Long-Term Stimulation of Adenosine A2b Receptors Begun After Myocardial Infarction Prevents Cardiac Remodeling in Rats

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Background—Adenosine inhibits proliferation of cardiac fibroblasts and hypertrophy of cardiomyocytes, both of which may play crucial roles in cardiac remodeling. In the present study, we investigated whether chronic stimulation of adenosine receptors begun after myocardial infarction (MI) prevents cardiac remodeling.

Methods and Results—MI was produced in Wistar rats by permanent ligation of the left anterior descending coronary artery. One week after the onset of MI, animals were randomized into 8 groups: vehicle, dipyridamole (DIP; the adenosine uptake inhibitor, 50 mg/kg), 2-chroloadenosine (CADO; the stable analogue of adenosine, 2 mg/kg), and CADO in the presence of the nonselective adenosine receptor antagonist 8-sulfophenyltheophylline (8-SPT) or the selective antagonist for adenosine A1, A2a, A2b, or A3 receptor. Three weeks after treatment, hemodynamic and echocardiographic parameters in the DIP and CADO groups were significantly improved compared with the vehicle group. These hemodynamic and echocardiographic improvements were blunted by either 8-SPT or the selective adenosine A2b antagonist MRS1754 but not by the selective antagonists for other subtypes of adenosine receptors. The collagen volume fraction was smaller, and gene expression of the molecules associated with cardiac remodeling such as matrix metalloproteinase in noninfarcted areas was reduced in the DIP and CADO groups compared with the vehicle group, both of which were attenuated by either 8-SPT or MRS1754.

Conclusions—Long-term stimulation of adenosine A2b receptors begun after MI attenuates cardiac fibrosis in the noninfarcted myocardium and improves cardiac function. Drugs that stimulate adenosine A2b receptors or increase adenosine levels are new candidates for preventing cardiac remodeling after MI. (***Circulation.*** **2006;114:1923-1932.)**

Key Words: adenosine ■ heart failure ■ myocardial infarction ■ remodeling

Chronic heart failure (CHF) is a major complication of myocardial infarction (MI) that substantially worsens its prognosis.[1,2] Although several major therapeutic advances have been made in the management of MI, postinfarction CHF remains a common cause of high morbidity, hospitalization, and cardiac death.[3,4] Worsening of cardiac functions after MI is followed by a complex sequence of structural changes of the left ventricle (LV), referred to as postinfarction remodeling.[3,4] These changes include progressive chamber dilatation, cardiac hypertrophy, and fibrosis.[4,5] Recent studies have highlighted the importance of fibrosis in noninfarcted areas remote from the site of infarction for the pathogenesis of postinfarction cardiac dysfunction.[3–7]

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Adenosine is a nucleoside that exerts multiple functions through specific subtypes of adenosine receptors.[8,9] Four subtypes of adenosine receptors (A1, A2a, A2b, and A3) have been cloned and pharmacologically characterized.[10,11] Adenosine triggers/mediates cardioprotection against short-term ischemia/reperfusion injury mainly through adenosine A1, A2a, and A3 receptors.[12–14] In addition, adenosine inhibits cardiac hypertrophy as a result of pressure overload through adenosine A1 receptors.[15] Adenosine also inhibits proliferation of cultured myocardial fibroblast through adenosine A2b receptors,[16] suggesting that adenosine may play an important role in cardiac remodeling. Recently, we found that plasma adenosine levels increase in patients with CHF[17] and that a further elevation of plasma adenosine levels resulting from either dipyridamole (DIP) or dilazep reduces the severity of CHF.[18] However, the long-term effects of adenosine that start after the completion of the necrotic process following MI on cardiac remodeling are unclear. Furthermore, it has

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not been determined which subtypes of adenosine receptors are involved in cardiac remodeling. In the present study, we investigated whether the long-term stimulation of adenosine receptors prevents cardiac remodeling in rat MI model and, if so, which subtype of adenosine receptors are involved in this condition.

Methods

Animals

All procedures were performed in conformity with the *Guide for the Care and Use of Laboratory Animals* (NIH publication No. 85–23, 1996 revision) and were approved by the Osaka University Committee for Laboratory Animal Use.

Materials

DIP, the adenosine uptake inhibitor, was kindly provided by Boehringer-Ingelheim (Ingelheim, Germany). 2-Chroloadenosine (CADO; the stable analogue of adenosine), 8-sulfophenyltheophylline (8-SPT; the nonselective antagonist of adenosine receptors), 1,3-diethyl-8-phenylxanthine (DPCPX; the selective adenosine A1 antagonist), 5-amino-7-(phenylethyl)-2-(2-furyl)-pyrazolo[4,3-e]-1,2,4-triazolo[1,5-c] pyrimidine (SCH58261; the selective adenosine A2a antagonist), 8-[4-[((4-cyanohenyl)carbamoylmethyl)oxy]phenyl]-1, 3-di(n-propyl)xanthine (MRS1754; the selective adenosine A2b receptor antagonist), and (5-propyl-2-ethyl-4-propyl-3-(ethylsulfanylcarbonyl)-6-phenylpyridine-5-carboxylate) (MRS1523; the selective adenosine A3 receptor antagonist) were purchased from Sigma Chemical Co (St Louis, Mo). DIP, CADO, and 8-SPT were dissolved in saline. DPCPX, MRS1754, and MRS1523 were dissolved in a solution of 50% dimethyl sulfoxide in distilled water and diluted immediately before use in saline. SCH58261 was dissolved in Tween 80 aqueous suspension (5 mL/kg). Antibodies for matrix metalloproteinase (MMP)-2 and MMP-9 were purchased from Santa Cruz Biotechnology (Santa Cruz, Calif).

Experimental Protocols

Male Wistar rats (age, 8 weeks; weight, 240 to 260 g; Japan Animals, Osaka, Japan) were used in these experiments. MI was induced by permanent ligation of the left anterior descending coronary artery as previously described.[19] Briefly, the rats were anesthetized with sodium pentobarbital (30 mg/kg IP), the thorax was opened, the heart was exteriorized, and a ligature was placed around the proximal left anterior descending coronary artery. The heart was returned to its normal position, and the thorax was closed. Mortality was 47% within the first 24 hours. The same surgical procedure was performed in a sham group of rats (n=8) except that the suture around the coronary artery was not tied.

Starting on the seventh postoperative day, sham rats received 2% dimethyl sulfoxide intraperitoneally, and surviving MI rats were randomly allocated to 1 of the following 8 treatment groups: vehicle (2% dimethyl sulfoxide), DIP (50 $mg \cdot kg^{-1} \cdot d^{-1}$ PO), CADO (2 $mg \cdot kg^{-1} \cdot d^{-1}$), CADO (2 $mg \cdot kg^{-1} \cdot d^{-1}$) plus 8-SPT (10 $mg \cdot kg^{-1} \cdot d^{-1}$), CADO (2 $mg \cdot kg^{-1} \cdot d^{-1}$) plus DPCPX (0.1 $mg \cdot kg^{-1} \cdot d^{-1}$), CADO (2$mg \cdot kg^{-1} \cdot d^{-1}$) plus SCH58261 (1 $mg \cdot kg^{-1} \cdot d^{-1}$), CADO (2 $mg \cdot kg^{-1} \cdot d^{-1}$) plus MRS1754 (1 $mg \cdot kg^{-1} \cdot d^{-1}$), or CADO (2 $mg \cdot kg^{-1} \cdot d^{-1}$) plus MRS1523 (1 $mg \cdot kg^{-1} \cdot d^{-1}$); these treatments were given to MI rats (n=8, respectively) for 3 weeks. DIP was given by gastric gavage twice daily. CADO and all adenosine receptor antagonists were given intraperitoneally twice daily. The administration of CADO at the dose used in the present study (1 mg/kg IP) into sham or MI rats (n=5 for both) caused maximal decrease 5 minutes after its administration in pulse rate (24±4% and 22±6% reduction from the baseline, respectively) and systolic blood pressure (26±5% and 27±3% reduction from the baseline, respectively), both of which returned to baseline 90 minutes after the administration was discontinued. Administration of 8-SPT (5 mg/kg IP) blocked the CADO-induced hemodynamic changes. Doses of antagonists for subtypes of adenosine receptors used in the in vivo study were chosen on the basis of previous studies of their efficacy.[15,20–25]

Noninvasive Blood Pressure and Pulse Rate

Both blood pressure and pulse rate were measured before MI and 1 and 4 weeks after MI by the tail-cuff method without the use of anesthesia (MK-2000, Muromachi, Tokyo, Japan).

Echocardiographic Studies

Rats were lightly anesthetized with sodium pentobarbital anesthesia (30 mg/kg IP).[26] Echocardiography was performed using a commercially available echocardiographic system equipped with a 15-MHz phased-array transducer (SONOS 5500, Hewlett Packard, Andover, Mass). A 2-dimensional short-axis view of the LV was obtained at the level of the papillary muscles. These studies were performed at both 1 and 4 weeks after MI.

Hemodynamic Studies

LV systolic pressure (LVSP), LV end-diastolic pressure (LVEDP), maximal rate of pressure rise and decline (LV dP/dt_{max} and LV dP/dt_{min}, respectively), and heart rate were measured with a 3.5F Millar pressure catheter 4 weeks after MI under sodium pentobarbital anesthesia (30 mg/kg IP).

Histology

The right ventricle and LV were separated in ice-cold saline and weighed. The LV was cut into 3 transverse sections: apex, mid ventricle, and base. For the light microscopic study, the specimens were fixed in 10% formaldehyde, embedded in paraffin, and cut into 4-μm-thick sections, which were stained with hematoxylin and eosin and Masson's trichrome stains (n=8 for both).

Infarct size was determined as previously described.[27] Briefly, infarct size was calculated as the average of all slices stained with Masson's trichrome and expressed as a percentage of the fibrotic length to the mean LV circumference. Rats with an infarct size <30% were excluded from analysis because they did not show typical LV remodeling.

The cross-sectional area of cardiomyocytes was measured as previously described.[26] One hundred cardiomyocytes per heart stained with hematoxylin and eosin were counted, and the average area was determined.

The collagen volume fraction was measured while omitting fibrosis of the perivascular, epicardial, and endocardial areas.[26] The collagen volume fraction was expressed as the average of connective tissue to the total tissue area of all slices stained with Masson's trichrome.

Quantitative Real-Time Reverse-Transcriptase Polymer Chain Reaction

Quantitative real-time reverse-transcriptase polymer chain reaction was performed as described previously.[28] Total RNA from the noninfarcted and infracted LV was extracted with RNA-Bee-RNA Isolation Reagent (Tel-Test, Friendswood, Tex). Then, 200 ng total RNA was reverse transcribed and amplified with an Omniscript RT Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Oligonucleotide primers and TaqMan probes for rat collagen type I, rat collagen type III, rat transforming growth factor-β1 (TGF-β1), rat MMP-2, rat MMP-9, rat tissue inhibitor of metalloproteinase (TIMP)-1, TIMP-2, rat atrial natriuretic factor (ANF), rat brain natriuretic peptide (BNP), and rodent glyceraldehyde phosphate dehydrogenase were purchased from Applied Biosystems (Forster City, Calif).

Immunoblotting

Immunoblotting was performed as described previously.[28] Immunoreactive bands were quantified by densitometry (Molecular Dynamics).

Collagen Synthesis in Cultured Cardiac Fibroblasts

Adult cardiac fibroblasts were prepared as described previously.[16] The effects of either DIP or CADO on collagen synthesis in cardiac fibroblasts were evaluated on confluent cultures by incorporating [^{3}H]proline into cells as previously described.[29] Then, either DIP or CADO with or without antagonists for subtypes of adenosine A1, A2a, A2b, or A3 receptors was added; [^{3}H]proline (0.5 μCi/mL) also was added. After the cells were incubated for 24 hours, the radioactivity of aliquots of the trichloroacetic acid–insoluble material was determined with a liquid scintillation counter. Cardiac fibroblasts in the first or second passage were used for all experiments. Doses of antagonist for subtypes of adenosine receptors used in the in vitro study were chosen on the basis of previous studies of their efficacy.[11,16]

Statistical Analysis

All data are expressed as mean±SEM. Comparisons of the time course changes between groups were performed by use of 2-way repeated-measures ANOVA. Comparisons of other data between groups were performed through the use of 1-way fractional ANOVA. The Bonferroni-Holm procedure was used to correct multiple comparisons. A value of $P<0.05$ was considered statistically significant.

The authors had full access to the data and take full responsibility for their integrity. All authors have read and agree to the manuscript as written.

Results

Effects of Adenosine and Antagonists for Subtypes of Adenosine Receptors on Infarct Size and Hemodynamic Parameters

Both blood pressure (110±8 mm Hg) and pulse rate (405±11 bpm) under baseline conditions in the sham group did not change throughout the protocol and were not different at any time points compared with the MI groups. In addition, body weight at baseline (250±5 g) and at the end of the protocol (424±3 g) did not differ from other groups. No difference in infarct size among groups tested was found (Figure 1).

Four weeks after the onset of MI, LVEDP in all MI groups was higher than in the sham group (Figure 2A). Both LV dP/dt_{max} and LV dP/dt_{min} in all MI groups were smaller than in the sham group (Figure 2B and 2C). Long-term adenosine receptor stimulation by either DIP or CADO decreased LVEDP and increased both LV dP/dt_{max} and LV dP/dt_{min}. The improvement in hemodynamic parameters as a result of CADO treatment was blunted by 8-SPT or MRS1754 but not by other antagonists for subtypes of adenosine receptors (Figure 2A through 2C). No statistical difference existed in LVSP (Figure 2D) or heart rate (sham, 372±8 bpm) among all groups when hemodynamic parameters such as LVEDP, LV dP/dt_{max}, and LV dP/dt_{min} were measured. The ratios of heart weight to body weight, ventricular weight to body weight, and lung weight to body weight increased in all MI groups compared with the sham group. Long-term stimulation of adenosine receptors by CADO decreased all 3 ratios compared with other MI groups (the Table). Either 8-SPT or MRS1754, but not the antagonist for other subtypes of adenosine receptors, blunted the decrease in all 3 ratios by CADO.

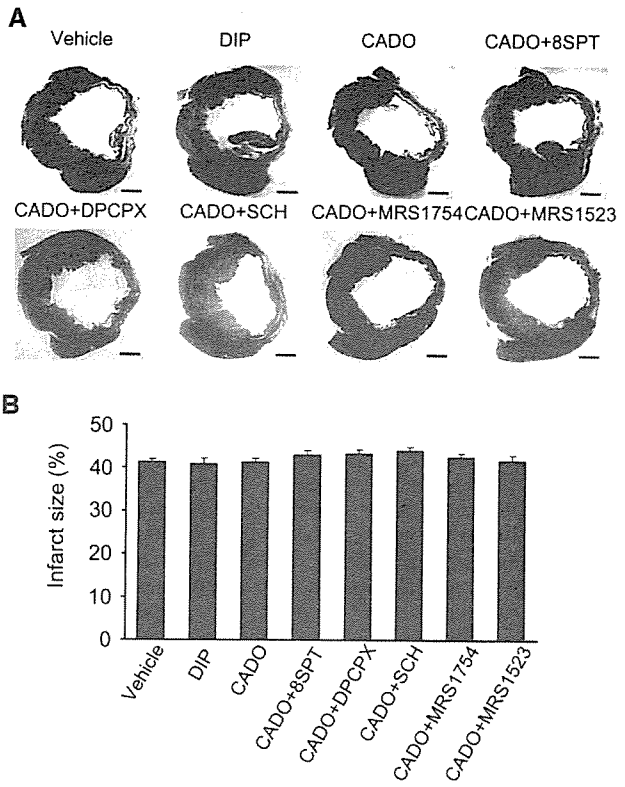

Figure 1. Effects of adenosine and antagonists for subtypes of adenosine receptors on infarct size. A, Representative LV cross sections. Scale bar=1 mm. B, Quantitative analysis of myocardial infarct size. Values are mean±SEM. No statistical difference was found among all groups (n=8).

Effects of Adenosine and the Antagonists for Subtypes of Adenosine Receptors on Echocardiographic Parameters

Figure 3A shows the M-mode view of all groups. Four weeks after the onset of MI, both LV end-diastolic dimension and LV end-systolic dimension in all MI groups were larger than in the sham group. Although no statistical difference existed in LV end-diastolic dimension among MI groups, both DIP and CADO reduced LV end-systolic dimension and increased fractional shortening. The improvement in echocardiographic parameters by CADO was blunted by either 8-SPT or MRS1754 but not by the antagonist for other subtypes of adenosine receptors (Figure 3B).

Effects of Adenosine and Antagonists for Subtypes of Adenosine Receptors on Cardiac Collagen Volume in Noninfarcted Areas

To clarify the pathophysiological mechanism of the improved cardiac performance caused by the long-term stimulation of adenosine receptors by either DIP or CADO, we examined the collagen volume fraction in noninfarcted areas that may affect cardiac remodeling. The collagen volume fraction in all MI groups increased more than in the sham group, and administration of either DIP or CADO attenuated an increase in morphometric collagen volume fraction in noninfarcted areas (Figure 4). Either 8-SPT or

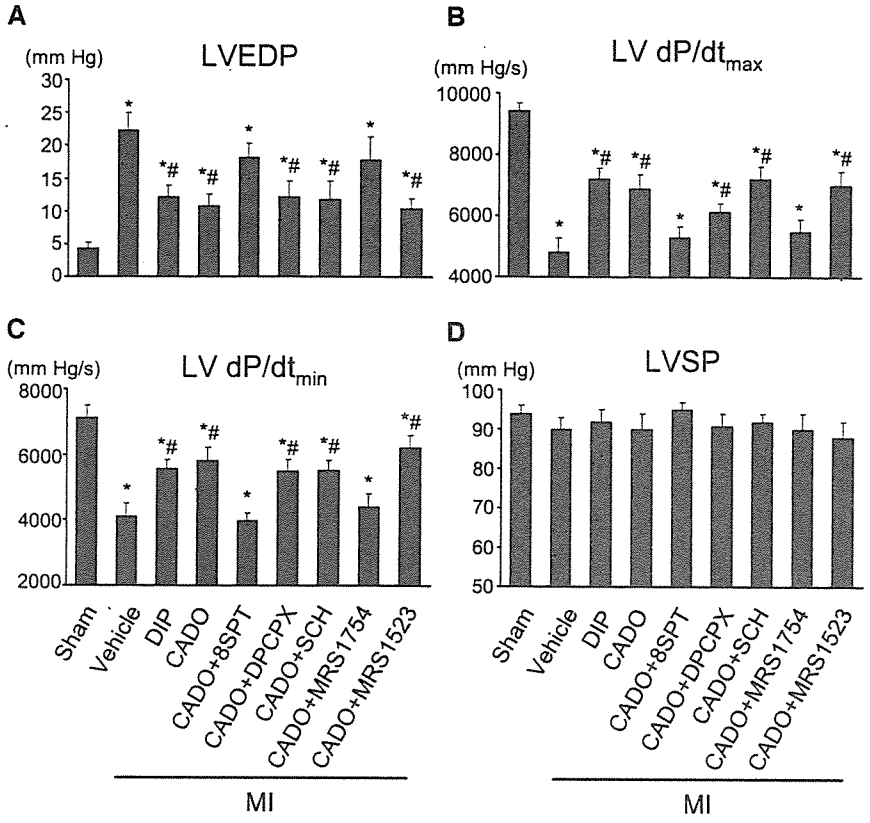

Figure 2. Effects of adenosine and antagonists for subtypes of adenosine receptors on hemodynamic parameters. A, LVEDP; B, LV dP/dt_{max}; C, LV dP/dt_{min}; D, LVSP. Values are mean±SEM. *$P<0.05$ vs sham; #$P<0.05$ vs vehicle (n=8).

MRS1754, but not antagonists for other subtypes of adenosine receptors, abolished the effects of CADO on the collagen volume fraction in the noninfarcted areas. CADO (93±4%) or DIP (90±5%) did not change the collagen volume fraction in infracted areas compared with vehicle (88±4%).

Effects of Adenosine and Antagonists for Subtypes of Adenosine Receptors on Cardiac Hypertrophy in Noninfarcted Areas

The cross-sectional area of cardiomyocytes in all MI groups increased more than in the sham group, and either DIP or CADO inhibited hypertrophy of cardiomyocytes in noninfarcted areas (Figure 5). Either 8-SPT or DPCPX, but not other adenosine receptor antagonists, abolished the effects of CADO on hypertrophy of cardiomyocytes.

Effects of Antagonists for Subtypes of Adenosine Receptors on Molecules Associated With Cardiac Remodeling in Noninfarcted Areas

To examine the molecular mechanisms by which adenosine attenuates cardiac fibrosis in the noninfarcted myocardium, we examined the mRNA levels of molecules associated with fibrosis and hypertrophy in noninfarcted areas after MI (Figure 6). The increased mRNA expressions of collagen type I, TGF-β1, MMP-2, and TIMP-1 after MI were suppressed by treatment with either DIP or CADO. Interestingly, either 8-SPT or MRS1754, but not the antagonist for other subtypes

Heart Weight and Lung Weight 4 Weeks After MI

		MI							
	Sham	Vehicle	DIP	CADO	CADO+8SPT	CADO+DPCPX	CADO+SCH58261	CADO+MRS1754	CADO+MRS1523
HW/BW, mg/g	2.93±0.06	4.05±0.11*	3.52±0.15*†	3.60±0.11*†	3.95±0.12*	3.58±0.09*†	3.51±0.12*†	3.89±0.06*	3.45±0.19*†
VW/BW, mg/g	2.54±0.06	3.65±0.11*	3.25±0.09*†	3.21±0.10*†	3.54±0.11*	3.17±0.08*†	3.11±0.12*†	3.48±0.07*	3.05±0.19*†
LVW/BW, mg/g	1.93±0.07	2.57±0.07*	2.62±0.12*	2.60±0.10*	2.60±0.17*	2.56±0.10*	2.50±0.11*	2.48±0.09*	2.47±0.19*
LW/BW, mg/g	3.3±0.10	8.2±1.0*	4.8±0.4*†	4.4±0.2*†	8.8±0.7*	5.1±0.4*†	4.4±0.6*†	7.7±0.7*	4.2±0.3*†

HW indicates heart weight; BW, body weight; VW, ventricular weight; LVW, LV weight; and LW, lung weight. Values are mean±SEM (n=8).
*$P<0.05$ vs sham.
†$P<0.05$ vs vehicle.

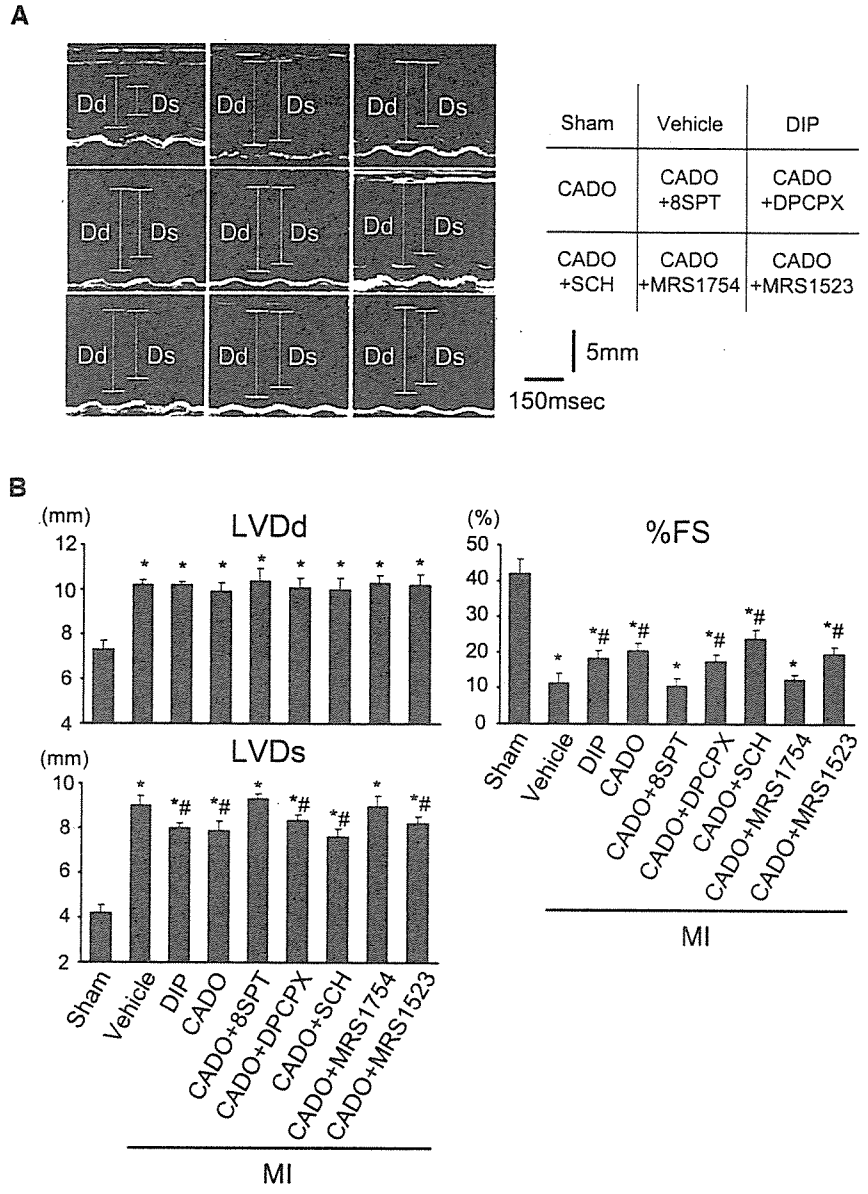

Figure 3. Effects of adenosine and antagonists for subtypes of adenosine receptors on echocardiographic parameters. A, Representative 2D echocardiograms. B, Quantitative analysis of echocardiographic parameters. Values are mean±SEM. LVDd indicates LV end-diastolic dimension; LVDs, LV end-systolic dimension; and %FS, fractional shortening. *$P<0.05$ vs sham; #$P<0.05$ vs vehicle (n=8).

of adenosine receptors, blunted the suppression of mRNA levels of collagen type I, TGF-β1, MMP-2, or TIMP-1 by CADO. However, neither DIP nor CADO changed the mRNA levels of MMP-9 or TIMP-2, each of which may also link with cardiac remodeling after MI.[30] Consistent with the mRNA levels of MMP-2 and MMP-9, CADO decreased MMP-2, but not MMP-9, protein levels in noninfarcted areas (Figure 7). Either DIP or CADO treatment resulted in suppression of the ANF and BNP mRNA levels, both of which are useful markers of cardiac hypertrophy, in noninfarcted areas (Figure 6G and 6H). 8-SPT or DPCPX, but not the antagonist for other subtypes of adenosine receptors, blunted the suppression of mRNA levels of ANF by CADO. 8-SPT, DPCPX, or MRS1754, but not the antagonist for other subtype of adenosine receptors, blunted the suppression of mRNA levels of BNP by CADO. In infarcted areas, all of the evaluated molecules increased in all MI groups compared with the sham group, but no difference existed in expression levels among MI groups (data not shown).

Cellular Mechanisms of the Antifibrosis Action of Adenosine

Both DIP and CADO decreased the incorporation of [^{3}H]proline into cardiac fibroblasts from rat adult rats in a dose-dependent manner, either of which was blocked by 8-SPT (Figure 8A). The decrease in [^{3}H]proline incorporation by CADO was completely blocked by MRS1754, a selective adenosine A2b receptor antagonist, at a concentration of 10^{-7} mol/L (Figure 8B). DPCPX, SCH58261, or MRS1523 at a dose from 10^{-8} to 10^{-6} mol/L did not affect the reduction in [^{3}H]proline incorporation by CADO (10^{-6} mol/L) (data not shown).

Discussion

Adenosine or adenosine receptor agonist administration before the onset of ischemia or during early reperfusion has been documented by several investigators not only to reduce infarct size but also to improve functional recovery after MI.[31–33] In the present study, we demonstrated that

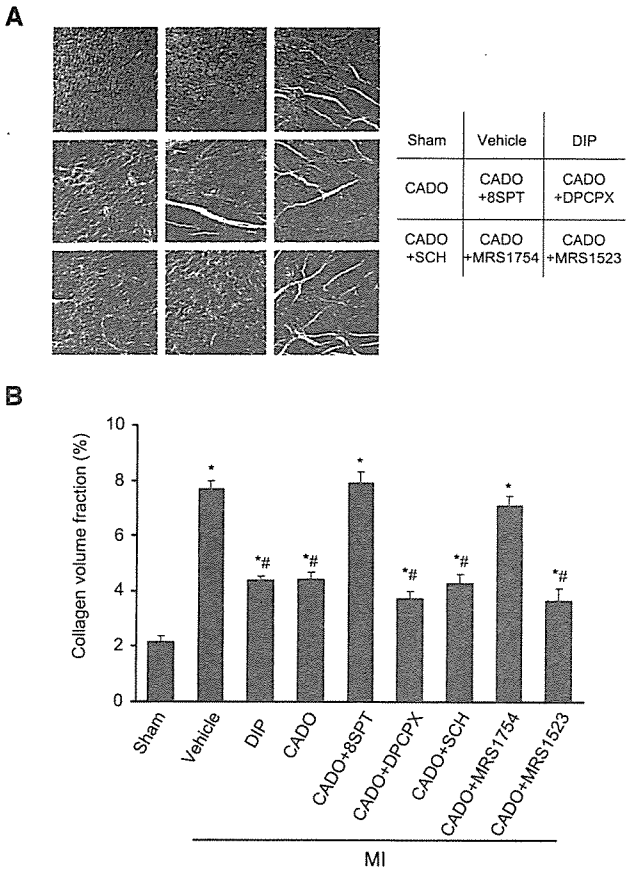

Figure 4. Effects of adenosine and antagonists for subtypes of adenosine receptors on cardiac collagen volume fraction in noninfarcted areas. A, Representative histological findings stained with Masson's trichrome in noninfarcted areas. Scale bar=200 μm. B, Quantitative analysis of collagen volume fraction in noninfarcted areas. Values are mean±SEM. *P<0.05 vs sham; #P<0.05 vs vehicle (n=8).

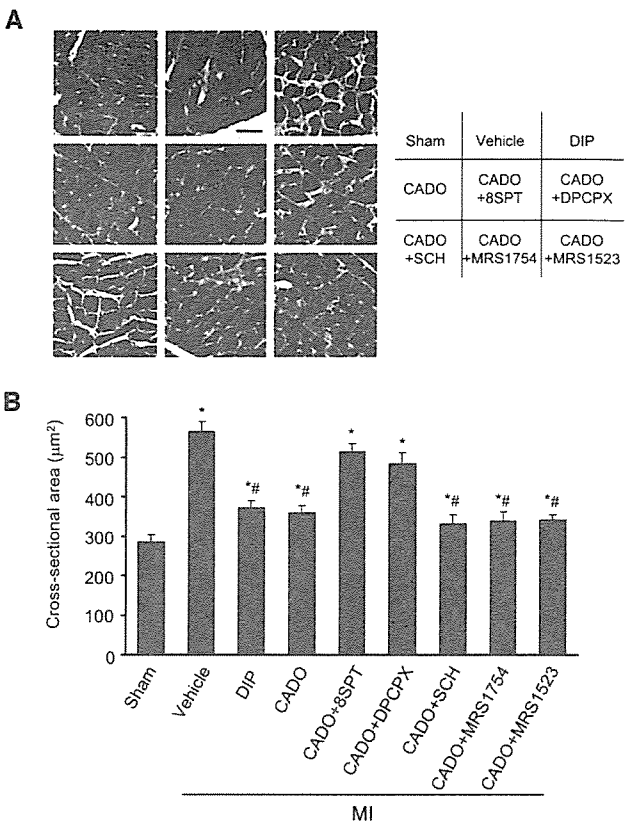

Figure 5. Effects of adenosine and antagonists for subtypes of adenosine receptors on cardiac hypertrophy in noninfarcted areas. A, Representative histological findings of cardiomyocytes stained with hematoxylin and eosin in noninfarcted areas. Scale bar=20 μm. B, Quantitative analysis of cross-sectional areas of cardiomyocyte. Values are mean±SEM. *P<0.05 vs sham; #P<0.05 vs vehicle (n=8).

the long-term administration of either DIP or CADO that starts 1 week after the onset of MI, during which time the necrotic process may be completed,[34] improves cardiac performance in MI rats, as indicated by hemodynamic and echocardiographic parameters. To the best of our knowledge, this study is the first to document that adenosine administered after the completion of the necrotic process exerts cardioprotective effects.

We also demonstrated that the attenuation of cardiac remodeling by adenosine was blunted by 8-SPT, indicating that the activation of adenosine receptors is responsible for the attenuation of cardiac remodeling after MI. Importantly, we further examined which subtype of adenosine receptor was involved in cardiac remodeling after MI. Because 4 subtypes of adenosine receptors have been cloned in the rat,[9,10] we used specific antagonists for subtypes of adenosine receptors: DPCPX for A1 adenosine receptors, A2a for SCH58161, A2b for MRS1754, and A3 for MRS1523.[20,22–25,35]

We found that the improvement in cardiac performance by long-term stimulation by CADO was blunted by MRS1754 but not by the antagonist of other subtypes of adenosine receptors. To the best of our knowledge, this study was the first to demonstrate the involvement of adenosine A2b receptor in cardiac remodeling.

Because the excess deposition of extracellular matrix proteins in noninfarcted areas has gained recognition as an important contributor to adverse remodeling and ventricular dysfunction after MI,[36,37] we have examined the in vivo effects of either DIP or CADO on the extent of fibrosis in noninfarcted areas. We found that either DIP or CADO significantly attenuates an increase in morphometric collagen volume fraction in noninfarcted areas. The attenuation in collagen volume fraction in noninfarcted areas by either DIP or CADO was blunted by 8-SPT or MRS1754 but not by the antagonist of other subtypes of adenosine receptors. The amount of myocardial collagen deposition after MI in infarcted and noninfarcted areas during the healing process was reported to influence and to be integral to the process of ventricular remodeling.[38] In addition, it has been shown that excessive accumulation of myocardial collagen may result in rigidity of the myocardium and severely impaired relaxation.[39] Our data strongly suggest that the reduction in collagen volume in noninfarcted areas through adenosine A2b receptors may contribute to the improvement in cardiac function after MI.

Multiple subtypes of adenosine receptors have been reported to contribute to the antihypertrophic effects of

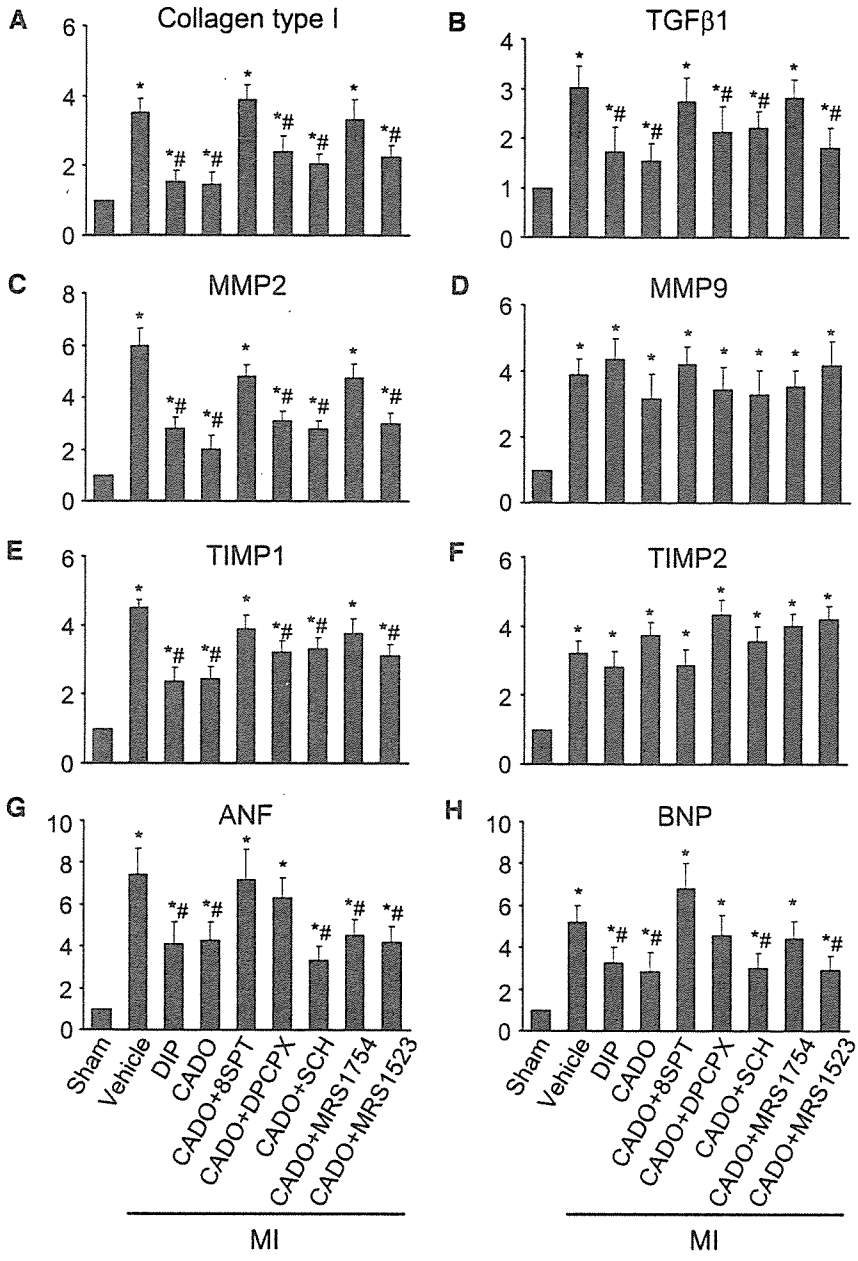

Figure 6. Effects of adenosine and antagonists for subtypes of adenosine receptors on molecules associated with cardiac remodeling in noninfarcted areas. Quantitative real-time reverse-transcriptase polymerase chain reaction analysis of collagen type I (A), TGF-β1 (B), MMP-2 (C), MMP-9 (D), TIMP-1 (E), TIMP-2 (F), ANF (G), and BNP (H). Each mRNA value was corrected for glyceraldehyde phosphate dehydrogenase mRNA value. Levels in sham group were arbitrarily assigned a value of 1.0. Values are mean±SEM. *$P<0.05$ vs sham; #$P<0.05$ vs vehicle. Results represent analysis of 3 independent experiments.

adenosine in cardiomyocytes.[40] We have recently demonstrated that adenosine inhibits cardiac hypertrophy through adenosine A1 receptor in pressure-overloaded hearts.[15] Interestingly, we found that either DIP or CADO attenuated hypertrophic changes in cardiomyocytes and mRNA levels of ANF and BNP in the noninfarcted LV, both of which were blunted by DPCPX, the antagonist of adenosine A1 receptors. However, DPCPX did not blunt the improvement in cardiac performance after MI by long-term stimulation of adenosine receptors during the experimental period, suggesting that long-term stimulation of the adenosine A1 receptor may not play an important role in cardiac remodeling after MI. One potential explanation for the discrepancy in the effects of adenosine A1 and A2 receptors on cardiac function is the difference in the pathophysiology in hearts, ie, hypertrophy versus MI. We need to consider the possibility that the long-term stimulation of adenosine A1 receptor may attenuate cardiac remodeling through its effects on cardiomyocytes.

To examine the molecular mechanism by which adenosine attenuates cardiac fibrosis in noninfarcted areas, we examined the gene expression of molecules associated with cardiac remodeling such as TGF-β1, collagen, MMPs, and TIMPs. CADO attenuated the expression of collagen type I, TGF-β1, MMP-2, and TIMP-1 in noninfarcted areas at 4 weeks after MI. In addition, the effect of CADO was blunted by either 8-SPT or MRS1754 but not by the antagonist of other subtypes of adenosine receptors. These results provide in vivo evidence that adenosine is a potent "fibrosis-inhibitory agent" after MI. Interestingly, either DIP or CADO failed to attenuate the mRNA levels of MMP-9 or TIMP-2. However, because the extracellular

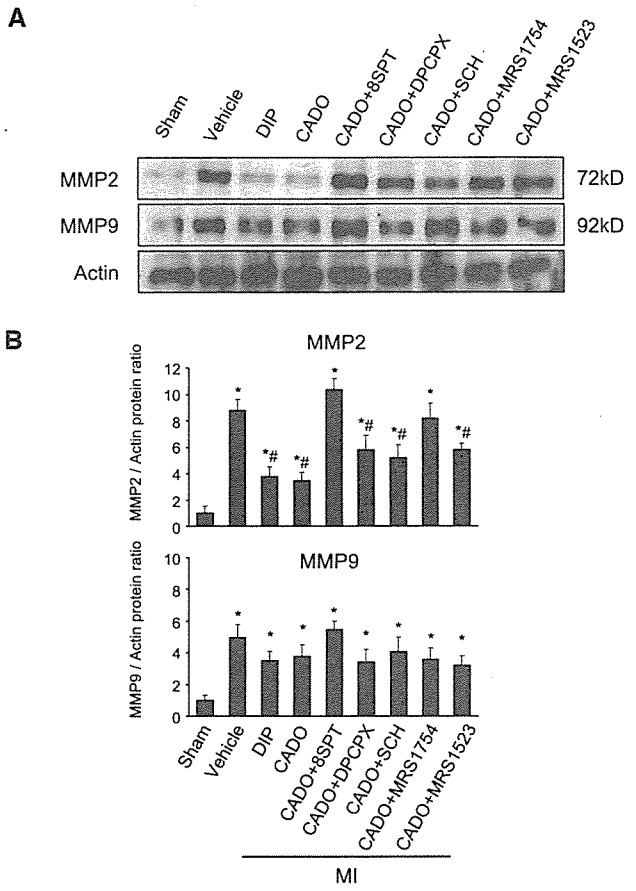

Figure 7. Effects of adenosine and antagonists for subtypes of adenosine receptors on MMP protein levels in noninfarcted areas. A, Representative immunoblot analysis of MMP-2 and MMP-9 proteins. B, Quantitative analysis of MMP-2 and MMP-9 proteins by densitometry. Each protein value was corrected for actin protein value. Levels in sham group were arbitrarily assigned a value of 1.0. Values are mean±SEM. *$P<0.05$ vs sham; #$P<0.05$ vs vehicle. Results represent analysis of 3 independent experiments.

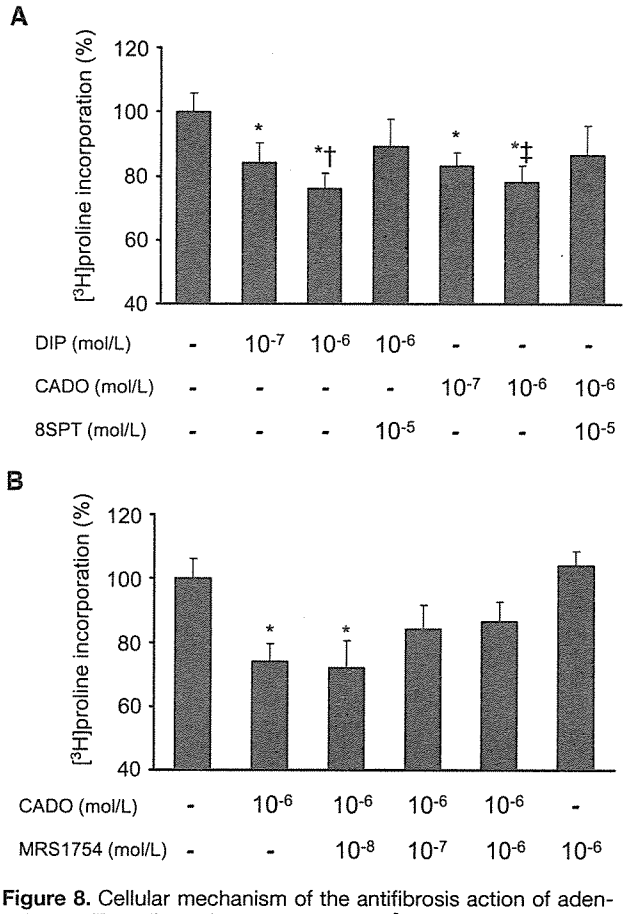

Figure 8. Cellular mechanism of the antifibrosis action of adenosine. A, The effect of DIP or CADO on [^{3}H]proline incorporation in adult cardiac fibroblasts with or without 8-SPT (10^{-5} mol/L). Results represent analysis of 3 independent experiments. B, [^{3}H]proline incorporation into adult cardiac fibroblasts with or without MSR1754, the selective A2b receptor antagonist, in the presence of CADO (10^{-6} mol/L). Values are mean±SEM. *$P<0.05$ vs no treatment; †$P<0.05$ vs DIP (10^{-7} mol/L); ‡$P<0.05$ vs CADO (10^{-7} mol/L).

matrix dramatically changes during the time course after MI,[41] we need to carefully consider the effects of adenosine on the molecules associated with cardiac remodeling. Further investigation is needed to clarify the effect of adenosine on the regulation of extracellular matrix after MI.

The activation of adenosine A2b receptors directly inhibits collagen production[42] and mitogenesis in cardiac fibroblasts from adult rats.[43,44] Consistent with the previous study,[42] our in vitro study using rat adult cardiac fibrosis showed that the decrease in [^{3}H]proline incorporation by either DIP or CADO was completely blocked by MRS1754, the selective A2b receptor antagonist, but not the antagonist of other subtypes of adenosine receptors. These findings support the idea that the activation of adenosine A2b receptors decreases the severity of myocardial fibrosis in the noninfarcted LV. Although we chose the specific antagonist for the each subtype of adenosine receptors, we must notice that these antagonists still have capacity to antagonize other subtype of adenosine receptors.[22,45] Future studies using genetically engineered animals are needed to clarify the exact role of each subtype of adenosine receptors.

The cause of CHF may not be unique, and several neurohumoral factors contribute largely to the pathophysiology of CHF.[3,4] Therefore, it is important in the treatment of CHF to attenuate these neurohumoral factors. Because adenosine is reported to attenuate the sympathetic nervous system, renin-angiotensin system, and cytokine system,[46–49] elevation of adenosine levels may contribute largely to the beneficial treatment of CHF by modulating neurohumoral factors. Recently, Yang et al[50] demonstrated the augmentation of proinflammatory cytokines such as TNFα in adenosine A2b receptor knockout mice. Further studies are needed to determine the contribution ratio of direct and indirect effects of adenosine A2b receptor on cardiac remodeling.

A number of therapeutic approaches to limiting ventricular remodeling in MI have been reported.[3,4] These agents include angiotensin-converting enzyme inhibitors, angiotensin II type 1 receptor blockers, β-adrenergic blockers, aldosterone antagonists, and MMP inhibitors.[3–5] Our findings suggest that the increased adenosine levels in CHF

may be compensatory against CHF, leading to the idea that further elevation of adenosine levels or long-term stimulation of adenosine A2b receptors may be a new strategy for treating CHF.

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Disclosures

None.

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CLINICAL PERSPECTIVE

Chronic heart failure is a major complication of myocardial infarction (MI) that substantially worsens its prognosis. Postinfarction remodeling includes progressive chamber dilatation, cardiac hypertrophy, and fibrosis. Fibrosis in the noninfarcted myocardium contributes to the pathogenesis of postinfarction cardiac dysfunction. Adenosine mediates cardioprotection against acute ischemia/reperfusion injury, mainly through adenosine A1, A2a, and A3 receptors. Adenosine also inhibits proliferation of cardiac fibroblasts and hypertrophy of cardiomyocytes, both of which may play crucial roles in cardiac remodeling. The present study demonstrated that long-term stimulation of adenosine receptors, begun 1 week after the onset of MI, improved hemodynamic and echocardiographic parameters. Long-term adenosine receptor stimulation reduced the collagen volume fraction and gene expression of the molecules associated with cardiac remodeling in noninfarcted myocardium. These improvements were blunted by the selective adenosine A2b antagonist. These data suggest that the long-term stimulation of adenosine A2b receptors begun after MI attenuates cardiac fibrosis in the noninfarcted regions and improves cardiac function. Drugs that stimulate adenosine A2b receptors or increase adenosine levels may be new candidates to attenuate post-MI cardiac remodeling.

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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.

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

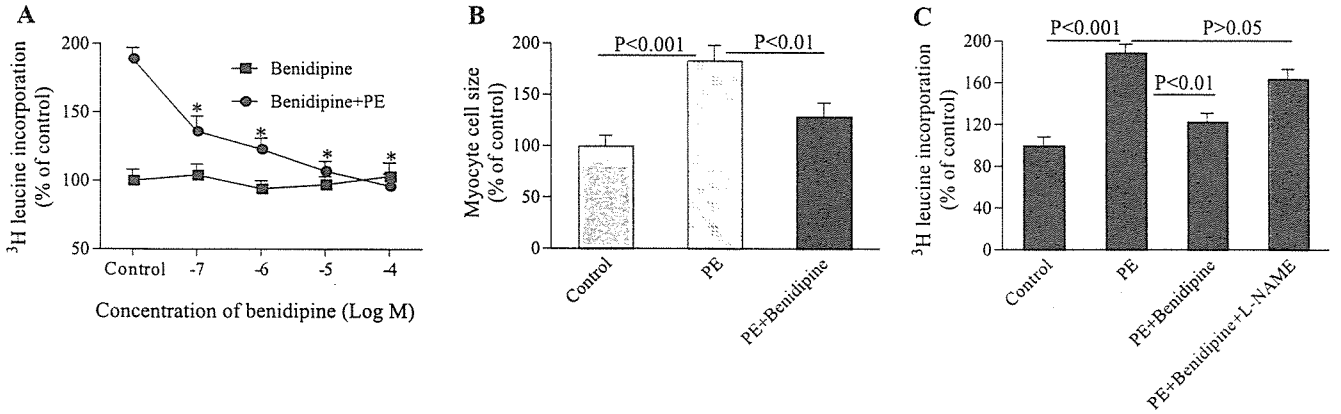

Fig. 1. Effect of benidipine on protein synthesis and the size of neonatal rat cardiac myocytes. (A) Protein synthesis stimulated by 10^{-4} M phenylephrine (PE) was inhibited by benidipine at concentrations ranging from 10^{-7}–10^{-4} M in a dose-independent fashion, and this concentration range did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

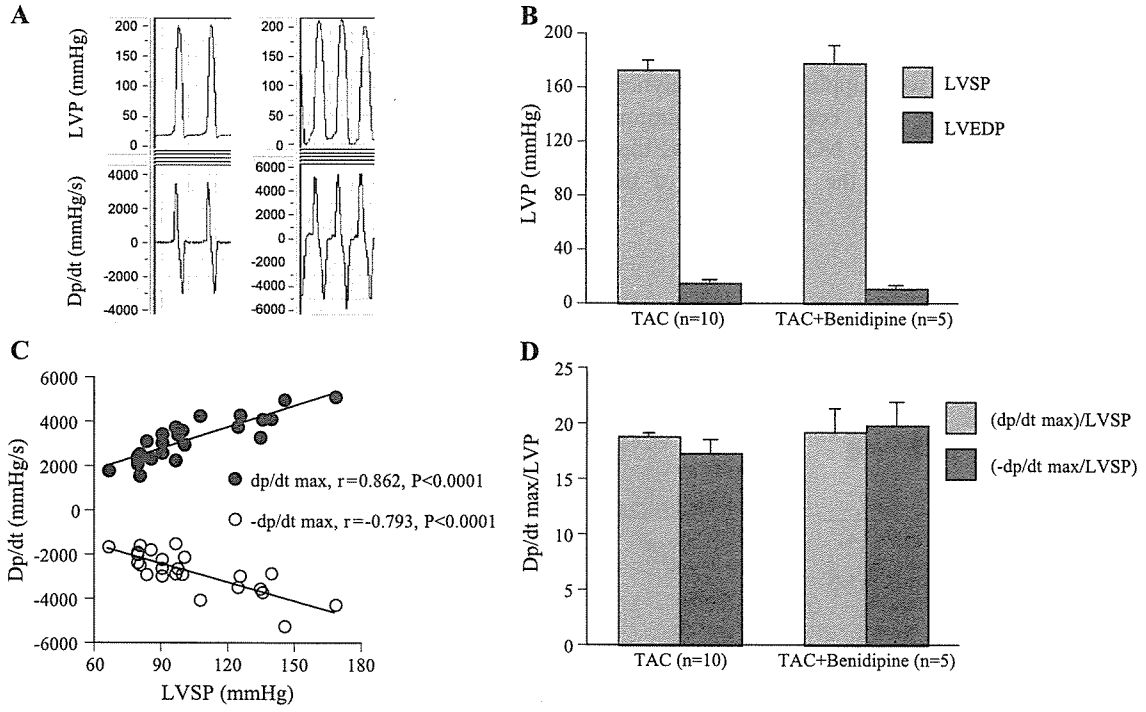

Fig. 2. Left ventricular (LV) hemodynamics measured with a Millar Catheter at 4 weeks after TAC. (A) LV pressure and dp/dt in the TAC and benidipine groups. (B) No significant differences of LV systolic pressure (LVSP) and LV end-diastolic pressure (LVEDP) were noted between TAC mice with or without 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.

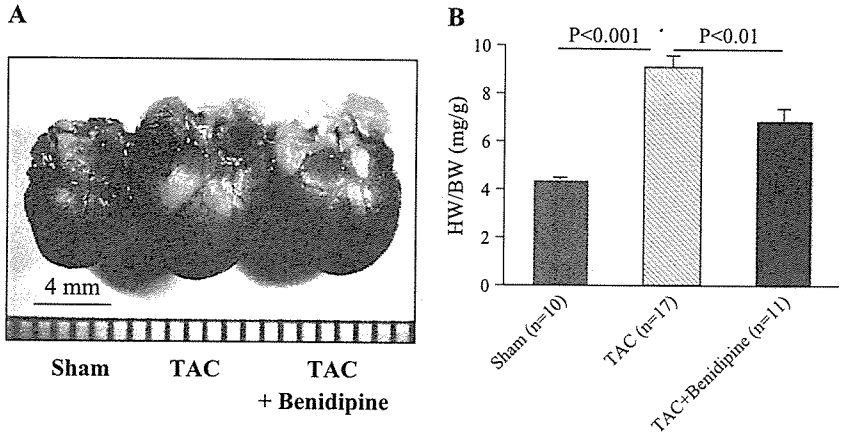

Fig. 3. Benidipine inhibits cardiac remodeling. (A) Representative pictures of whole hearts. (B) The heart to body weight ratio (HW/BW) was significantly decreased in TAC mice treated with benidipine (10 mg/kg/d) compared with untreated TAC mice.

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).

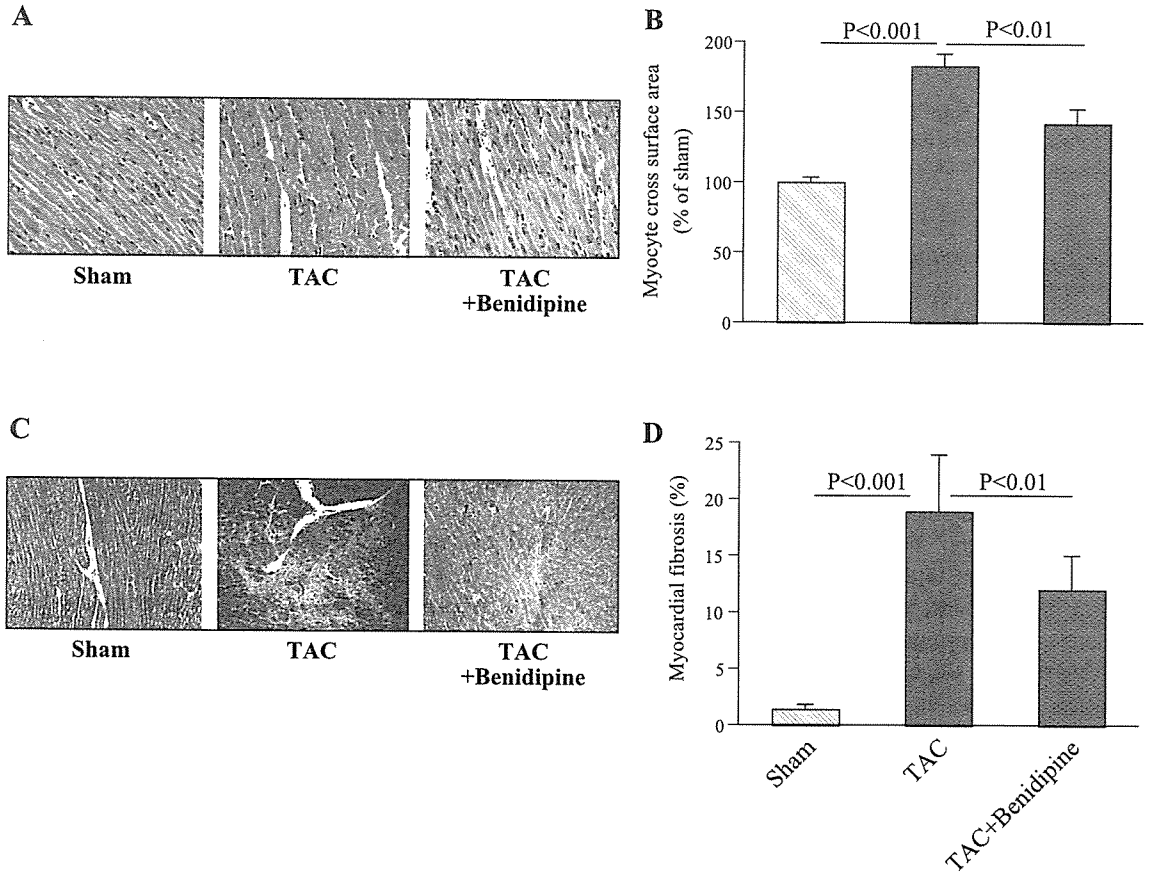

Fig. 4. Results of histological examination. (A) Representative images of the myocardium (HE stain×200). (B) The cross-sectional area of cardiac myocytes was significantly increased in TAC mice by pressure overload for 4 weeks, while treatment with benidipine blunted the enlargement of myocytes. (C)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

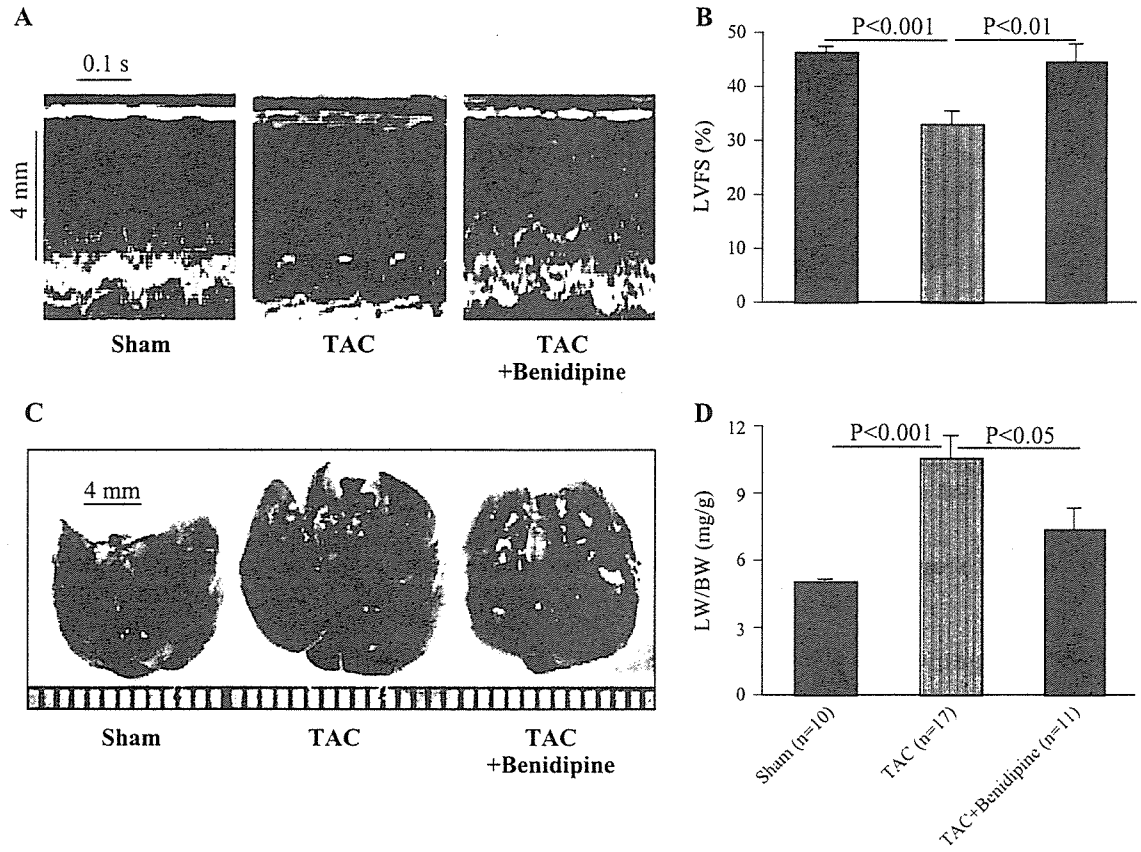

Fig. 5. Benidipine ameliorates heart failure induced by pressure overload. (A) Representative images obtained by echocardiography. (B) LV fractional shortening (LVFS) was increased by benidipine treatment. (C) Macroscopic views of lungs from each group. (D) The lung to body weight ratio (LW/BW) was significantly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

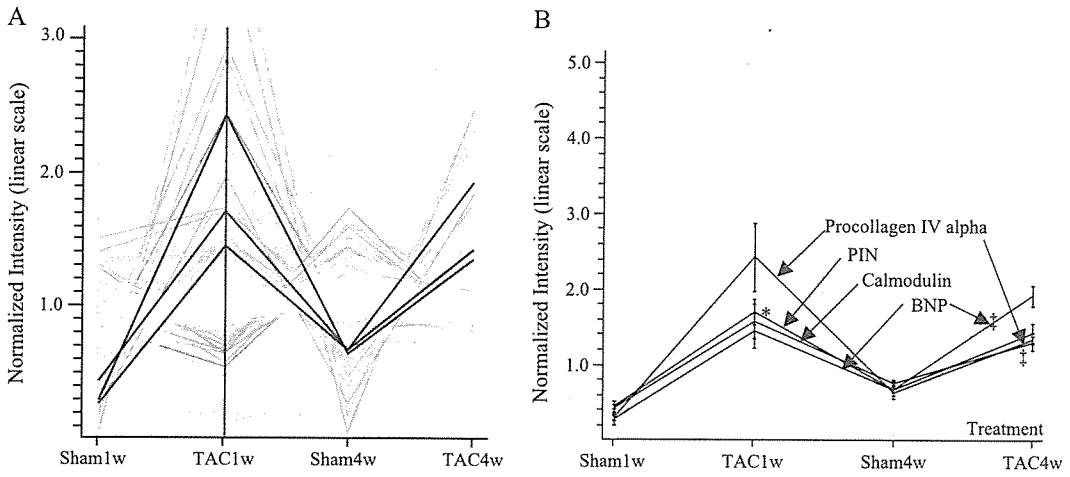

Fig. 6. cDNA microarray analysis of pressure-overload or sham-operated murine hearts. (A) From a total of 12,488 genes, three target genes were selected. These genes were functionally related to cardiac hypertrophy, heart failure, and nitric oxide signaling or fibrosis. (B) The three target genes were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

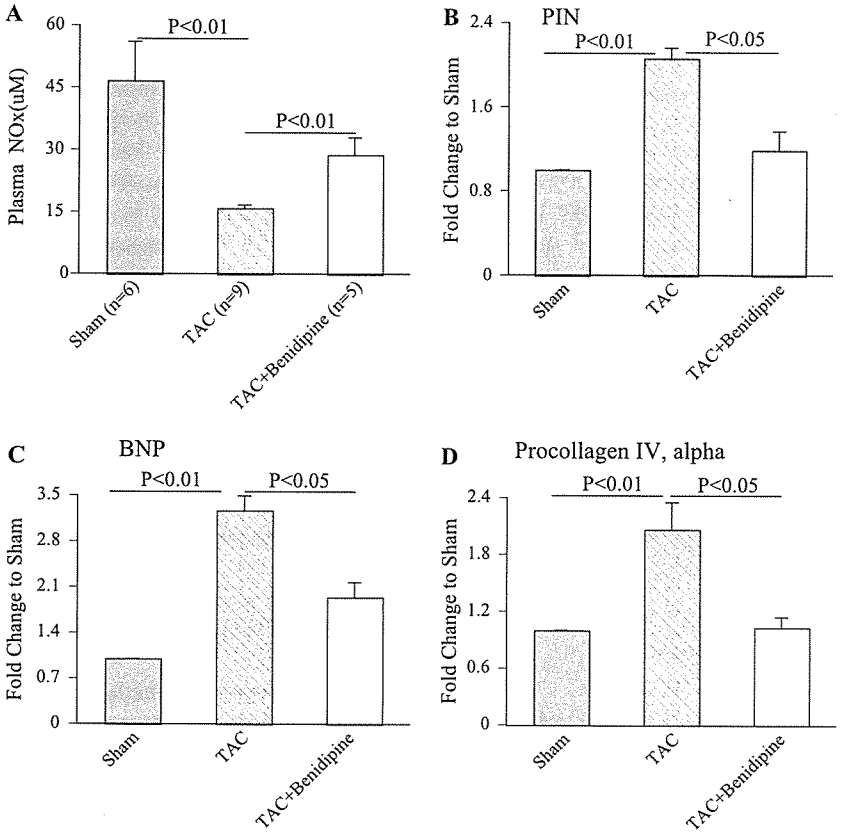

Fig. 7. Plasma nitric oxide level (A) and real-time PCR of the three target genes (B–D). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an endogenous control. PIN—protein inhibitor of neuronal nitric oxide synthase; BNP—natriuretic peptide precursor type B. $n=4$ per group for real-time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