

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 μ m, 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 \pm 5	23 \pm 0.2	112 \pm 4	644 \pm 26
TAC (n = 6)	157 \pm 9 ^b	22.4 \pm 0.3	100 \pm 5 ^b	670 \pm 24
TAC+amlodipine (n = 6)	161 \pm 8 ^b	20.1 \pm 0.8 ^{b,c}	93 \pm 3 ^b	675 \pm 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.

Acknowledgments

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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 dem-

onstrated 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 [³H] 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 [³H] 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 previously [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 (NO₂+NO₃) 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 NO₂ to NO₃, 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 [³H] leucine uptake by cardiac myocytes, but it inhibited PE-induced

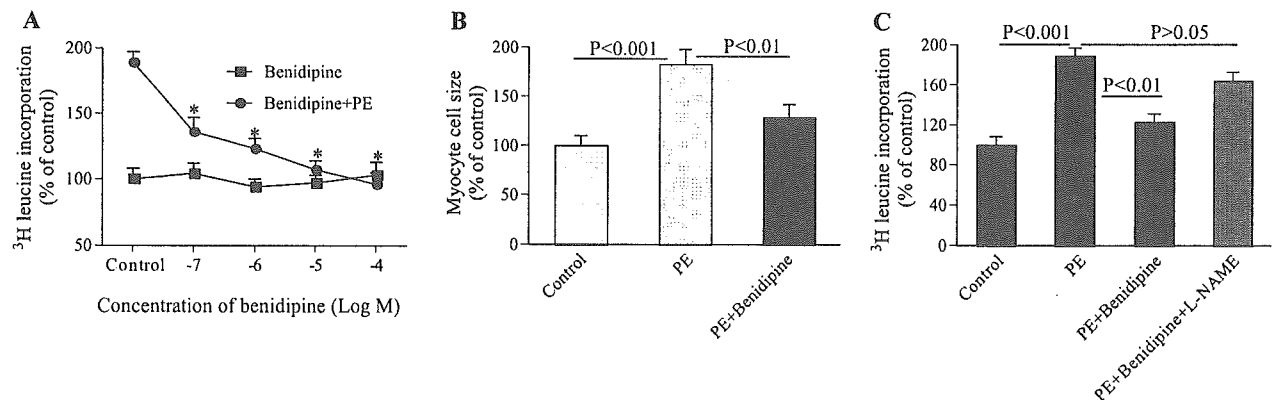


Fig. 1. Effect of benidipine on protein synthesis and the size of neonatal rat cardiac myocytes. (A) Protein synthesis stimulated by 10^{-4} M phenylephrine (PE) was inhibited by benidipine at concentrations ranging from 10^{-7} – 10^{-4} M in a dose-independent fashion, and this concentration range 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).

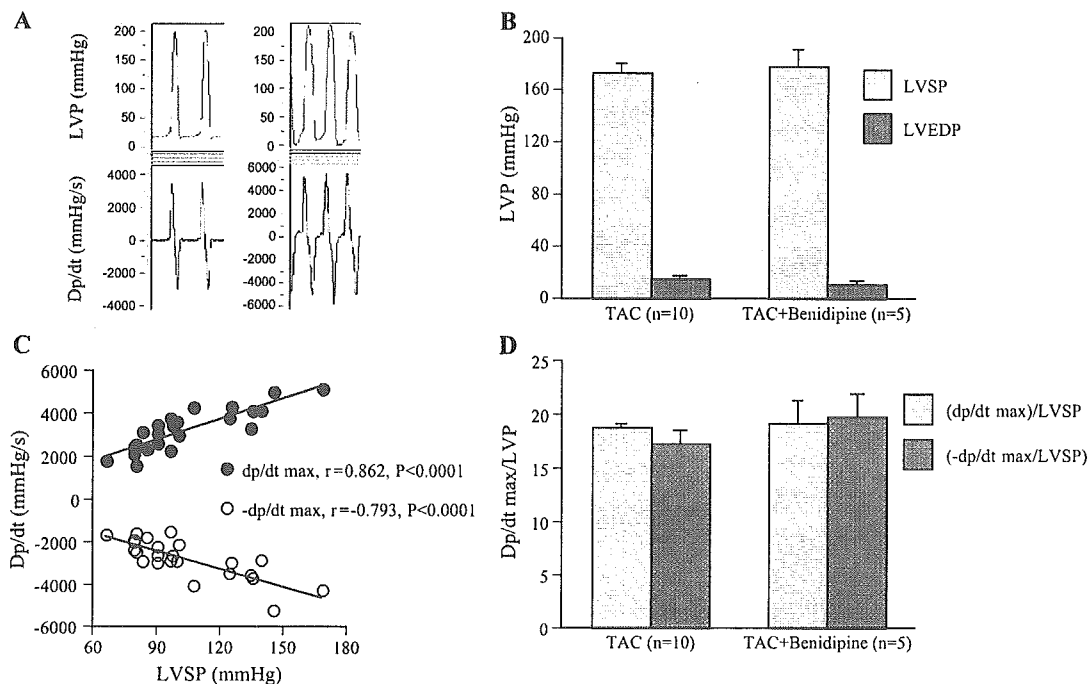


Fig. 2. Left ventricular (LV) hemodynamics measured with a Millar Catheter at 4 weeks after TAC. (A) LV pressure and dp/dt in the TAC and benidipine groups. (B) No significant differences of LV systolic pressure (LVSP) and LV end-diastolic pressure (LVEDP) were noted between TAC mice with or without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

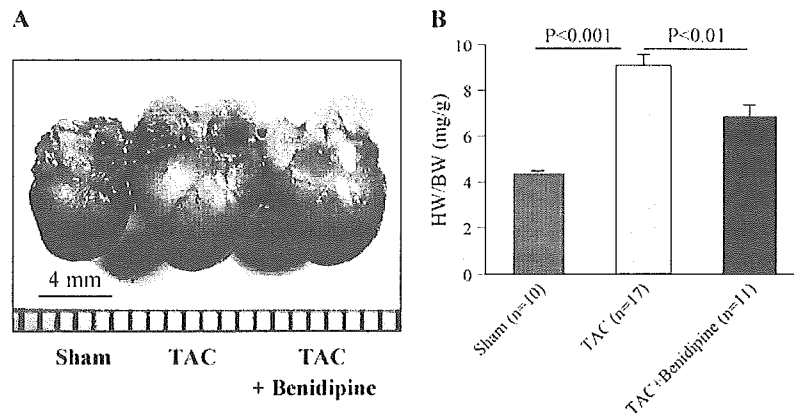


Fig. 3. Benidipine inhibits cardiac remodeling. (A) Representative pictures of whole hearts. (B) The heart to body weight ratio (HW/BW) was significantly decreased in TAC mice treated with benidipine (10 mg/kg/d) compared with untreated TAC mice.

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).

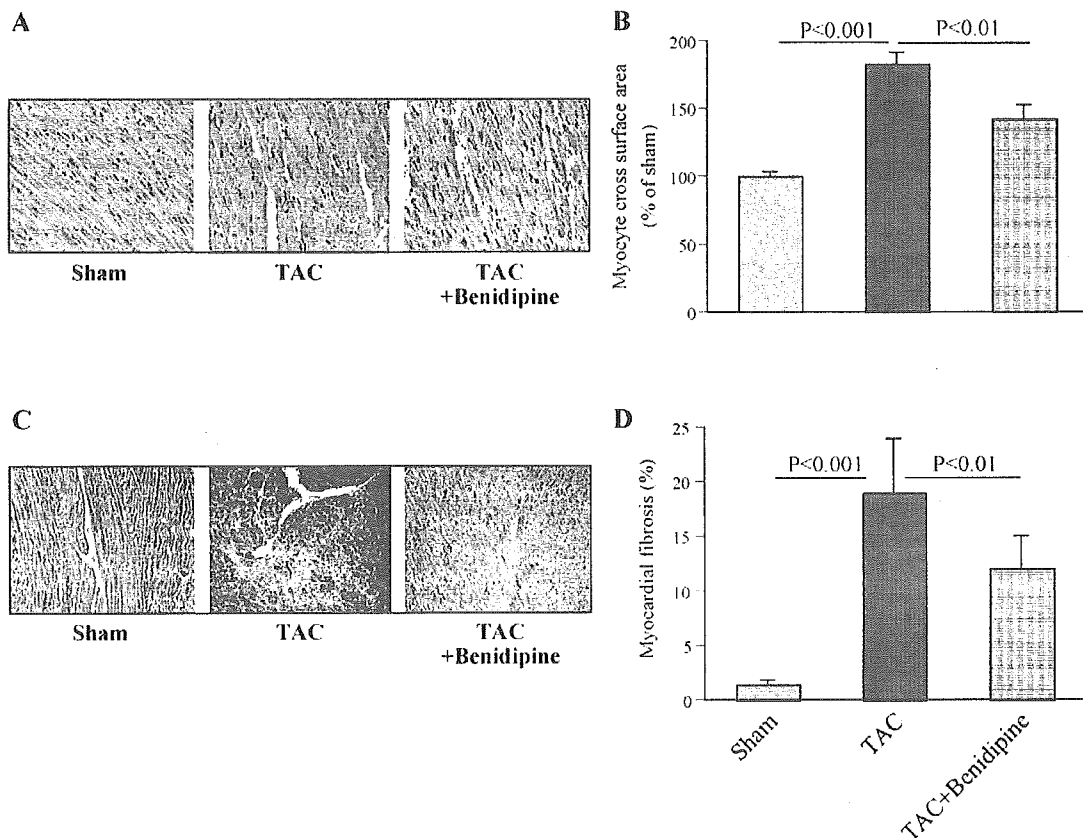


Fig. 4. Results of histological examination. (A) Representative images of the myocardium (HE stain×200). (B) The cross-sectional area of cardiac myocytes was significantly increased in TAC mice by pressure overload for 4 weeks, while treatment with benidipine blunted the enlargement of myocytes. (C) 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.

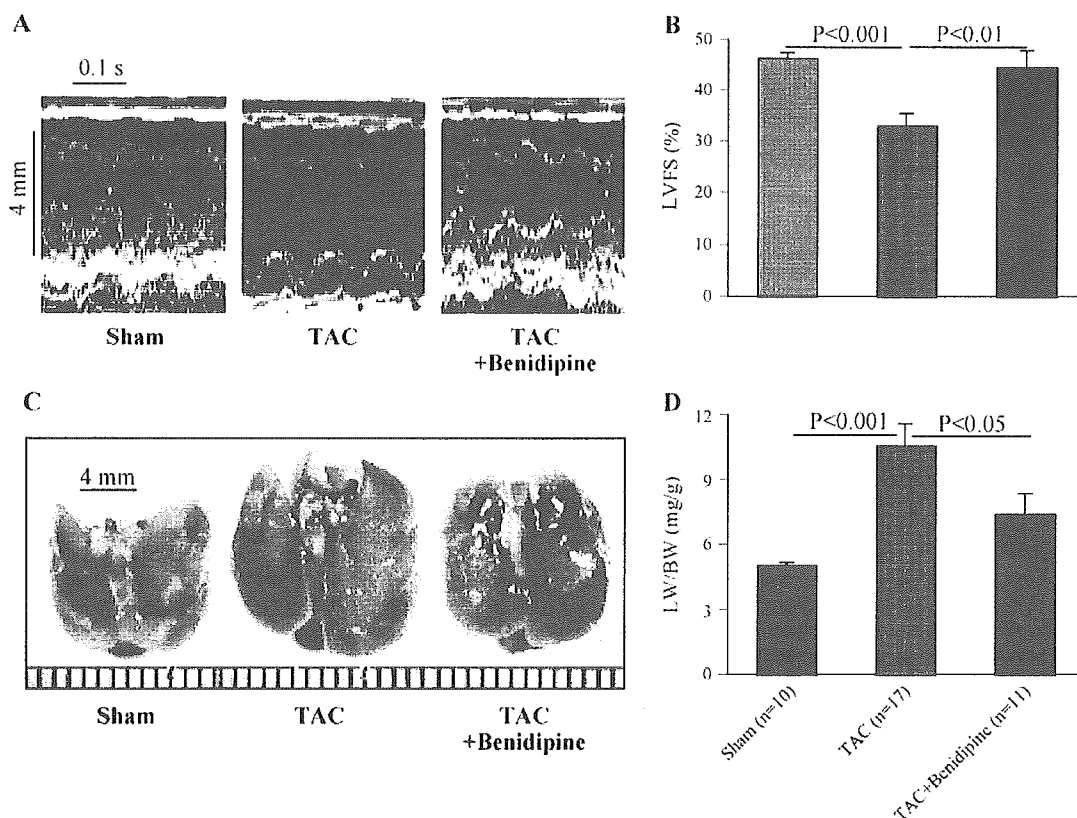


Fig. 5. Benidipine ameliorates heart failure induced by pressure overload. (A) Representative images obtained by echocardiography. (B) LV fractional shortening (LVFS) was increased by benidipine treatment. (C) Macroscopic views of lungs from each group. (D) The lung to body weight ratio (LW/BW) was significantly 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 alpha₁. 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.

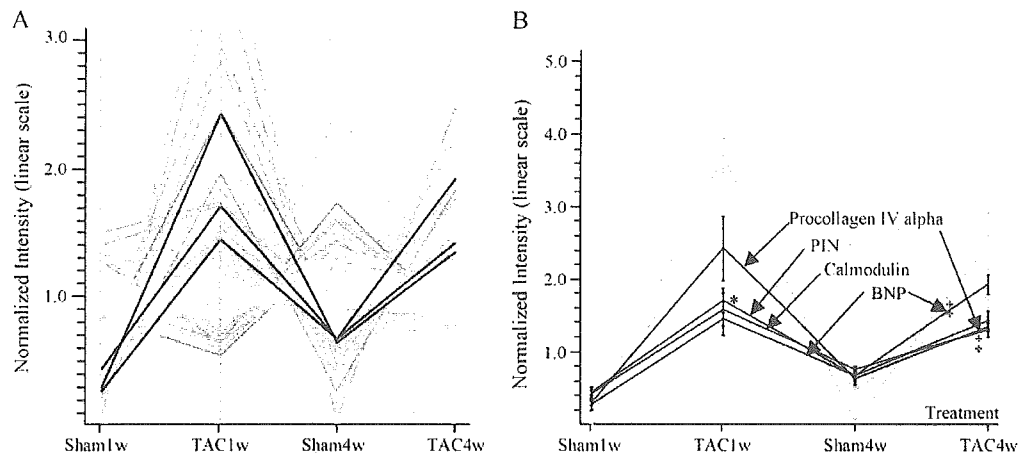


Fig. 6. cDNA microarray analysis of pressure-overload or sham-operated murine hearts. (A) From a total of 12,488 genes, three target genes were selected. These genes were functionally related to cardiac hypertrophy, heart failure, and nitric oxide signaling or fibrosis. (B) The three target genes were 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, † $P < 0.05$ 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

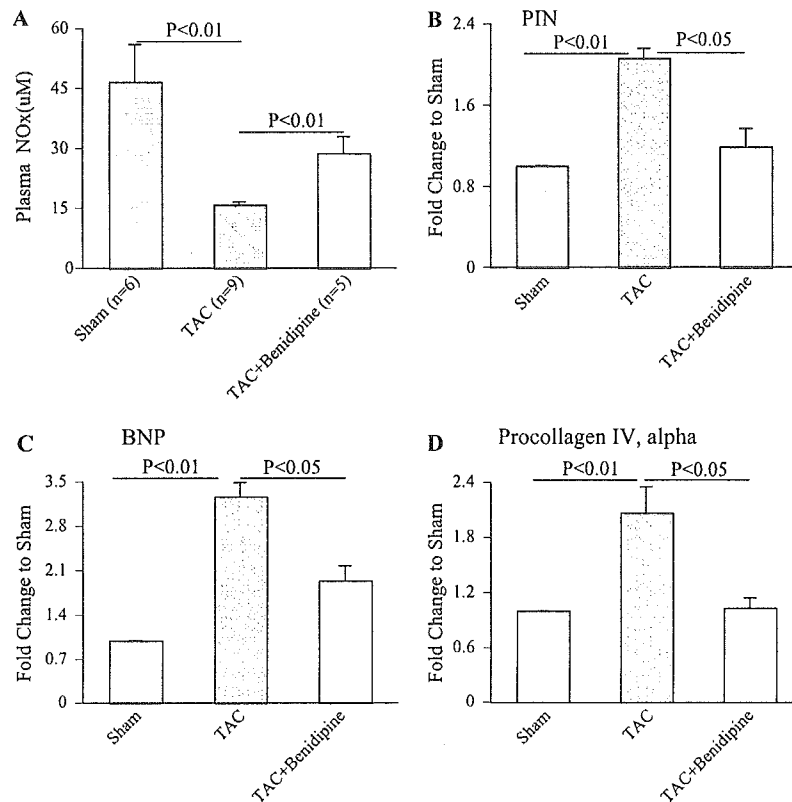


Fig. 7. Plasma nitric oxide level (A) and real-time PCR of the three target genes (B–D). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an endogenous control. PIN—protein inhibitor of neuronal nitric oxide synthase; BNP—natriuretic peptide precursor type B. $n = 4$ per group for real-time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

also been demonstrated to cause dose-dependent inhibition of α_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 α_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 μm², *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 reorganization³ 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 μ m) 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 \times 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, [³H]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) \times 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 Eclipse 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 \pm 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

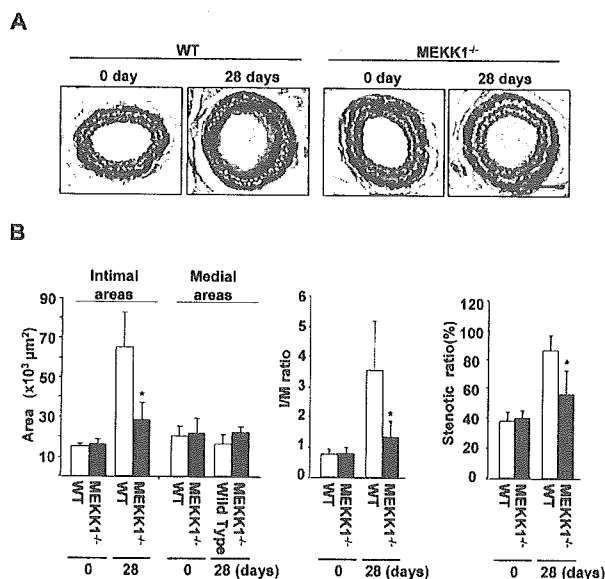


Figure 1. Photomicrographs and morphometry of intimal formation after ligation of carotid arteries. **A**, Representative cross sections of unligated and ligated carotid arteries on day 28 (elastica van Gieson's stain). Bar indicates 100 μm. **B**, Quantitative analysis of intimal and medial areas, I-M ratio, and stenotic ratio of ligated arteries. * $P < 0.05$ vs WT mice.

compared with those 1 day after ligation, but there were no significant differences between WT and MEKK1^{-/-} mice. It is noteworthy that 7 days after ligation, the number of PCNA-positive cells in the intima was significantly less in MEKK1^{-/-} arteries than in WT arteries, although there was no difference within other areas (Figure 2A and 2B). These findings suggest that migration of PCNA-positive cells from the media to the intima was impaired, whereas proliferation was not impaired in MEKK1^{-/-} mice. Consistent with *in vivo* data, no difference was observed in [³H]thymidine incorporation and cell number between WT and MEKK1^{-/-} AoSMCs after stimulation with PDGF-BB *in vitro* (Figure 2C). Treatment with EGF or FGF-2 instead of PDGF-BB yielded identical results (data not shown).

Effects of Ablation of MEKK1 on MAPK Activities of AoSMCs

Ablation of MEKK1 does not affect the total protein expression of extracellular signal-regulated kinase (ERK), c-Jun NH₂-terminal kinase (JNK), or p38. We examined the effects of ablation of MEKK1 on MAPK activities of AoSMCs. FGF-2-induced JNK and ERK but not p38 activation in AoSMCs from MEKK1^{-/-} mice was less than that from WT mice (Figure 3A). We confirmed that addition of MEKK1 restored MEKK1 protein levels in MEKK1^{-/-} AoSMCs (Figure 3B). Addition of MEKK1 restored JNK and ERK activation in response to FGF-2 in MEKK1^{-/-} AoSMCs (Figure 3C and 3D). Treatment with EGF or PDGF-BB instead of FGF-2 yielded identical results (data not shown).

MEKK1 Ablation Inhibited AoSMC Migration and Invasion

In the scrape wound-induced migration assays, the average number of and distance that MEKK1^{-/-} AoSMCs migrated

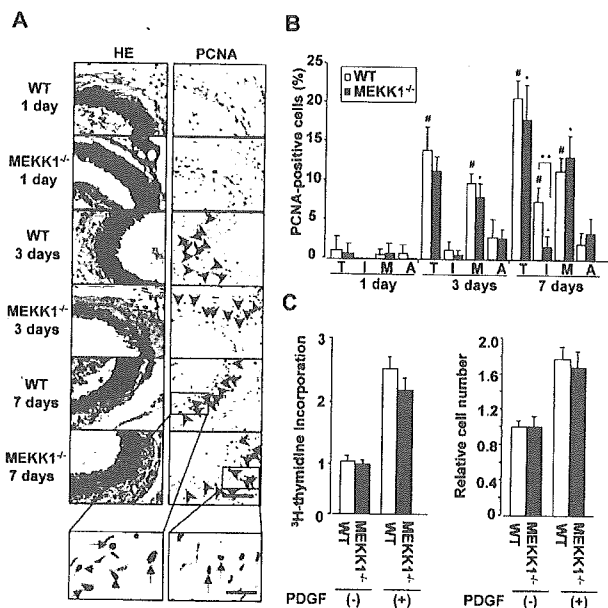


Figure 2. Effects of ablation of MEKK1 on cell proliferation. **A**, Immunohistochemical analysis of hematoxylin and eosin staining and PCNA staining 1, 3, and 7 days after ligation. Bar indicates 100 μm. Photographs of WT and MEKK1^{-/-} mice 7 days after ligation were enlarged, and bar indicates 30 μm. Red arrowheads indicate PCNA-positive cells. **B**, Quantification of ratio of PCNA-positive cells to total cell number in arterial wall (T), intima (I), media (M), and adventitia (A) of ligated arteries at 1, 3, and 7 days. Black bars and white bars indicate WT and MEKK1^{-/-} mice, respectively. # $P < 0.05$ vs day 1 in WT mice; * $P < 0.05$ vs day 1 in MEKK1^{-/-} mice; ** $P < 0.05$ vs day 7 in WT mice. **C**, Effects of MEKK1 on PDGF-BB-induced [³H]thymidine incorporation and cell number in AoSMCs. Data are indicated as percentages relative to control group and are mean ± SEM of 10 wells in each group of at least 3 replicates.

from the wound edge (white dotted line) in the absence of any stimulus was similar to those in WT mice; however, the FGF-2-induced increase in cell number and distance was significantly suppressed in MEKK1^{-/-} AoSMCs compared with WT cells. Addition of MEKK1 restored the number and distance of MEKK1^{-/-} AoSMCs to normal levels (Figure 4A and 4B).

In the aortic explant assays, the number of AoSMCs migrating from MEKK1^{-/-} explants at 10 days was considerably lower than those from WT explants (Figure 4C). Moreover, the number of MEKK1^{-/-} aortic explants that showed migrating cells was significantly smaller than in WT explants (27.2 ± 2.2% versus 60.0 ± 2.5%, $P < 0.05$).

In the transwell Matrigel-coated chamber invasion assays, the number of invading MEKK1^{-/-} AoSMCs in response to FGF-2 was suppressed markedly compared with WT cells. Addition of MEKK1 restored the number of MEKK1^{-/-} AoSMCs to normal levels (Figure 4D). Treatment with EGF or PDGF-BB instead of FGF-2 yielded identical results (data not shown).

MEKK1 Ablation Impaired Lamellipodia Formation

There were no morphological differences between WT and MEKK1^{-/-} AoSMCs under basal conditions; however, typi-

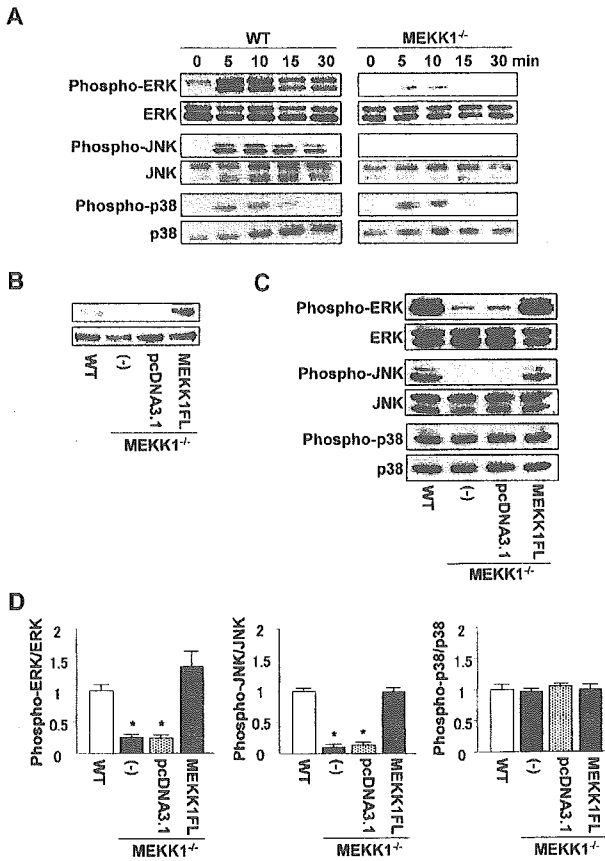


Figure 3. Effects of ablation of MEKK1 on MAPK activities in AoSMCs. A, Time course of JNK, ERK, and p38 activities followed by FGF-2 stimulation. B, Addition of MEKK1 experiments in MEKK1^{-/-} AoSMCs. C, Western blot analysis of MAPKs in MEKK1^{-/-} AoSMCs transfected with empty vector (pcDNA3.1) or full-length forms of MEKK1 vector (MEKK1FL) followed by FGF-2 stimulation. D, Cumulative data for MAPK in Figure 3C. **P*<0.05 vs WT AoSMCs.

cal lamellipodia formation was induced by treatment with EGF in WT AoSMCs but was seldom induced in MEKK1^{-/-} AoSMCs (Figure 5A). The percentage of MEKK1^{-/-} AoSMCs showing lamellipodia was significantly lower than in WT AoSMCs (*P*<0.05). Addition of MEKK1 restored the lamellipodia-forming capacity of MEKK1^{-/-} AoSMCs (Figure 5B). Treatment with FGF-2 or PDGF-BB instead of EGF yielded identical results (data not shown).

MEKK1 Ablation Decreased uPA Expression

uPA expression began to increase on day 1 and reached a peak on day 3, after which it decreased gradually by 7 days after ligation in WT mice. In contrast, there was only weak positive staining for uPA up to 7 days in MEKK1^{-/-} mice (Figure 6A). In vitro immunofluorescence staining revealed that PDGF-BB-induced uPA expression was decreased in MEKK1^{-/-} AoSMCs compared with those of WT mice (Figure 6B). Western blotting showed a dramatic reduction of FGF-2-induced uPA expression in MEKK1^{-/-} AoSMCs, which was restored by addition of MEKK1 (Figure 6C).

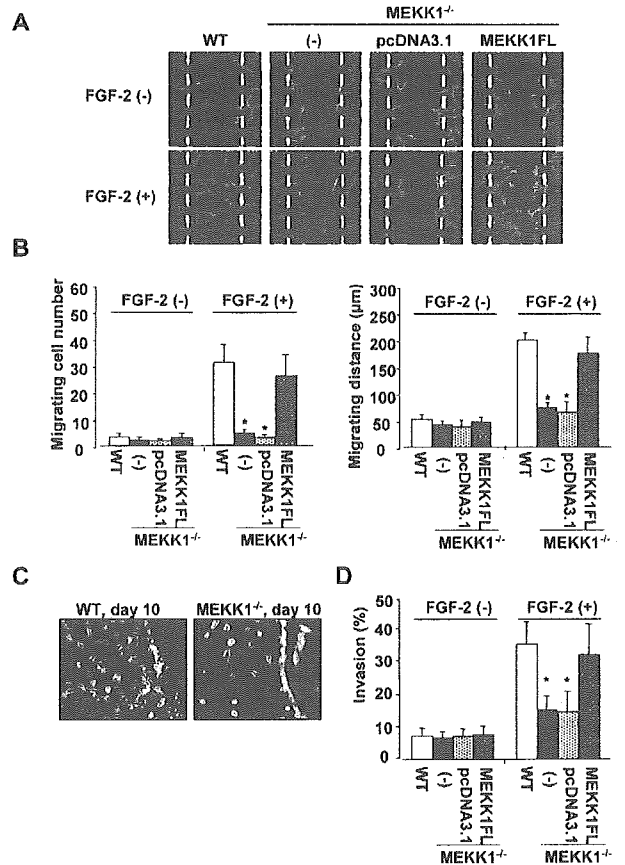


Figure 4. Migration and invasion of AoSMCs. A, Scrape wound-induced migration assay. Immunofluorescence microscopy of migrating cells from wound edge when AoSMCs were cultured for 24 hours with or without FGF-2. F-actin was stained with rhodamine-phalloidin (red), and nuclei were counterstained with DAPI (blue; not visible). B, Quantification of means of number and distance of migrating cells. **P*<0.05 vs WT AoSMCs. C, Migration of AoSMCs from aortic explants on day 10. D, Quantification of percent invasion of AoSMCs by transwell Matrigel-coated chamber invasion assay. **P*<0.05 vs WT AoSMCs.

Discussion

Several lines of evidence suggest that MEKK1 is implicated in diverse biological responses.⁶⁻⁸ Recently, the unique role of MEKK1 in regulating the migration of several cell types has aroused widespread attention^{9,12,27} and inspired us to study its involvement in cardiovascular diseases. Up to now, there has been no previous report on the role of MEKK1 in the development of vascular remodeling, during which invasion and proliferation of SMCs play key roles. In the present study, we investigated the mechanism whereby MEKK1 regulates vascular remodeling in a well-established, blood-flow cessation model in MEKK1^{-/-} mice. We found that MEKK1 is essential for intimal hyperplasia after cessation of blood flow.

In this study, we clearly demonstrated that intimal areas, I-M ratios, and stenotic ratios of the ligated arteries were significantly lower in MEKK1^{-/-} mice relative to WT mice, indicating that MEKK1 is implicated in intimal hyperplasia. Although an angioplasty/balloon injury model would have yielded greater applicability to the clinical situation, we used

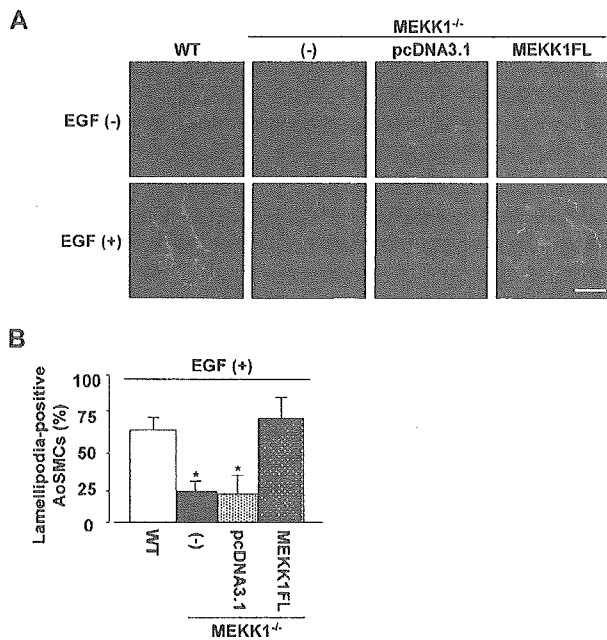


Figure 5. Lamellipodia formation in AoSMCs. A, Representative photomicrographs of lamellipodia formation in AoSMCs with or without EGF stimulation. Bar indicates 25 μ m. B, Percentage of lamellipodia-positive cells (n=100) in AoSMCs with EGF. *P<0.05 vs WT AoSMCs.

the ligation model because of its excellent reproducibility. To clarify the mechanism(s) of MEKK1 involvement, we first examined cell proliferation within the arterial wall after ligation and found no statistically significant difference in the

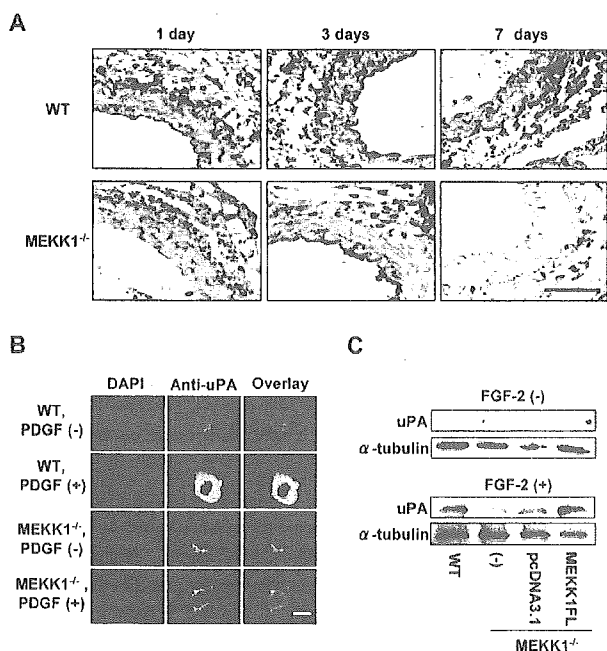


Figure 6. Expression of uPA in AoSMCs. A, Immunohistochemical analysis of time course of uPA expression 1, 3, and 7 days after ligation of carotid arteries. Bar indicates 100 μ m. B, Immunofluorescence staining for uPA in AoSMCs stimulated with PDGF-BB. Bar indicates 10 μ m. C, Western blots of uPA in AoSMCs stimulated with FGF-2.

number of PCNA-positive cells between WT and MEKK1^{-/-} mice. Results from this ligation model are consistent with the fact that MEKK1^{-/-} mice have no overt defects in growth and fertility.⁹ There was also no difference in the proliferation of WT and MEKK1^{-/-} AoSMCs evaluated by [³H]thymidine incorporation and cell number. These findings suggest that SMC proliferation might not contribute to the reduced intimal hyperplasia in MEKK1^{-/-} mice. Then we investigated SMC migration and uPA expression, both of which are important factors for intimal hyperplasia.

We demonstrated that there were significantly fewer intimal PCNA-positive cells 7 days after ligation in MEKK1^{-/-} mice than in WT mice. Because PCNA-positive cells are believed to migrate from the media to the intima,² this finding suggests that SMC migration is impaired in MEKK1^{-/-} mice. To directly assess the effects of MEKK1 ablation on SMC migration, we used several migration and invasion assays in vitro. We observed comparable migration or invasion of SMCs under control condition between WT and MEKK1^{-/-} AoSMCs; however, significant impairment of migration or invasion was observed after stimulation with FGF-2 in MEKK1^{-/-} AoSMCs, which was restored by addition of full-length MEKK1. These findings indicate that ablation of MEKK1 impairs invasion and migration, both of which may contribute to reduced intimal hyperplasia wherein growth factors may play a vital role.

Lamellipodia formation is essential for cell migration.¹³ Exogenous stimuli, such as PDGF or FGF-2, induce lamellipodia formation that can help to complete the first step of the motility cycle.^{14,28} Therefore, we examined whether MEKK1 is involved in lamellipodia formation in AoSMCs. Our results showed that the percentage of lamellipodia-positive cells was significantly smaller in MEKK1^{-/-} AoSMCs compared with WT AoSMCs in the presence of EGF. This finding suggests that the impairment of migration in MEKK1^{-/-} AoSMCs may be due to inhibited formation of lamellipodia. Indeed, addition of MEKK1 to MEKK1^{-/-} AoSMCs recovered their capacity to form lamellipodia and migrate. On the other hand, it has been reported that loss of MEKK1 disrupts focal adhesion composition, with decreased vinculin content and focal adhesion kinase (FAK) cleavage.²⁹ Because disruption of focal adhesion composition will affect cell migration, further investigation will be needed to clarify its role in MEKK1^{-/-} mice.

There is evidence that uPA is induced after arterial injury.^{5,28,30,31} uPA enhances vascular remodeling by transforming plasminogen into plasmin, which can activate metalloproteinases and in turn degrade ECM proteins.^{5,30,31} We found that uPA expression began to increase at 1 day and reached a peak 3 days after ligation in WT mice, whereas uPA staining appeared to be significantly lower in MEKK1^{-/-} arteries at corresponding times. In vitro, PDGF-BB- and FGF-2-induced uPA expression as detected by immunofluorescence staining and Western blotting was also significantly decreased in MEKK1^{-/-} AoSMCs. Thus, in addition to impaired lamellipodia formation, inhibited uPA expression by MEKK1 ablation may also contribute to impairment of SMC invasion. Addition of full-length MEKK1 restored uPA expression in MEKK1^{-/-} AoSMCs.

Although it has been reported that MEKK1 is required for FGF-2-induced signals to control uPA expression in fibroblasts,¹⁵ further investigations will be needed to elucidate the mechanism by which MEKK1 regulates uPA expression in arteries after ligation.

Recent studies have demonstrated that JNK and ERK transduction pathways may regulate cell migration^{32,33} and uPA expression.^{34,35} In the present study, we demonstrated that JNK and ERK activation after growth factor stimulation was blunted in MEKK1^{-/-} AoSMCs. Thus, it is possible that ablation of MEKK1 may inhibit cell migration and uPA expression by interfering with the downstream signaling pathways JNK and/or ERK. MEKK1 also has been reported to be associated with cytoskeletal reorganization^{11,12} and to be necessary for uPA upregulation,¹⁵ suggesting another possibility that ablation of MEKK1 directly inhibits lamellipodia formation and uPA expression. The stimulus for remodeling after ligation is also influenced by the resultant vascular ischemia. Because MEKK1 is activated by hypoxic stimuli as well as growth factors,³⁶ we must consider the possibility that the resultant hypoxic stimuli are also important during vascular remodeling in the ligation model.

Izumi et al²² demonstrated that activation of apoptosis signal-regulating kinase 1 (ASK1), another member of the MAP3K family, also plays a key role during intimal hyperplasia in the carotid artery balloon injury model. Unlike MEKK1, ablation of ASK1 blunted both JNK and p38 but not ERK activation in AoSMCs after serum stimulation. In addition, ablation of ASK1 caused impairment of both SMC migration and proliferation. Thus, although the methods or models used to evaluate functions of MEKK1 and ASK1 were not the same, both MEKK1 and ASK1 may contribute to the development of intimal hyperplasia by different mechanisms.

In conclusion, we have demonstrated that MEKK1 plays a critical role during intimal hyperplasia in a mouse carotid blood-flow cessation model. Intimal hyperplasia is greatly lessened, possibly due to a reduction of SMC invasion by an impairment of their migration and reduced uPA expression. We propose that MEKK1 is a potential target for drug development to prevent vascular remodeling.

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Aldosterone Nongenomically Worsens Ischemia Via Protein Kinase C-Dependent Pathways in Hypoperfused Canine Hearts

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Abstract—Rapid nongenomic actions of aldosterone independent of mineralocorticoid receptors (MRs) on vascular tone are divergent. Until now, the rapid nongenomic actions of aldosterone on vascular tone of coronary artery and cardiac function in the in vivo ischemic hearts were not still fully estimated. Furthermore, although aldosterone can modulate protein kinase C (PKC) activity, there is no clear consensus whether PKC is involved in the nongenomic actions of aldosterone on the ischemic hearts. In open chest dogs, the selective infusion of aldosterone into the left anterior descending coronary artery (LAD) reduced coronary blood flow (CBF) in the nonischemic hearts in a dose-dependent manner. Also, in the ischemic state that CBF was decreased to 33% of the baseline, the intracoronary administration of aldosterone (0.1 nmol/L) rapidly decreased CBF (37.4 ± 3.8 to 19.3 ± 5.2 mL/100 g/min; $P < 0.05$), along with decreases in fractional shortening (FS) (8.4 ± 0.7 to $5.4 \pm 0.4\%$; $P < 0.05$) and lactate extraction rate (LER) (-31.7 ± 2.9 to $-41.4 \pm 3.7\%$; $P < 0.05$). The decrease in CBF was reproduced by the infusion of bovine serum albumin-conjugated aldosterone. Notably, these aldosterone-induced deteriorations of myocardial contractile and metabolic functions were blunted by the co-administration of GF109203X, an inhibitor of PKC, but not spironolactone. In addition, aldosterone activated vascular PKC. These results indicate that aldosterone nongenomically induces vasoconstriction via PKC-dependent pathways possibly through membrane receptors, which leads to the worsening of the cardiac contractile and metabolic functions in the ischemic hearts. Elevation of plasma or cardiac aldosterone levels may be deleterious to ischemic heart disease through its nongenomic effects. (*Hypertension*. 2005;46:113-117.)

Key Words: aldosterone ■ ischemia ■ protein kinases

Aldosterone modulates cardiovascular function in addition to the crucial role in sodium and potassium homeostasis through binding to the intracellular mineralocorticoid receptors (MRs).¹ These receptor-mediated effects with transcriptional modulation of target genes are termed genomic effects.² Recently, another pathway possibly mediated by the specific membrane receptors through which aldosterone acts, ie, nongenomic effects of aldosterone, has been investigated.²⁻⁴ Although this nongenomic action of aldosterone was shown in a variety of tissues such as vascular smooth muscle cells or cardiomyocytes, the effects of aldosterone on vascular tone are divergent.⁴⁻⁹ Aldosterone increased systemic vascular resistance in humans, as shown in the first report on the nongenomic effects of aldosterone, whereas aldosterone inhibits vasoconstriction in renal afferent arterioles.⁷⁻⁹ Until now, the rapid nongenomic effects of aldosterone on vascular tone of coronary artery and cardiac

functions in vivo under ischemia are not still fully estimated. Moreover, several rapid aldosterone-induced changes of the concentration of intracellular second messengers have been described.^{6,10,11} Aldosterone is reported to activate protein kinase C (PKC) in distal colon cells and cultured kidney cells and to decrease its activity stimulated by phorbol-12-myristate-13-acetate in rat neonatal cardiomyocytes.¹²⁻¹⁴ However, there is no clear consensus whether PKC is involved in the nongenomic effects of aldosterone on the vascular tone of coronary arteries in the ischemic hearts. Thus, this study was undertaken to investigate the nongenomic effects of aldosterone on coronary vascular tone and cardiac functions in the in vivo ischemic hearts and a role of PKC in this nongenomic effect in canine hearts.

Materials and Methods

Both aldosterone and spironolactone were purchased from Sigma (St. Louis, Mo), and a specific PKC inhibitor, GF109203X, was

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