Benidipine inhibits cardiac remodeling. (A) Representative pictures of whole hearts. (B) The heart to body weight ratio (HW/BW) was significantly decreased in TAC mice treated with benidipine (10 mg/kg/d) compared with untreated TAC mice.

3.3. Benidipine prevents progression from hypertrophy to heart failure

TAC induced congestive heart failure with a reduction in LVFS and increase of pulmonary congestion. LVFS measured by echocardiography was significantly higher in benidipine-treated mice than in TAC mice (Fig. 5A,B). Compared with the value for sham-operated mice, the lung weight to body weight ratio (LW/BW) was increased by about 108% in TAC mice, but only rose by 46% in benidipine-treated mice (Fig. 5C,D).

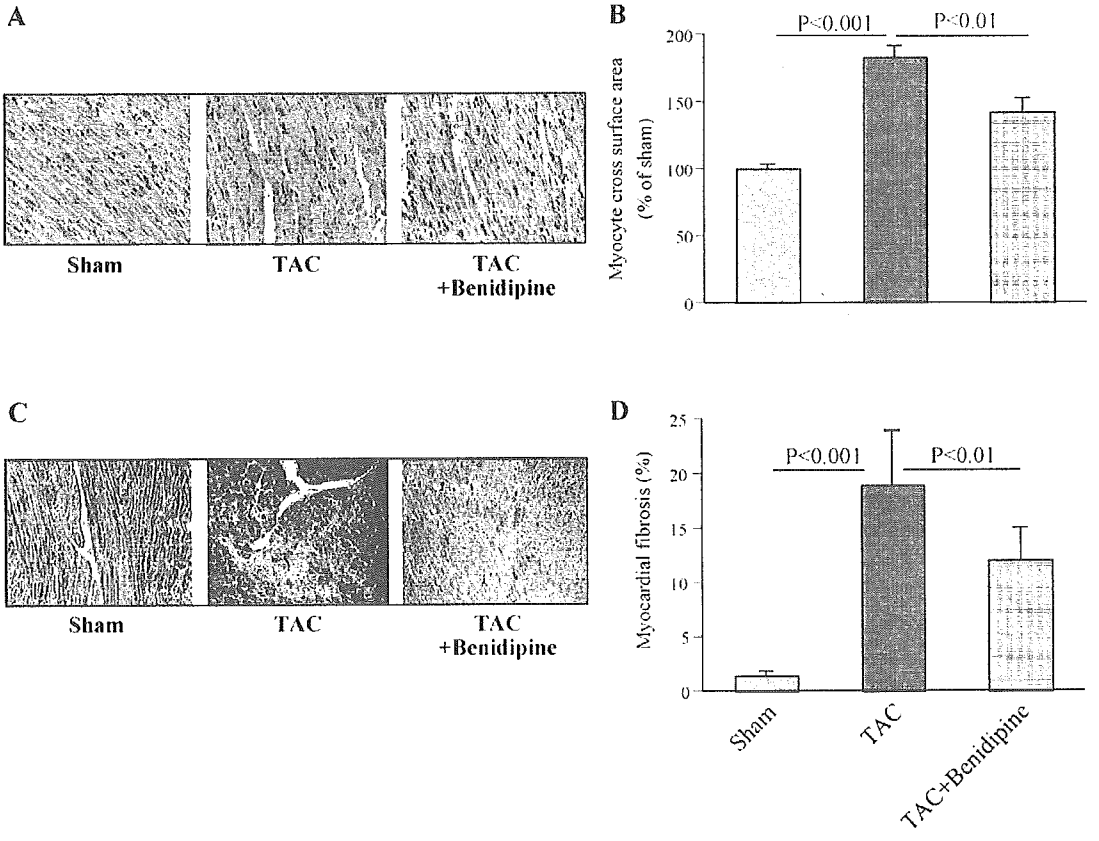

Fig. 4. Results of histological examination. (A) Representative images of the myocardium (HE stain×200). (B) The cross-sectional area of cardiac myocytes was significantly increased in TAC mice by pressure overload for 4 weeks, while treatment with benidipine blunted the enlargement of myocytes. (C) Representative pictures of myocardial fibrosis (Azan–Mallory stain×100). (D) Quantitative analysis showed that benidipine significantly inhibited myocardial fibrosis due to pressure overload for 4 weeks. Three hearts per group were used to determine the cross-sectional area of cardiac myocytes and the extent of myocardial fibrosis.

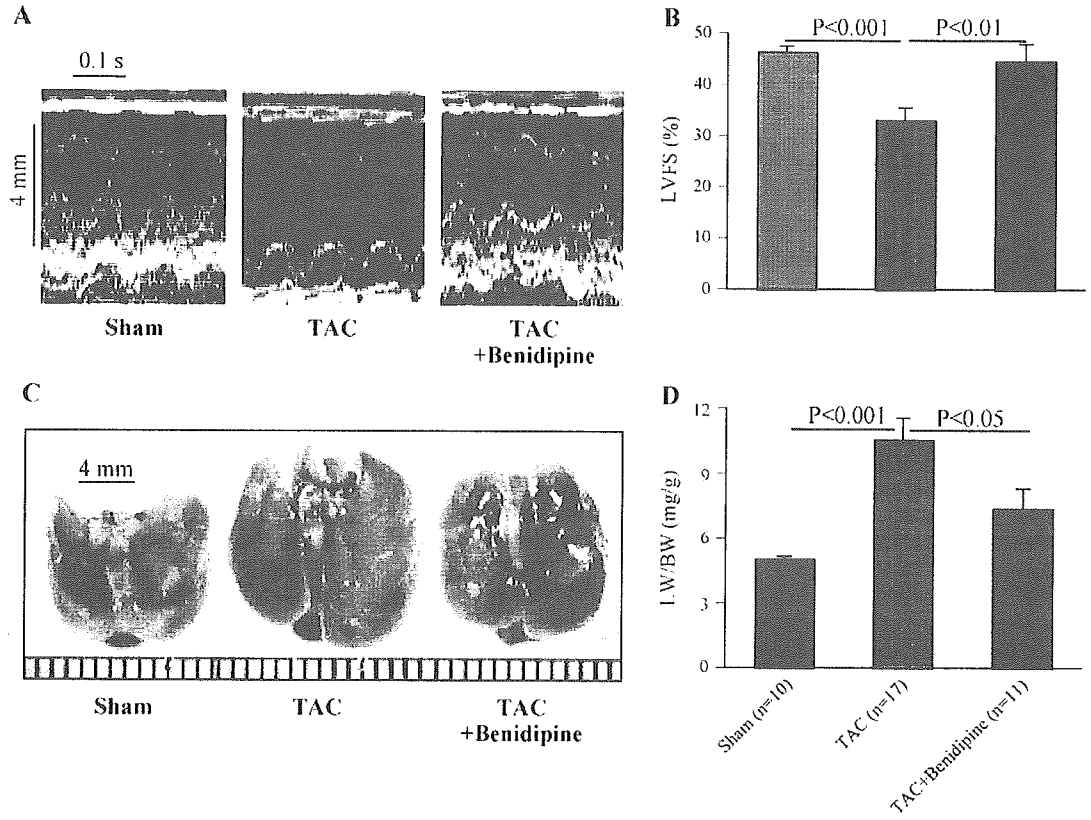

Fig. 5. Benidipine ameliorates heart failure induced by pressure overload. (A) Representative images obtained by echocardiography. (B) LV fractional shortening (LVFS) was increased by benidipine treatment. (C) Macroscopic views of lungs from each group. (D) The lung to body weight ratio (LW/BW) was significantly decreased in TAC mice treated with benidipine in comparison with untreated mice.

3.4. BNP, PIN, and procollagen IV are up-regulated in cardiac hypertrophy

Based on evidence from our laboratory and other investigators that BNP is an important molecular marker of cardiac hypertrophy or heart failure, and that both NO and fibrosis play an important role in cardiac remodeling, we assessed the expression of the BNP, PIN, and procollagen IV alpha genes in pressure-overloaded murine hearts, using microarray analysis. We found that a series of hypertrophy-related genes were up-regulated (Fig. 6A), including the BNP, PIN, and procollagen IV alpha genes, which were consistently up-regulated at both 1 and 4 weeks after TAC. Expression of calmodulin and five other procollagen genes was also increased by pressure overload (Fig. 6B).

3.5. Benidipine increases plasma NOx and down-regulates BNP, PIN, and procollagen IV alpha

As shown in Fig. 7A, the plasma level of NOx was markedly decreased in TAC mice at 4 weeks and was significantly increased in TAC mice treated with benidipine. Quantitative RT-PCR (Fig. 7B–D) demonstrated that benidipine decreased the level of BNP, a molecular marker for hypertrophy, and also down-regulated the expression of PIN and procollagen IV alpha$_1$. These changes supported our other findings in vitro and in vivo that benidipine inhibits cardiac hypertrophy and improves cardiac function partly by increasing the release of NO.

4. Discussion

4.1. Major findings

The present study is the first to evaluate the inhibitory effect of benidipine on cardiac remodeling induced by TAC in mice. The major findings of this study include the observations that (1) benidipine inhibits the increase of protein synthesis by cardiac myocyte stimulated by phenylephrine; (2) cardiac hypertrophy, myocardial fibrosis, and heart failure in pressure-overload mice were ameliorated by treatment with benidipine; and (3) an NO synthase inhibitor partially blocked the beneficial effect of benidipine on myocyte hypertrophy, while benidipine down-regulated protein inhibitor of neuronal nitric oxide synthase and increased the plasma NO level. These findings suggest that benidipine improves cardiac remodeling via an effect on the NO signaling pathway.

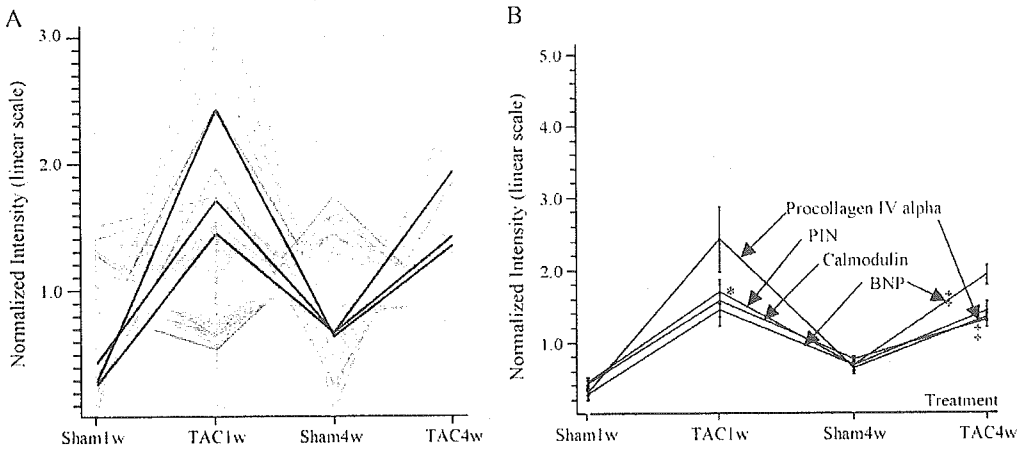

Fig. 6. cDNA microarray analysis of pressure-overload or sham-operated murine hearts. (A) From a total of 12,488 genes, three target genes were selected. These genes were functionally related to cardiac hypertrophy, heart failure, and nitric oxide signaling or fibrosis. (B) The three target genes were significantly up-regulated at 1 and 4 weeks after TAC relative to the levels in corresponding sham mice. Calmodulin and five other procollagen genes also showed up-regulation in response to pressure overload. The number of mice tested in each group was two. $^{*}P<0.05$ vs. sham at 1w, $^{\ddagger}P<0.05$ vs. sham at 4W (ANOVA).

4.2. Role of NO in cardiac remodeling

NO has been recognized as an important regulator of cardiac remodeling since it can influence both cardiac hypertrophy and heart failure. NO has been reported to exert an antihypertrophic effect in the hearts of spontaneously hypertensive rats without changing the blood pressure [18], which is in agreement with the results of this study. It is generally recognized that hemodynamic factors regulate cardiac myocyte hypertrophy [19], but exceptions have also been frequently reported. We previously reported that hydralazine significantly reduces the systemic blood pressure but does not have any effect on cardiac hypertrophy. In contrast, some drugs inhibit cardiac myocyte hypertrophy in the absence of a significant effect on hemodynamic, as we have reported previously [11,12,14]. Exogenous NO has

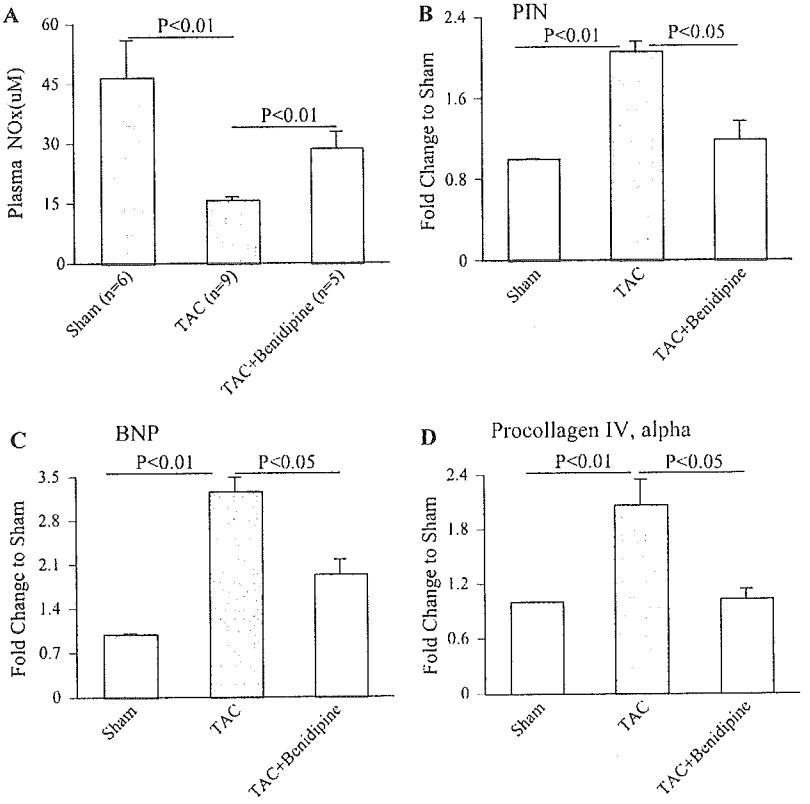

Fig. 7. Plasma nitric oxide level (A) and real-time PCR of the three target genes (B–D). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an endogenous control. PIN—protein inhibitor of neuronal nitric oxide synthase; BNP—natriuretic peptide precursor type B. $n=4$ per group for real-time PCR.

also been demonstrated to cause dose-dependent inhibition of alpha$_1$-adrenoceptor-stimulated protein synthesis in neonatal rat myocytes [7]. These results support our finding that benidipine caused a concentration-dependent decrease of PE (an alpha$_1$-adrenoceptor agonist)-stimulated protein synthesis by cardiac myocytes, and that this effect was blunted by NO synthase inhibitor. In addition, benidipine attenuated cardiac hypertrophy in pressure-overload mice without a significant change of blood pressure, and this antihypertrophic effect was at least partially mediated via the down-regulation of myocardial PIN. PIN has been demonstrated to regulate three types of NO synthase (NOS) [20]. Since both neural NOS (nNOS) and endothelial NOS (eNOS) are constitutively expressed in the myocardium, consistent up-regulation of PIN during the progression of cardiac hypertrophy, as noted in this study, is likely to decrease the release of NO. Interestingly, our data showed that benidipine significantly increased circulating NO levels, providing direct evidence for the abovementioned hypothesis that NO may play an important role in regulating cardiac hypertrophy. Although we did not monitor the blood concentration of benidipine, the dose that we used was effective for increasing the production of NO and consequently for attenuating cardiac hypertrophy.

We also found that benidipine could ameliorate progression from cardiac hypertrophy to heart failure, as confirmed by echocardiography, assessment of pulmonary congestion, and measurement of BNP expression. These results are partially attributable to the increase in NO production. Indeed, we have previously reported that benidipine increases coronary blood flow and reduces the severity of myocardial ischemia via an NO-dependent mechanism [5], and benidipine also improves cardiac remodeling induced by the eNOS inhibitor L-NAME [4]. Studies using genetically engineered mice have provided substantial evidence for a critical role of NO in cardiac remodeling. After myocardial infarction, LV dilation is more marked, heart function is more severely impaired, and long-term mortality is higher in eNOS-deficient mice compared with wild-type mice [8]. In contrast, congestive heart failure is less severe, and survival is increased in eNOS transgenic mice receiving coronary ligation [21]. It is worth noting that the preventive effect of benidipine on progression to heart failure may be secondary to its antihypertrophic effect. Further studies are needed to examine whether benidipine is effective in animals or humans with chronic heart failure.

4.3. Fibrosis and cardiac remodeling

Fibrosis of the myocardium plays a pivotal role in the process of cardiac remodeling. In the present study, we found that benidipine could significantly inhibit myocardial fibrosis in pressure-overload mice, a result that agrees with previous findings [9]. Although collagen type I and collagen type III produced by cardiac fibroblasts are the major components of the myocardial collagen matrix, type IV collagen is also expressed by both cardiac myocytes and fibroblasts and is a major component of the basement membrane [22,23]. Type IV collagen was reported to be increased in the hearts of diabetic rat [24] and is found in the fibrotic cardiac lesions of patients with DCM [25]. The angiotensin II-induced increase of fibronectin mRNA in the myocardium is accompanied by a similar increase of type I collagen, type IV collagen, and atrial natriuretic factor steady-state mRNA [26]. In this study, cDNA microarray analysis showed significant up-regulation of procollagen IV alpha at both 1 and 4 weeks after TAC, suggesting that this may be a potentially important gene in cardiac remodeling. Down-regulation of this gene by benidipine might have made an important contribution to the inhibition of cardiac remodeling.

4.4. Benidipine and cardiac sympathetic activity

Long-term cardiac sympathetic activation is detrimental to the heart, so one of the major aims of antihypertensive therapy is to reduce sympathetic tone. Differences in the formulations and pharmacokinetics of CCBs have various clinical influences, altering the effect of these drugs on blood pressure, heart rate, and cardiac sympathetic activity. Short-acting dihydropyridine CCBs enhance noradrenaline release from the sympathetic nerves [27]. In contrast, evidence suggests that long-acting calcium antagonists do not significantly affect sympathetic tone and may exert a more favorable clinical effect [28–30]. Our data showed that benidipine did not increase the heart rate. Moreover, benidipine prevented progression from cardiac hypertrophy to failure, suggesting that it does not enhance sympathetic tone. It is even possible that benidipine counteracts sympathetic activation in cardiac hypertrophy by increasing the release of NO because a reduced action of NO often contributes to overall sympathetic excitation in heart failure (review [31]).

4.5. Perspectives

In summary, this study provided evidence of the beneficial effect of a long-acting calcium antagonist, benidipine, on cardiac remodeling. Benidipine inhibited cardiac myocyte hypertrophy both in vitro and in vivo and also inhibited progression from cardiac hypertrophy to failure due to LV pressure overload. These effects were potentially mediated via an influence on the NO signaling pathway.

The question of whether CCB therapy increases cardiovascular events has attracted worldwide attention. Recent clinical trials have largely settled this question [29,30,32], but CCBs are still linked with a slightly increased risk of heart failure. However, the PRAISE trial revealed that amlodipine, a long-acting CCB, was not associated with increased mortality or morbidity in patients with severe

CHF [29]. Our studies and other investigations have consistently confirmed that amlodipine increases NO production [4,10,33,34]. Benidipine may also be beneficial for patients with hypertension-induced CHF, but a well-designed clinical trial is needed to investigate this point.

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Ablation of MEK Kinase 1 Suppresses Intimal Hyperplasia by Impairing Smooth Muscle Cell Migration and Urokinase Plasminogen Activator Expression in a Mouse Blood-Flow Cessation Model

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Background—Migration, proliferation, and matrix-degrading protease expression of smooth muscle cells (SMCs) are major features of intimal hyperplasia after vascular injury. Although MEK kinase 1 (MEKK1) has been shown to regulate cell migration and urokinase plasminogen activator (uPA) expression, the precise role of MEKK1 in this process remains unknown.

Methods and Results—We triggered a vascular remodeling model by complete ligation of the right common carotid artery in wild-type (WT) and MEKK1-null (MEKK1$^{-/-}$) mice. The intimal areas 28 days after ligation were significantly decreased in the ligated MEKK1$^{-/-}$ arteries compared with WT arteries (28 ± 8 versus 65 ± 17 μm^2, $P<0.05$). There were no differences in the ratios of proliferating cell nuclear antigen (PCNA)–positive cells to total cells within the arterial wall between WT and MEKK1$^{-/-}$ arteries. Proliferation capacity also did not differ between WT and MEKK1$^{-/-}$ cultured aortic smooth muscle cells (AoSMCs). In contrast, the number of intimal PCNA-positive cells 7 days after ligation was significantly smaller in MEKK1$^{-/-}$ arteries. Three different migration assays revealed that migration and invasion of MEKK1$^{-/-}$ AoSMCs were markedly impaired. Addition of full-length MEKK1 restored the migration capacity of MEKK1$^{-/-}$ AoSMCs. The number of MEKK1$^{-/-}$ AoSMCs showing lamellipodia formation by epithelial growth factor was significantly smaller compared with those of WT SMCs. Furthermore, uPA expression after ligation was markedly decreased in MEKK1$^{-/-}$ arteries.

Conclusions—MEKK1 is implicated in vascular remodeling after blood-flow cessation by regulating the migration and uPA expression of SMCs. MEKK1 is a potential target for drug development to prevent vascular remodeling. (***Circulation.*** **2005;111:1672-1678.)**

Key Words: remodeling ■ muscle, smooth ■ vasculature ■ restenosis

Blood vessels respond to damaging stimuli by activating a remodeling mechanism that leads to intimal hyperplasia.[1,2] Accumulating evidence has shown that the underlying causes of intimal hyperplasia are the invasion and proliferation of vascular smooth muscle cells (SMCs), both of which processes are triggered and controlled by numerous growth factors and mitogens.[2] Cell invasion involves migration by cytoskeletal reorganization[3] and activation of a cascade of proteases that degrade various extracellular matrix (ECM) components.[2] Urokinase-type plasminogen activator (uPA) is responsible for degradation of the ECM.[4] Recent studies in uPA-deficient mice have demonstrated that the number of neointimal SMCs after injury is markedly reduced compared with those in wild-type (WT) mice, suggesting that uPA plays a critical role in cell invasion during vascular remodeling.[5]

MEK kinase 1 (MEKK1) is a 196-kDa, mitogen-activated protein kinase kinase kinase (MAP3K) that acts as an upstream regulator of several MAPK pathways.[6–8] MEKK1 has been implicated in diverse and cell type–specific biological responses, including cardiac hypertrophy,[6] cell survival,[7]

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and apoptosis.[8] Recent studies on MEKK1-null ($MEKK1^{-/-}$) mice have uncovered a unique function for this protein kinase in cell migration.[9,10] $MEKK1^{-/-}$ mice were found to exhibit impairment of embryonic eyelid closure, a process involving epithelial cell migration.[9] MEKK1 is also involved in growth factor–induced embryonic stem cell migration and contributes to fibroblast and epithelial cell migration in vitro.[10] Furthermore, endogenous and overexpressed MEKK1 was reported to colocalize with the α-actinin cytoskeleton along actin stress fibers in focal adhesions.[11] Conversely, cytoskeletal reorganization can also lead to MEKK1 activation.[12] This close relationship between MEKK1 and the cytoskeleton implies that MEKK1 may be essential for regulating morphological changes such as the formation of lamellipodia, which proceeds to cell migration.[3,13,14] Furthermore, MEKK1 is necessary for uPA upregulation in response to fibroblast growth factor-2 (FGF-2).[15] These findings indicate that MEKK1 might play an important role in SMC migration and uPA production, both of which will affect SMC invasion during vascular remodeling. Herein, using a blood-flow cessation model in mice with targeted ablation of the *MEKK1* gene, we tested our hypothesis that MEKK1 plays a pivotal pathophysiological role in arterial remodeling by regulating SMC migration and uPA expression.

Methods

Animals and Blood-Flow Cessation Model

All animal studies were conducted in accordance with guidelines of the National Institutes of Health (Bethesda, Md) and institutional Animal Care and Use Committees. $MEKK1^{-/-}$ mice were generated as described previously.[9] The blood-flow cessation model was performed as reported previously.[16] Carotid arteries were harvested immediately for biochemical analysis or after fixation for morphological and immunohistological analysis 1, 3, 7, and 28 days after surgery.

Morphometric and Histological Examinations

Standard hematoxylin-eosin staining, elastica van Gieson's staining, immunostaining for uPA (1:100, American Diagnostica), and proliferating cell nuclear antigen (PCNA) staining (1:50, Santa Cruz) were performed on serial sections (5 μm) within 0.5 mm proximal to the site of ligation of right common carotid arteries, as reported previously.[17,18] The luminal, internal elastic lamina (IEL), and external elastic lamina areas were measured with Scion Image software (Scion Corp). Morphological parameters were calculated as described previously.[19] In brief, the intimal area was calculated as the IEL area minus luminal area, and the medial area was the external elastic lamina area minus IEL area. The ratio of intima to media area (I/M) was calculated as intimal area/medial area, and the stenotic ratio was calculated as the intimal area/IEL area×100.

Primary Culture of AoSMCs and Transfection

Primary culture of aortic SMCs (AoSMCs) was performed and characterized as described previously.[20] Cells were identified by positive immunostaining for α-smooth muscle actin (American Research Products). AoSMCs were transiently transfected with lipofectamine and either a full-length form of MEKK1 vector (MEKK1FL) or an empty vector (pcDNA3.1).[21] After 48 hours, SMCs were studied in migration assays or for uPA expression.

AoSMC Proliferation Assay

AoSMCs (5×10^3) were cultured for 24 hours in 96-well plates with or without epithelial growth factor (EGF, 20 ng/mL), FGF-2 (20 ng/mL, R&D Systems, Inc), or platelet-derived growth factor-BB (PDGF-BB, 25 ng/mL, Sigma). Then, [^{3}H]thymidine incorporation and cell number were measured, as previously reported.[22]

Quantification of Scrape Wound–Induced Migration Assay

Dense monolayers of overconfluent AoSMCs grown on Laboratory-Tek chamber slides were scraped with a sterile scraper as described previously.[23] After the wound was created, cells were incubated for 24 hours with or without FGF-2 (20 ng/mL) and visualized with rhodamine-phalloidin (R-451, Molecular Probes). The number of and distance that AoSMCs migrated from the wound line were calculated as the mean of 6 different fields.

Aortic Explant Migration Assay

Preparation of aortic explants was performed as described previously.[24] Explants were counted as positive when >1 cell was observed and identified by positive staining for α-smooth muscle actin.

Transwell Matrigel-Coated Chamber Invasion Assay

Cell invasion was analyzed with a BioCoat Matrigel invasion chamber (BD Biosciences Corp), as described previously.[25] Inserts without a Matrigel coating were used as controls. FGF-2 (20 ng/mL) was added to the lower chambers as a chemoattractant. The number of invading cells was manually counted per high-power field for each condition (10 fields for each filter). The percentage of invasion was calculated as (invading cells in Matrigel inserts/migrated cells of control inserts) × 100.

Immunofluorescence Confocal Microscopy

Immunofluorescence staining with rhodamine-phalloidin and a monoclonal antibody for uPA (1:50) was performed as described previously.[26] Staining was examined with a Nikon Eclipase TE2000-U confocal scanning electron microscope.

Protein Extraction and Western Blotting Analysis

Protein extraction and immunoblotting were performed as described previously.[15,21,22] Protein phosphorylation levels were normalized to the matching densitometric values of nonphosphorylated proteins.

Statistical Analysis

All data were expressed as mean±SEM. Significant differences were analyzed by an unpaired Student *t* test, Fisher exact test, or ANOVA followed by the Bonferroni post hoc test. A value of $P<0.05$ was considered statistically significant.

Results

MEKK1 Ablation Inhibits Intimal Hyperplasia

There was no intimal thickening in unligated arteries of either WT or $MEKK1^{-/-}$ mice. Significant neointimal growth was observed 28 days after ligation in WT mice, whereas it was much less in $MEKK1^{-/-}$ mice (Figure 1A). Morphometric analysis of ligated arteries on day 28 revealed that the average intimal area was significantly smaller in $MEKK1^{-/-}$ mice than in WT mice, whereas no difference in medial area was observed between WT and $MEKK1^{-/-}$ mice (Figure 1B). Consequently, I-M ratios and stenotic ratios were significantly decreased in $MEKK1^{-/-}$ mice compared with those in WT mice (Figure 1B).

MEKK1 Ablation Prevented Increases in the Numbers of Intimal PCNA-Positive Cells

In both WT and $MEKK1^{-/-}$ mice, the ratios of PCNA-positive cells to total cells within the arterial wall, intima, and media were significantly increased 3 days after ligation