obtained from Calbiochem (La Jolla, Calif).[15] Bovine serum albumin-conjugated aldosterone was purchased from Steraloids Inc. (Newport, RI). The primary antibody and the positive control against PKCα were commercially obtained from BD Biosciences (San Jose, Calif). Aldosterone was initially dissolved in 99.5% ethanol and diluted in saline.

Instrumentation

All procedures were performed in careful conformance with the *Guide for the Care and Use of Laboratory Animals* published by the National Institutes of Health (NIH publication No. 85-23, revised 1996). Experimental protocols were approved by the Osaka University Ethical Committee for Laboratory Animal Use.

Fifty-nine hybrid beagle dogs weighing 14 to 22 kg were anesthetized with pentobarbital sodium (30 mg/kg intravenously). The dogs were prepared as previously described.[16] Briefly, the trachea was intubated and the dog was ventilated with room air mixed with oxygen. The chest was opened through the left fifth intercostal space, and the heart was suspended in a pericardial cradle. After heparinization (500 U/kg), the proximal portion of the left anterior descending coronary artery (LAD) was cannulated and perfused with blood via the carotid artery through an extracorporeal bypass tube. Either coronary perfusion pressure (CPP) or coronary blood flow (CBF) was monitored at this tube. A small collecting tube was inserted into a small coronary vein near the perfused area to sample coronary venous blood. The drained venous blood was collected in a reservoir placed at the level of the left atrium. A pair of ultrasonic crystal probes was placed in the center of the perfused area to allow the measurement of myocardial segment length with an ultrasonic dimension gauge (5 MHz; Schuessler, Cardiff by the Sea, Calif). End-diastolic length was determined at the R wave of the ECG, and end-systolic length was determined at the minimal dP/dt. Fractional shortening (FS) was calculated by the formula [(end-diastolic length)−(end-systolic length)]/(end-diastolic length), and served as an index of myocardial contractility of the perfused area.

Experimental Protocols

Protocol I: Effects of an Intracoronary Administration of Aldosterone on Systemic and Coronary Hemodynamics in the Nonischemic Hearts

First of all, to clarify dose-dependent effects of aldosterone on CBF, 20 dogs were used in this protocol. Vehicle and 3 different doses (0.05, 0.1, and 0.2 nmol/L; n=5 each) of aldosterone were randomly and selectively administered into the LAD through the extracorporeal bypass tube. We continuously infused 60 ng aldosterone in 10 mL saline into the LAD so that the final concentration of this infused aldosterone in coronary circulation became 0.1 nmol/L in the nonischemic hearts for 60 minutes. Hemodynamic parameters including heart rate, CPP, and CBF were measured 5, 10, 20, 30, 45, and 60 minutes after drug infusion.

Protocol II: Effects of an Intracoronary Administration Aldosterone on Coronary Hemodynamic and Metabolic Parameters in the Ischemic Hearts (Constant Low CPP Model)

After hemodynamic stabilization, CPP was reduced so that CBF was decreased to 33% of the control CBF using an occluder attached at the extracorporeal bypass tube. After a low level of CPP was obtained, the occluder was manually adjusted to keep CPP constant. All of the hemodynamic parameters were measured 5 minutes after the onset of hypoperfusion. Both coronary arterial and venous blood were sampled for metabolic analysis. Then, we administrated aldosterone (0.1 nmol/L, n=7) into the LAD through the extracorporeal bypass tube. The dose of 0.1 nmol/L of aldosterone was chosen because this dose of aldosterone was the minimal dose to induce the maximal coronary vasoconstriction in protocol I. In other dogs, to test the involvement of PKC in regulating CBF, we infused aldosterone with either a PKC inhibitor, GF109203X (300 ng/kg per minute; n=5), or a MR antagonist spironolactone (10 μg/kg per minute, n=5) in the ischemic hearts. An intracoronary infusion of GF109203X at this dose was reported to inhibit PKC activation without changing the coronary hemodynamic and metabolic parameters.[17]

Protocol III: Effects of Aldosterone on the Activation of PKC of Coronary Artery With and Without Ischemia

To check effects of aldosterone on PKC activation in coronary arteries, we used 4 dogs in this protocol. After the 15-minute intracoronary infusion of vehicle or aldosterone (0.1 nmol/L) with and without ischemia, the hearts were excised and the vascular segments from the LAD were modestly separated and quickly placed into liquid nitrogen (LN_2) and stored at −80°C. Then, the vascular segments obtained were separated into membrane and cytosolic fractions and the activity of PKC was checked by Western blot analysis as previously described.[18]

Biochemical Analysis

Lactate concentration was assessed by an enzymatic assay.[19] Lactate extraction ratio (LER) was calculated by multiplying the coronary arteriovenous difference in the lactate concentration by 100 and dividing it by the arterial lactate concentration.

Statistical Analysis

The time course of changes in hemodynamic parameters in each group was compared by 1-way repeated measures ANOVA, followed by the Fisher test. The time course of changes in hemodynamic parameters between groups was compared by repeated measures ANOVA, followed by the Fisher test. All values are expressed as mean±SEM, and $P<0.05$ was considered significant.

Results

Effects of an Intracoronary Administration of Aldosterone on Systemic and Coronary Hemodynamics in the Nonischemic Hearts

In the nonischemic hearts, either heart rate or CPP was not significantly changed during the infusion of aldosterone (Figure 1A and 1B). The infusion of vehicle did not change CBF throughout 60 minutes. Aldosterone at the dose of 0.1 nmol/L gradually decreased CBF from 5 minutes and reached the maximal decrease of CBF 30 minutes after the onset of hypoperfusion and did not further change CBF. Aldosterone at the dose of either 0.1 nmol/L or 0.2 nmol/L caused comparable decrease in CBF, but aldosterone at the dose of 0.05 nmol/L decreased CBF to a lesser extent than did 0.1 nmol/L (Figure 1C).

Effects of an Intracoronary Administration of Aldosterone on Coronary Hemodynamics and Cardiac Functions in the Ischemic Hearts

Before and during coronary hypoperfusion, both heart rate and CPP were unchanged with or without pharmacological interventions. There were no significant differences in baseline hemodynamics among all groups. The infusion of aldosterone (0.1 nmol/L) decreased CBF gradually from 5 minutes and reached maximal decrease at 30 minutes (Figure 2A). In the ischemic hearts, both FS (23.7±1.5% to 8.4±0.7%) and LER (41.4±3.0% to -31.7±2.9%) 30 minutes after the onset of hypoperfusion were decreased ($P<0.05$) compared with the baseline. Furthermore, the intracoronary infusion of aldosterone further decreased both FS (5.4±0.4%) and LER (−41.4±3.7%) in the ischemic hearts. Co-administration of GF109203X completely blunted the aldosterone-induced decrease in CBF (38.1±2.9 mL/100g

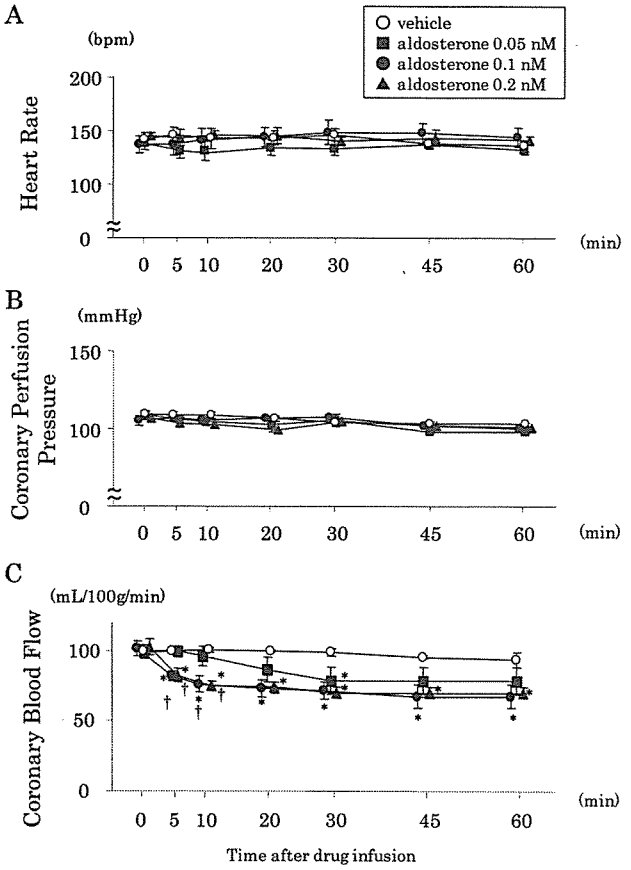

Figure 1. Nongenomic effects of aldosterone on coronary hemodynamics under nonischemic hearts. Plots of changes in heart rate (upper panel, A), coronary perfusion pressure (middle panel, B), and coronary blood flow (lower panel, C) in the nonischemic hearts during intracoronary administration of vehicle or aldosterone at the 3 doses (0.05, 0.1, and 0.2 nmol/L). *P<0.05 vs vehicle, †P<0.05 vs at the corresponding time. Data are presented as mean±SEM (n=5, each).

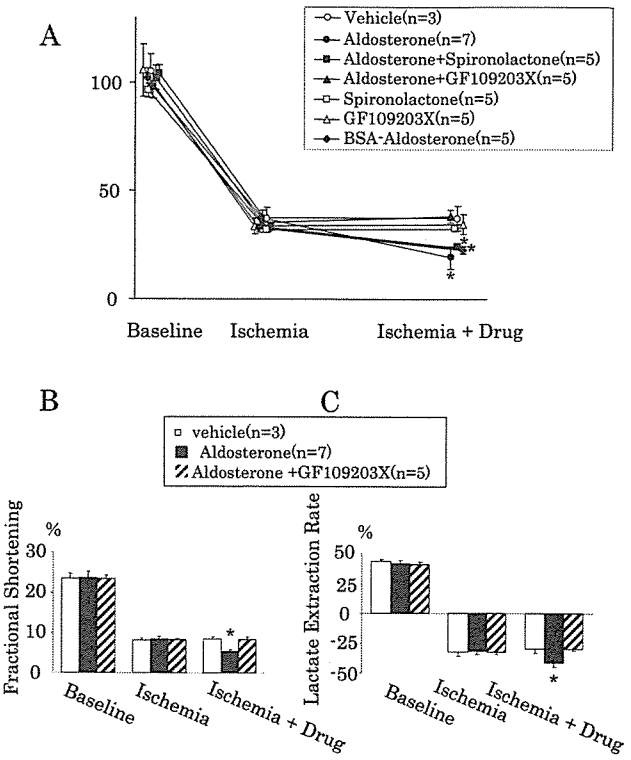

Figure 2. Nongenomic effects of aldosterone on coronary hemodynamics and myocardial contractile and metabolic functions under ischemic hearts. A, Plots of changes in coronary blood flow in the ischemic hearts with intracoronary administration of aldosterone (0.1 nmol/L) with and without either GF109203X or spironolactone, GF109203X alone, spironolactone alone, and bovine serum albumin-conjugated aldosterone. *P<0.05 vs vehicle. B and C, Changes in fractional shortening (left panel, B) and lactate extraction ratio (right panel, C) during the intracoronary administration of vehicle and aldosterone (0.1 nmol/L) with and without GF109203X. *P<0.05 vs vehicle.

per minute) (Figure 2A). This agent also blunted the aldosterone-induced decreased in both FS (8.3±0.7%) and LER (−30.2±1.3%) in the ischemic hearts (Figure 2B and 2C). The infusion of GF109203X alone (n=5) did not change CBF (33.8±3.7 to 34.5±4.3 mL/100 g per minute), FS (8.4±1.0 to 8.2±1.0%), or LER (−32.4±3.2 to −33.6±2.8%) in the ischemic hearts. Co-administration of spironolactone (n=5) did not prevent the aldosterone-induced decrease in CBF (24.0±0.5 mL/100 g per minute). The infusion of bovine serum albumin-conjugated aldosterone decreased CBF gradually from 5 minutes and reached maximal decrease at 30 minutes (22.5±0.9 mL/100 g per minute).

Effects of Aldosterone on the Activation of PKC With and Without Ischemia

As shown in Figure 3, in the nonischemic condition, aldosterone induced the translocation of PKCα from cytosolic to membrane fraction in the vascular segments of the LAD. Moreover, the ischemic insult itself induced the translocation of PKCα from cytosolic to membrane fraction and aldosterone further augmented the translocation of PKCα in the vascular segments under ischemia.

Discussion

We demonstrated here that the intracoronary administration of aldosterone rapidly decreased CBF in the ischemic as well as the nonischemic hearts in vivo. Moreover, aldosterone further worsened the contractile and metabolic functions gauged by FS and LER, respectively, in the ischemic hearts. These decreases in CBF, FS, and LER in the ischemic hearts were blunted by a PKC inhibitor but not an MR antagonist. In addition, bovine serum albumin-conjugated aldosterone reduced CBF under ischemic conditions, suggesting that the

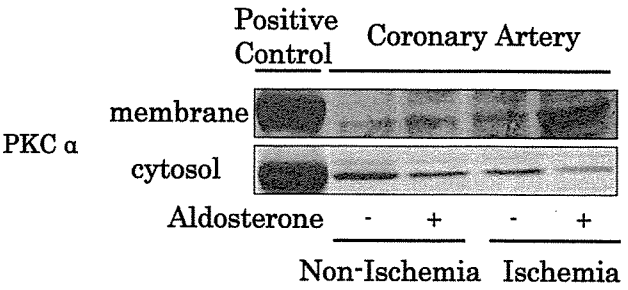

Figure 3. Effects of aldosterone on PKCα activation in coronary arteries. The representative cases in Western blotting against PKCα in the vascular tissues from the LAD.

reduction in CBF by aldosterone was mediated through possible membrane receptors, not intracellular MR. 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 function in the ischemic hearts.

Rapid Aldosterone-Induced Coronary Vasoconstriction in the Nonischemic and Ischemic Hearts

In this study, in the nonischemic hearts, the intracoronary administration of aldosterone decreased CBF within 30 minutes, suggesting that aldosterone nongenomically reduces CBF. Moreover, in the ischemic hearts, we observed the rapid coronary vasoconstriction induced by aldosterone along with the decrease in FS and LER, both of which indicated the contractile and metabolic deterioration, respectively. These findings suggest that the rapid decrease in CBF induced by aldosterone may cause the worsening of ischemia in the in vivo hypoperfused hearts.

Involvement of PKC in the Aldosterone-Induced Coronary Vasoconstriction

Aldosterone is reported to activate 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] In our study, this nongenomic effect of aldosterone on CBF was completely blunted by the PKC inhibitor, GF109203X, confirming the involvement of the PKC activation. The dose of aldosterone at 0.1 nmol/L was reported to increase intracellular Ca^{2+} in cultured rat and rabbit vascular smooth muscle cells.[3] Consistent with this report, we showed that aldosterone activated Ca^{2+}-dependent PKCα in the vascular segments of the ischemic heart. There are some reports that endothelium nitric oxide synthase is a PKC substrate and PKC-mediated phosphorylation inhibits endothelium nitric oxide synthase activity.[20,21] Because nitric oxide is widely known to be a vasodilative agent,[22] decreased nitric oxide activity could attenuate the vascular tone, leading the decrease in CBF. Thus, there is a possibility that aldosterone induced vasoconstriction because of decreased endothelium nitric oxide synthase activity by PKC activation. Because we could not obtain antibodies that react with canine Ca^{2+} -independent subtypes of PKC, possible involvement of other subtypes of PKC was not investigated in the present study.

The Possibility of Transmembrane Receptors of Aldosterone in Canine Hearts

We demonstrated that spironolactone, a classical antagonist of intracellular MR, did not prevent aldosterone-induced vasoconstriction. In addition, bovine serum albumin-conjugated aldosterone induced vasoconstriction. Because bovine serum albumin–conjugated aldosterone would not permeate into the cytoplasm, the effects of bovine serum albumin–conjugated aldosterone on vascular tone were not mediated through intracellular MR, but rather possible membrane receptors. Arima et al suggested that aldosterone caused vasoconstriction in renal microcirculation mediated via membrane-bound receptors.[23] Although further investigation to identify the transmembrane receptors directly will be needed, these findings might support the possibility of the presence of the transmembrane receptors. These coronary vasoconstriction effects of aldosterone were categorized into AII-b according to Mannheim classifications indicating direct steroid action via nonclassical receptors.[24]

Perspectives

Recent large clinical trials resolutely established the beneficial effects of chronic blockade of aldosterone receptor using for patients with chronic heart failure after myocardial infarction.[25,26] In this study, we showed that in the ischemic hearts the nongenomic effect of aldosterone deteriorated ischemia and that this effect was blunted by the inhibition of PKC, not a MR antagonist. Our data suggest that elevated levels of aldosterone may worsen myocardial ischemia via nongenomic as well as genomic pathways in the ischemic hearts. Thus, we believe that this report throws a light on the novel clinical drug development to target nongenomic effects of aldosterone in the ischemic hearts, as well as the chronic inhibition of genomic effects of aldosterone using an antagonist against intracellular MR.

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Prevention of Left Ventricular Remodeling by Long-Term Corticosteroid Therapy in Patients With Cardiac Sarcoidosis

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Forty-three patients with cardiac sarcoidosis were studied echocardiographically before and after (mean follow-up 88 months) steroid therapy to determine the effectiveness of corticosteroids to prevent left ventricular (LV) remodeling and improve LV contractility. In patients with initial LV ejection fractions (LVEFs) ≥55%, long-term steroid therapy showed preventive effects for LV remodeling and LV function. Patients with LVEF <54% showed significant reductions of LV volumes and LVEF improvement. However, in patients with LVEFs <30%, steroid therapy resulted in neither LV volume reductions nor improved LVEFs. In the early or middle stage of the disease, steroid therapy may be protective or therapeutic in preventing LV remodeling and preserving LV function. However, it may not be as effective in the late stage. ©2005 by Excerpta Medica Inc.

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Sarcoidosis is a systemic granulomatous disease of undefined cause involving multiple organs.[1] Although its cardiac involvement has been demonstrated in 20% to 50% in autopsy studies, clinical cardiac manifestations have been seen in only about 5% of patients with sarcoidosis.[1–3] Cardiac sarcoidosis has significant morbidity and mortality due to fatal arrhythmia, atrioventricular conduction disturbance, and refractory congestive heart failure, but its diagnosis has not always been easy.[4,5] Therefore, early suspicion and diagnosis of cardiac sarcoidosis should be desirable in patients with cardiac symptoms[6,7] with abnormal echocardiographic or scintigraphic findings.[8–10] In patients with definite diagnoses or strong probabilities of cardiac sarcoidosis, corticosteroid therapy should be started, even if myocardial biopsy results are negative.[1,3] Previous reports have shown that steroid therapy for sarcoidosis is more effective for the heart than for other organs.[11,12] Although the long-term benefit of steroid therapy in reducing clinical morbidity and mortality has been demonstrated recently,[13] the effect of steroids on left ventricular (LV) morphology and function has not been defined clearly. Using echocardiography, we investigated if steroid therapy could prevent LV remodeling and improve LV function.

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Patients who were diagnosed with cardiac sarcoidosis and underwent long-term steroid therapy in our institution from January 1990 to June 2001 were analyzed retrospectively. We diagnosed patients with cardiac sarcoidosis according to the proposal by the Specific Diffuse Pulmonary Disease Research Group, Sarcoidosis Division (Japanese Ministry of Health and Welfare).[8,9,14,15] Briefly, the diagnostic criteria consist of histologic confirmation and electrocardiographic, echocardiographic, or myocardial scintigraphic abnormalities compatible with cardiac sarcoidosis. After excluding 9 patients without steroid therapy or regular follow-up, 43 patients met these criteria (16 men and 27 women; mean age 48 ± 14 years, range 21 to 71). All patients had coronary angiography, and those who had coronary heart disease were excluded. Right ventricular endomyocardial biopsies were performed on 28 patients, and 12 showed positive results. Pulmonary involvement was noted in 11 patients, skin lesions in 10, and eye involvement in 14. The occurrence of cardiac events (conduction disturbance, lethal arrhythmia, congestive heart failure, and sudden cardiac death) and mortality were investigated during follow-up.

All patients underwent echocardiographic examinations before and after steroid therapy (88 ± 48 months, range 1 to 196) with commercially available ultrasound systems. LV end-diastolic and end-systolic dimensions were determined from M-mode or B-mode echocardiograms. LV end-diastolic and end-systolic volumes and LV ejection fractions (LVEFs) were measured by the modified Simpson's method. LV volumes were corrected by body surface area to obtain LV volume indexes.

All patients were given prednisolone every other day according to a standard protocol.[13] The starting dose of prednisolone was 60 mg every other day for 2 months, which was tapered gradually to the final maintenance dose of 10 mg every other day.

All data are expressed as mean ± SD. Statistical analyses were performed using SPSS 10.0 software (SPSS, Inc., Chicago, Illinois). Comparisons of

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TABLE 1 Patient Characteristics

Characteristic	All	Group A (LVEF ≥55%, n = 22)	Group B (30 ≤LVEF <55%, n = 10)	Group C (LVEF <30%, n = 11)	p Value
Age (yrs)	48 ± 14	44 ± 15	45 ± 9	58 ± 9	0.018
Men/women	16/27	10/12	3/7	3/8	0.533
Patients with myocardial biopsy	28	10	8	10	
Patients with positive biopsy results		3 (33%)	4 (50%)	5 (50%)	
LVEF (%)	51 ± 22	69 ± 7	40 ± 10	22 ± 7	<0.0001
LV end-diastolic diameter (mm)	53 ± 10	46 ± 4	56 ± 7	64 ± 7	<0.0001
LV end-systolic diameter (mm)	39 ± 14	29 ± 3	45 ± 7	58 ± 7	<0.0001
LVEDVI (ml/m^2)	85 ± 43	59 ± 19	92 ± 19	133 ± 42	<0.0001
LV end-systolic volume index (ml/m^2)	49 ± 33	19 ± 5	57 ± 22	104 ± 37	<0.0001

TABLE 2 Comparison of Echocardiographic Parameters Before and After Steroid Therapy

Parameter	Before Steroid Therapy	After Steroid Therapy	p Value
LVEF (%)	51 ± 22	52 ± 23	0.127
LV end-diastolic diameter (mm)	53 ± 10	54 ± 10	0.445
LV end-systolic diameter (mm)	39 ± 14	39 ± 15	0.978
LVEDVI (ml/m^2)	85 ± 43	82 ± 41	0.371
LV end-systolic volume index (ml/m^2)	49 ± 33	47 ± 32	0.448

continuous variables between 2 groups were made by the unpaired Student's *t* test. Comparisons of variables before and after steroid therapy were made by the paired Student's *t* test. The analysis of variance test was used for comparisons among 3 groups. Long-term survival was estimated by Kaplan-Meier analysis, and differences in survival were assessed using a log-rank test. Bivariate analysis of the correlations was performed by Pearson's correlation coefficient analysis. A p value <0.05 was considered statistically significant.

Basic data and echocardiographic parameters are listed in Table 1. Patients were divided into 3 groups according to the LVEF before steroid therapy: group A (n = 22), normal LVEFs (≥55%); group B (n = 10), mildly to moderately reduced LVEFs (30% to 54%); and group C (n = 11), severely reduced LVEFs (<30%). Patients in group C were older than those in the other 2 groups (p = 0.018). LV diameters and volumes became larger as LV function deteriorated. Bivariate analysis showed that age was significantly positively correlated with LV end-systolic diameter (r = 0.396, p = 0.012) and LV end-systolic volume index (r = 0.339, p = 0.032) and negatively correlated with LVEF before steroid therapy (r = −0.427, p = 0.006), suggesting that cardiac sarcoidosis tended to be more advanced in older patients.

Overall, there were no significant differences in LV volumes and function before and after steroid therapy in all patients (Table 2). However, when we examined the data in each group, they showed different courses of changes (Figure 1). In group A, the LV end-diastolic volume index (LVEDVI) decreased (from 59 ± 19 to 57 ± 19 ml/m^2, p = 0.347) and the LVEF did not change (69 ± 7% to 69 ± 5%, p = 0.277) after long-term steroid therapy. In group B, however, the LVEDVI decreased significantly (from 92 ± 29 to 78 ± 25 ml/m^2, p = 0.018) and the LVEF increased significantly (from 40 ± 10% to 51 ± 12%, p = 0.008) at follow-up. In group C, neither the LVEDVI (133 ± 42 to 134 ± 33 ml/m^2, p = 0.918) nor the LVEF (22 ± 7% to 19 ± 5%, p = 0.082) changed substantially.

Because echocardiographic data before steroid therapy were different among the 3 groups by definition, we calculated percent changes of each parameter. The percent change in the LVEDVI after steroid therapy was most remarkable in group B compared with the other groups, although it did not reach statistical significance (−2 ± 10% in group A, −15 ± 13% in group B, and 7 ± 38% in group C, p = 0.125; Figure 2). The percent change of the LVEF was significantly larger in patients in group B than those in the other groups (3 ± 9% in group A, 28 ± 20% in group B, and −10 ± 21% in group C, p <0.0001; Figure 2), suggesting that patients in group B benefited the most from steroid therapy.

Fourteen patients required permanent pacemakers, and 3 patients required implantable-cardioverter defibrillators. Angiotension-converting enzyme inhibitors were administered to 9 patients in group B and to 6 patients in group C. Beta blockers were given to 4 patients in group B and to 7 patients in group C. Intravenous catecholamines were used in 9 patients in group C who had severe refractory congestive heart failure. The total survival rate for all 43 patients was relatively good, at 98% after 1 year, 93% after 3 years, 90% after 5 years, and 84% after 10 years. However, when analyzed on a subgroup basis, there was a difference (Figure 3). There were no cardiac deaths in group A during the follow-up period of 10 years. The survival rate was 100% in group B after 1, 3, and 5 years and 67% after 10 years. The survival rate in group C was clearly lower: 91% after 1 year, 72% after 3 years, 57% after 5 years, and 19% after 10 years. A log-rank test revealed a significant difference in the long-term survival rate between groups A and C (log-rank 16.470, p <0.0001) and between groups B

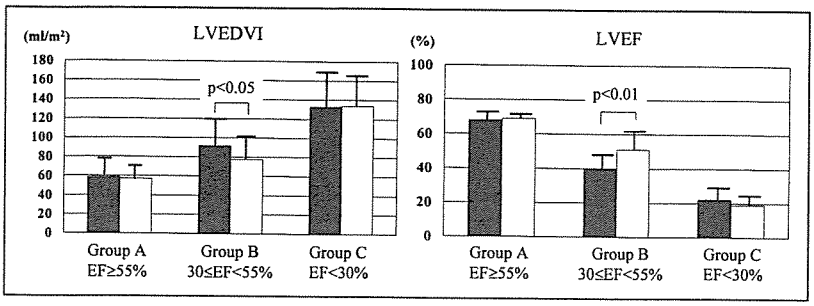

FIGURE 1. Comparison of the LVEDVI and LVEF among 3 groups. *Black and white columns*, before and after steroid therapy, respectively.

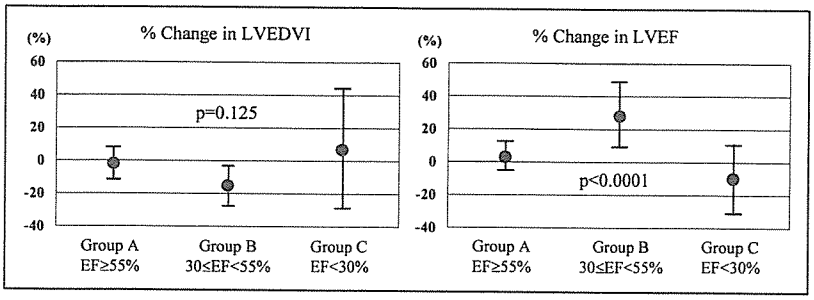

FIGURE 2. Comparison of percent changes in the LVEDVI and LVEF among 3 groups.

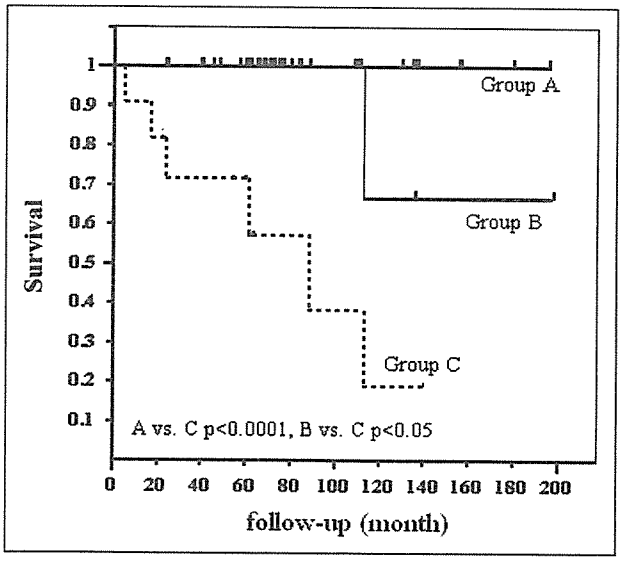

FIGURE 3. Comparison of survival curves among the 3 groups.

and C (log-rank 4.683, $p < 0.05$). After age matching, the log-rank test still revealed a significant difference in survival between groups A and C (log-rank 10.482, $p < 0.005$) and a marginal difference between groups B and C (log-rank 3.811, $p = 0.051$).

• • •

In the present study, for the first time, we showed that the effect of corticosteroid therapy on LV remodeling and function depended on the initial LV size and function and that steroid therapy seemed to have evident response in selected patients. In the early stage of the disease, when LV function and volume were normal, steroid therapy seemed to have the protective effects of preventing LV remodeling and the deterioration of cardiac function. In the middle stage of the disease, the clinical effect of steroid therapy was most obvious and potent, because LV size decreased and LVEF improved significantly. However, in the late stage, steroid therapy appeared not to have a significant beneficial effect, with unchanged LV size and LVEF. The different responses to steroid therapy may reflect the degree of irreversible myocardial damage and fibrosis caused by inflammation.

From the analysis of percent changes in the LVEDVI and LVEF after steroid therapy, it was also demonstrated that patients in the middle stage (group B) had most benefit from steroid therapy. This was supported by the Kaplan-Meier analysis showing better survival in group B than in group C. A recent report has similarly shown that patients with LVEFs >50% had better survival than those with LVEFs <50%.[13]

In the present study, we investigated mortality but not morbidity in patients with sarcoidosis. Patients with cardiac sarcoidosis sometimes present with congestive heart failure or lethal arrhythmia requiring hospitalization. Further, steroid therapy always has risks for infection, bleeding from peptic ulcers, osteoporosis, and so on.[12] Although we showed the prevention of LV remodeling by early steroid therapy, one should always pay attention to these possible complications. We found that patients in the early stage showed no substantial changes in LV size and function after corticosteroid therapy. However, we could not tell if this was due to the effect of steroid therapy or the natural course of the disease. To clarify this, another prospective study should be performed in which patients are randomly given steroids or a placebo. This was a retrospective study, and only patients with echocardiographic studies before and after long-term steroid therapy were enrolled. Therefore, some patients with suboptimal echocardiographic images or those without regular echocardiographic follow-up were excluded, resulting in a relatively small number of patients.

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Amlodipine ameliorates myocardial hypertrophy by inhibiting EGFR phosphorylation

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Abstract

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

Keywords: Calcium channel blocker; Cardiomyocyte; Hypertrophy; Epidermal growth factor; Phosphorylation; Mouse

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

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

Materials and methods

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

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

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

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

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

Results and discussion

Amlodipine attenuates the induction of cardiomyoctyte protein synthesis by epinephrine

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

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

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

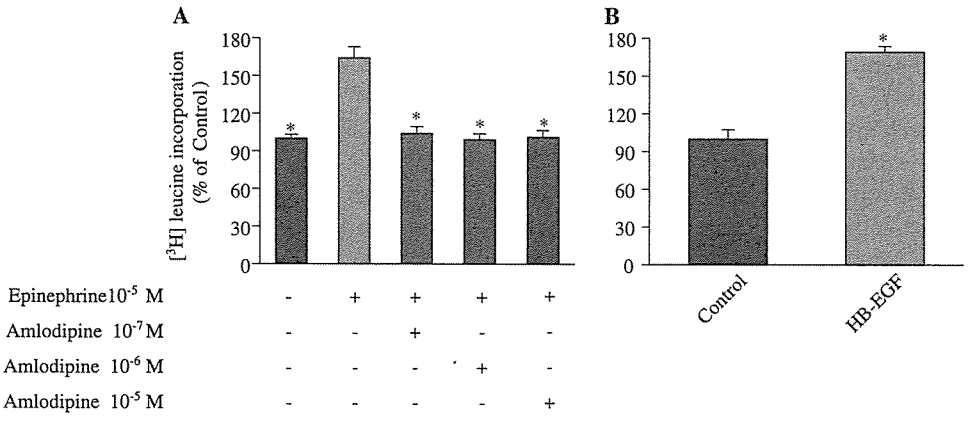

Fig. 1. Effect of amlodipine and HB-EGF on protein synthesis in rat cardiomyocytes. (A) Protein synthesis stimulated by epinephrine (10^{-5} M) was inhibited by amlodipine at concentrations ranging from 10^{-7} to 10^{-5} M. *$P < 0.01$ vs. epinephrine alone. (B) HB-EGF (10^{-8} M) significantly increased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

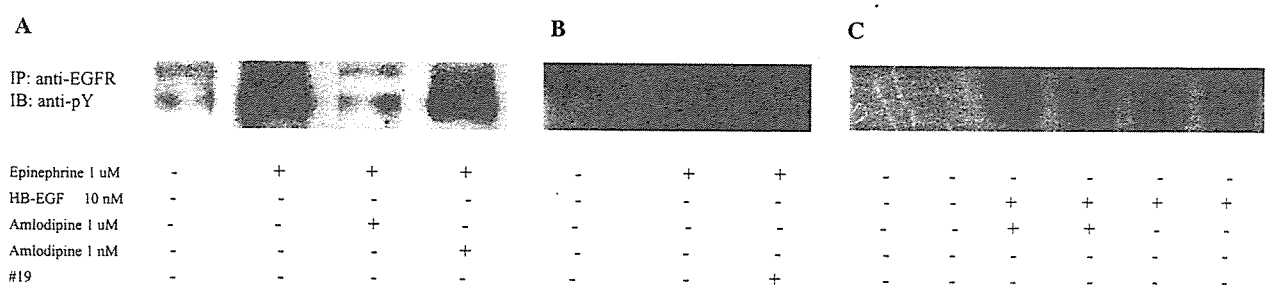

Fig. 2. EGFR phosphorylation and release of HB-EGF. (A) EGFR phosphorylation showed concentration-dependent inhibition by amlodipine. (B) HB-EGF neutralizing antibody #19 blocked epinephrine-induced EGFR phosphorylation. (C) Amlodipine did not influence EGFR phosphorylation induced by HB-EGF. Each experiment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

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

Amlodipine inhibits epinephrine-induced release of HB-EGF

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

Amlodipine inhibits myocardial hypertrophy in vivo

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

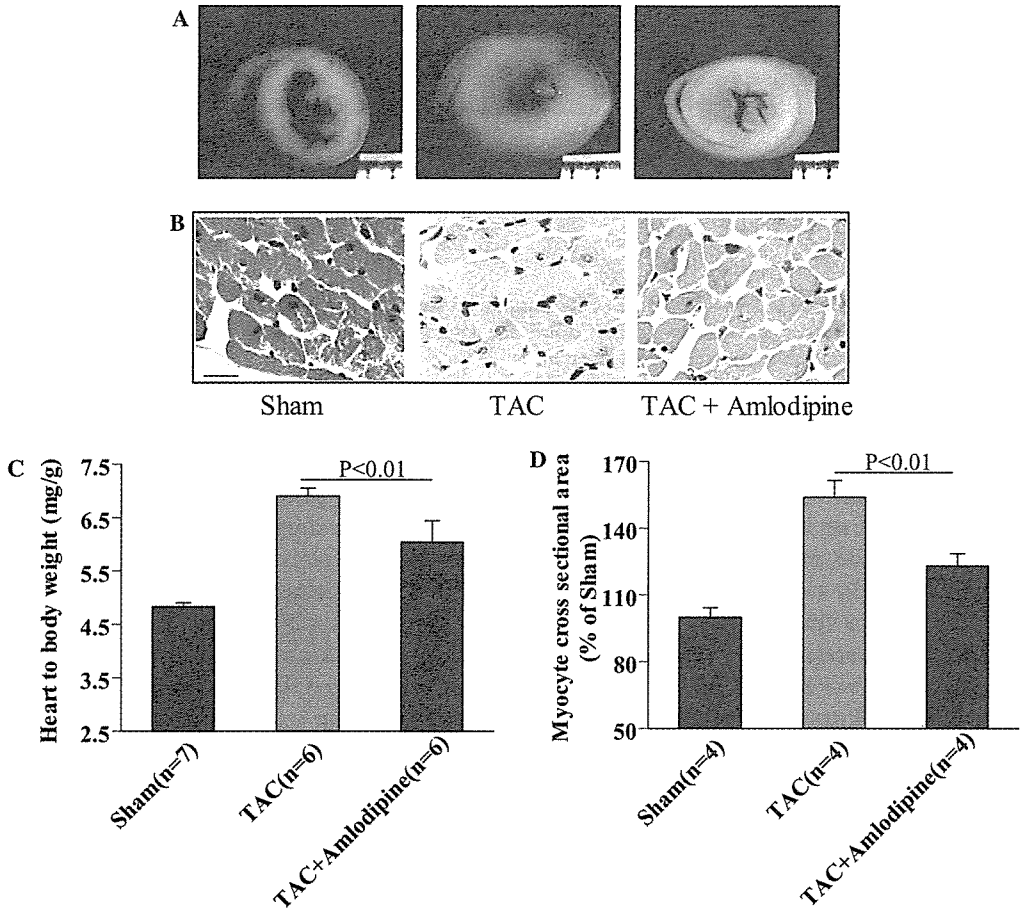

Fig. 3. Effects of amlodipine on cardiac hypertrophy induced by pressure overload in mice. (A) Representative cross-sections of hearts from the three groups. (B) Histological examination showed that cardiomyocyte hypertrophy was less severe in hearts of amlodipine-treated mice (Bar, 20 μm, HE stain).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

Table 1
General characteristics in three experimental groups

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

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

[a] $n = 2$ in each group.

[b] $P < 0.05$ vs. Sham.

[c] $P < 0.05$ vs. TAC.

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

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

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

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

Acknowledgments

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A role of opening of mitochondrial ATP-sensitive potassium channels in the infarct size-limiting effect of ischemic preconditioning via activation of protein kinase C in the canine heart

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Abstract

The opening of mitochondrial ATP-sensitive K^+ (mitoK_{ATP}) channels triggers or mediates the infarct size (IS)-limiting effect of ischemic preconditioning (IP). Because ecto-5′-nucleotidase related to IP is activated by PKC, we tested whether the opening of mitoK_{ATP} channels activates PKC and contributes to either activation of ecto-5′-nucleotidase or IS-limiting effect. In dogs, IP procedure decreased IS and activated ecto-5′-nucleotidase, both of which were mimicked by transient exposure to either cromakalim or diazoxide, and these effects were blunted by either GF109203X (a PKC inhibitor) or 5-hydroxydecanoate (a mitoK_{ATP} channel blocker), but not by HMR-1098 (a surface sarcolenmal K_{ATP} channel blocker). Either cromakalim or diazoxide activated both PKC and ecto-5′-nucleotidase, which was blunted by either GF109203X or 5-hydroxydecanoate, but not by HMR-1098. We concluded that the opening of mitoK_{ATP} channels contributes to either activation of ecto-5′-nucleotidase or the infarct size-limiting effect via activation of PKC in canine hearts.

Keywords: Ischemic preconditioning; Mitochondrial K_{ATP} channels; Infarct size; Ecto-5′-nucleotidase; PKC

Brief periods of ischemia which precede sustained ischemia limit infarct size markedly, a phenomenon known as ischemic preconditioning (IP) [1–3]. The mechanisms underlying this phenomenon have been studied extensively [4–6], and several lines of evidence support the idea that the activation of either protein kinase C (PKC) or p38MAP kinase plays an essential role in IP of canine hearts [7,8]. Activation of PKC opens ATP-sensitive K^+ (K_{ATP}) channels [9,10], which was believed to be a mediator of cardioprotection of IP. However, recently, it is also reported that the opening of mitochondrial K_{ATP} (mitoK_{ATP}) channels increases the production of oxidative stress, which activates p38 MAP kinase [11,12] and triggers the infarct size-limiting effect of IP. Although the opening of K_{ATP} channels plays a major role for a cardioprotection of IP [10,13–16], it has not been shown that the opening of mitoK_{ATP} channels activates PKC and contributes to the infarct size-limitation of IP. To test this idea, since cromakalim opens both mitoK_{ATP} and surface sarcolenmal K_{ATP} (sarcoK_{ATP}) channels, and diazoxide opens mitoK_{ATP} channels, we administered either cromakalim or diazoxide with and without a selective blocker of mitoK_{ATP} channels (5-hydroxydecanoate, 5HD), a selective blocker of sarcoK_{ATP} channels (HMR-1098), or a selective inhibitor of PKC (GF109203X) into canine coronary artery and we examined PKC activity and the infarct size-limiting effect. Furthermore, since we have shown that ecto-5′-

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nucleotidase is phosphorylated and activated by PKC [7], we measured the activity of ecto-5′-nucleotidase of the myocardium with or without cromakalim, diazoxide, 5HD, HMR-1098, or GF109203X.

Materials and methods

All procedures were performed in careful conformance with the *Guide for the Care and Use of Laboratory Animals* published by the National Institutes of Health (NIH Publication No. 85-23, revised 1985), and have been approved by the Osaka University Ethical Committee for Laboratory Animal Use. We purchased diazoxide, cromakalim, 5HD, HMR-1098, and GF109203X from Sigma Chemical, St. Louis, MO, and these agents were dissolved in saline.

Instrumentation

Beagle dogs weighing 8–14 kg were anesthetized with sodium pentobarbital (30 mg/kg, intravenous) and prepared as described previously [7,8]. We cannulated and perfused the left anterior descending coronary artery (LAD) with blood from the left carotid artery through an extracorporeal bypass tube, and aortic blood pressure (ABP) was monitored at this tube. In all experiments, mean ABP, heart rate (HR), and pO_2 in the systemic arterial blood under control conditions averaged 100 ± 3 mmHg, 139 ± 2 beats per minute, and 105 ± 2 mmHg, respectively. Both ABP and HR were measured continuously during the experiment to confirm the physiological states of each dog.

Experimental protocols

Protocol I: The effect of the administration of GF109203X, 5HD, or HMR-1098 on the infarct size-limiting effect induced by either cromakalim or diazoxide

In the open chest dogs, both CPP and CBF were measured continuously. After hemodynamic stabilization, four cycles of 5 min of administration of either cromakalim (0.4 μg/kg/min, ic, 0.024 mg/ml with an infusion rate of 0.0167 ml/kg/min, $n = 7$, the cromakalim group) or diazoxide (200 μg/kg/min, ic, 12 mg/ml with an infusion rate of 0.0167 ml/kg/min, $n = 7$, the diazoxide group) were performed with 5 min of discontinuation of either drug to precondition the myocardium. As a control, instead of pharmacological interventions, after 45 min of hemodynamic stabilization with saline infusion as the same timing of the cromakalim or diazoxide group, the coronary artery was occluded for 90 min and reperfused for 6 h ($n = 7$, the control group). Seven other dogs received the IP procedure (4 cycles of 5-min coronary occlusion and 5-min reperfusion) just prior to sustained ischemia and subsequent 90-min sustained ischemia followed by 6-h reperfusion (IP group).

In 14 dogs, a constant infusion of GF109203X (100 ng/kg/min, ic ($n = 7$ each), 6.0 mg/ml with an infusion rate of 0.0167 ml/kg/min) into the LAD coronary artery was performed 5 min prior to and during the administration of either cromakalim or diazoxide (the cromakalim + GF group, and the diazoxide + GF group). In 7 dogs, 100 ng/kg/min of GF109203X was infused into the LAD coronary artery for 45 min prior to ischemia without administration of either cromakalim or diazoxide.

In 14 dogs, a constant infusion of 5HD (300 μg/kg/min, ic ($n = 7$ each), 18 mg/ml with an infusion rate of 0.0167 ml/kg/min) into the LAD coronary artery was performed 5 min prior to and during the administration of either cromakalim or diazoxide (the cromakalim + 5HD group, and the diazoxide + 5HD group). In 7 dogs, 300 μg/kg/min of 5HD was infused into the LAD coronary artery for 45 min prior to ischemia without administration of either cromakalim or diazoxide.

In 14 dogs, a constant infusion of HMR-1098 (2 μg/kg/min, ic ($n = 7$ each), 0.12 mg/ml with an infusion rate of 0.0167 ml/kg/min) into the LAD coronary artery was performed 5 min prior to and during the administration of either cromakalim or diazoxide (the cromakalim + HMR-1098 group, and the diazoxide+HMR-1098 group). In 7 dogs, 2 μg/kg/min of HMR-1098 was infused into the LAD coronary artery for 45 min prior to ischemia without administration of either cromakalim or diazoxide.

Protocol II: The effect of the administration of GF109203X on the activation of myocardial ecto-5′-nucleotidase induced by either cromakalim or diazoxide

We used 65 other dogs in this protocol. After an administration of saline, cromakalim, or diazoxide, or the IP procedure with and without an administration of GF109203X, 5HD, or HMR-1098 as in Protocol I, we sampled endomyocardium to measure either ecto-5′-nucleotidase or PKC activity.

Measurements of collateral blood flow, risk area, and infarct size. In Protocols I and II, we measured myocardial collateral blood flow at 80 min of ischemia by the non-radioactive microsphere method, and evaluated both risk area (% of left ventricule) and infarct size (% of risk area) by the dual staining (Evans-blue and 2,3,5-triphenyl tetrazolium chloride (TTC)) as described previously [7,8,16–18]. Briefly, after 6 h of reperfusion, LAD was re-occluded and Evans-blue dye was injected intravenously to determine the risk area. The heart was then quickly removed, sliced, and incubated for TTC staining to determine the infarct area.

Criteria for exclusion. To ensure that all of the animals included in the data analysis were healthy and exposed to similar extents of ischemia, the criteria for exclusion as described previously [7,8,16–18] were used: (1) subendocardial collateral flow greater than 15 ml/100 g/min, (2) heart rate greater than 170 beats/min, (3) mean blood pressure over 170 mmHg or below 60 mmHg, and (4) more than two consecutive attempts required to convert ventricular fibrillation with low-energy direct current pulses applied directly to the heart.

Measurement of activity of either 5′-nucleotidase or PKC. A biopsy specimen of the myocardium (1–2 g) supplied by LAD was obtained in Protocol II. This sample was subdivided into endocardial and epicardial myocardium, and the myocardial tissue samples (0.5–1 g each) were frozen and stored under liquid nitrogen. We used endomyocardial sample. The myocardium was separated into membrane and cytosolic fractions as reported previously [18]. Ecto-5′-nucleotidase activity was defined as the activity of membrane fractions and assessed by the enzymatic assay technique [19,20]. PKC activity was also measured as reported previously [21]. We measured PKC activity of membrane fraction with both Ca^{2+} and phospholipids, since we have previously reported that Ca^{2+} and phospholipid sensitive PKC is responsible for IP-induced cardioprotection [7]. The protein concentration was measured by the method of Lowry et al. [22] using bovine serum albumin as a standard.

Statistical analysis

Statistical analyses were performed using paired and unpaired t tests [23,24], and the significance level was adjusted according to a modified Bonferroni's method. In order to compare the data among the groups, a modified Bonferroni test was used to determine significance at the $P < 0.05$ level for group pairs that exhibited statistically significant differences [23,24]. Analysis of covariance (ANCOVA) by regional collateral flow in the inner half LV wall as the covariate was used to account for the effect of collateral blood flow on infarct size. Each value was expressed as mean ± SEM, with $P < 0.05$ considered significant.

Results

Mortality and exclusions

Table 1 shows that among the 138 dogs in Protocol I, 17 and 15 dogs met the exclusion criteria of ventricular fibrillation during sustained ischemia and during reperfusion, respectively. Fifteen other dogs were also excluded because

Table 1
Mortality and exclusion in each group

Group	Initial *n*	Lethal arrhythmia		Excessive collateral flow	Final *n*
		Ischemia	Reperfusion		
Control	11	1	2	1	7
IP	10	0	1	2	7
Cromakalim	8	0	0	1	7
Cromakalim + GF	11	2	1	1	7
Cromakalim + 5HD	12	2	1	2	7
Cromakalim + HMR	9	0	1	1	7
Diazoxide	10	1	1	1	7
Diazoxide + GF	11	2	2	0	7
Diazoxide + 5HD	13	2	2	2	7
Diazoxide + HMR	10	2	1	0	7
GF	11	1	1	2	7
5HD	13	3	1	2	7
HMR	9	1	1	0	7

IP indicates ischemic preconditioning. GF indicates GF109203X, a specific PKC inhibitor. 5HD indicates 5-hydroxydecanoate, a specific inhibitor of mitochondrial ATP-sensitive K^+ channels. HMR indicates HMR-109S, a specific inhibitor of surface ATP-sensitive K^+ channels.

Table 2
Risk area and collateral blood flow during myocardial ischemia

Group	Risk area, % of left ventricle	Collateral flow during ischemia (mL/100 g/min)
Control	40 ± 2	7.2 ± 1.5
IP	42 ± 3	7.0 ± 1.7
Cromakalim	41 ± 4	7.3 ± 1.8
Cromakalim + GF	42 ± 3	8.5 ± 1.4
Cromakalim + 5HD	39 ± 6	8.0 ± 1.7
Cromakalim + HMR	41 ± 5	7.8 ± 1.0
Diazoxide	43 ± 3	7.1 ± 1.7
Diazoxide + GF	41 ± 3	7.7 ± 1.7
Diazoxide + 5HD	43 ± 5	8.1 ± 0.9
Diazoxide + HMR	43 ± 3	6.8 ± 1.9
GF	40 ± 7	7.3 ± 1.4
5HD	40 ± 5	7.5 ± 1.4
HMR	41 ± 4	7.4 ± 1.7

Data are presented as mean values ± SE and did not differ significantly among groups. IP indicates ischemic preconditioning. GF, 5HD, and HMR indicate GF109203X, 5-hydroxydecanoate, and HMR-1098, respectively.

of the excessive myocardial collateral blood flow (>15 ml/100 g/min). Therefore, the remaining 91 dogs completed the protocols satisfactorily and were used for data analysis (Table 1).

The effects of administration of GF109203X, 5HD, or HMR-1098, on the opening of mitoK$_{ATP}$ channels-induced infarct size-limiting effect

Systolic (~137 mmHg) and diastolic (~81 mmHg) ABP and HR (~137/min) before, during, and after 90 min of myocardial ischemia were not significantly changed either among the 13 groups or throughout the study. In the IP, cromakalim, or diazoxide group, coronary hyperemic flow 5 min after 5 min of coronary occlusion or during administration of each chemical was observed (89 ± 2 to 327 ± 11 ml/100 g/min for IP; 88 ± 2 to 357 ± 6 ml/100 g/min for cromakalim; 87 ± 2 to 243 ± 10 ml/100 g/min for or diazoxide, $P < 0.001$ each). Infusions of GF109203X, 5HD, and HMR-1098 did not affect either basal CBF (88 ± 2 to 89 ± 3, 87 ± 2, and 89 ± 2 ml/100 g/min) or hyperemic CBF (cromakalim-induced hyperemia: 357 ± 6 to 348 ± 5, 353 ± 4, and 366 ± 6 ml/100 g/min; diazoxide-induced hyperemia: 243 ± 10 to 231 ± 6, 234 ± 7, and 251 ± 4 ml/100 g/min), respectively.

Table 2 shows the risk area and collateral flow among each group. The risk area and collateral flow were comparable in all of the groups. Fig. 1 shows infarct size in these groups. IP, cromakalim, or diazoxide attenuated infarct size, and the infarct size-limiting effect of either IP or cromakalim group was more than the diazoxide group. The infarct size-limiting effect of cromakalim was partially attenuated and the infarct size-limiting effect of diazoxide was completely abolished by either GF109203X or 5HD; HMR-1098 did not affect the infarct size-limiting effect of either cromakalim or diazoxide, suggesting that the opening of mitoK$_{ATP}$ limits infarct size via PKC-dependent mechanisms. Table 3 shows the regression analysis between collateral flow and infarct size because infarct size depends on the collateral flow. This analysis verifies the observation of Fig. 1.

Changes in either myocardial PKC and ecto-5′-nucleotidase activity

Either transient administration of either cromakalim or diazoxide increased PKC activity of the myocardium, which was blunted by the removal of Ca^{2+} (Fig. 2), indicating that activated PKC is Ca^{2+}-dependent. We also found that ecto-5′-nucleotidase is activated by either cromakalim or diazoxide to the identical extent, and the administration of either GF109203X or 5HD blunted these increases in ecto-5′-nucleotidase activity (Fig. 2). However, HMR-1098 did not affect the cromakalim- or diazoxide-induced activation of both PKC and ecto-5′-nucleotidase. These results suggest that activation of mitoK$_{ATP}$ channels activates PKC and this activated PKC activates ecto-5′-nucleotidase.

Discussion

The infarct size-limiting effect of the opening of K$_{ATP}$ channels: the role of PKC activation

In the present study, we showed that the inhibitor of PKC or the blocker of mitoK$_{ATP}$ channels blunted either the infarct size-limiting effect of either cromakalim or diazoxide or the activation of both PKC and ecto-5′-nucleotidase in the canine hearts.

Although PKC is reported to open the mitoK$_{ATP}$ channels [9,10] and the opening of mitoK$_{ATP}$ channels is considered as a final mediator of IP, Pain et al. [11] reported that the opening of mitoK$_{ATP}$ channels activates p38MAP

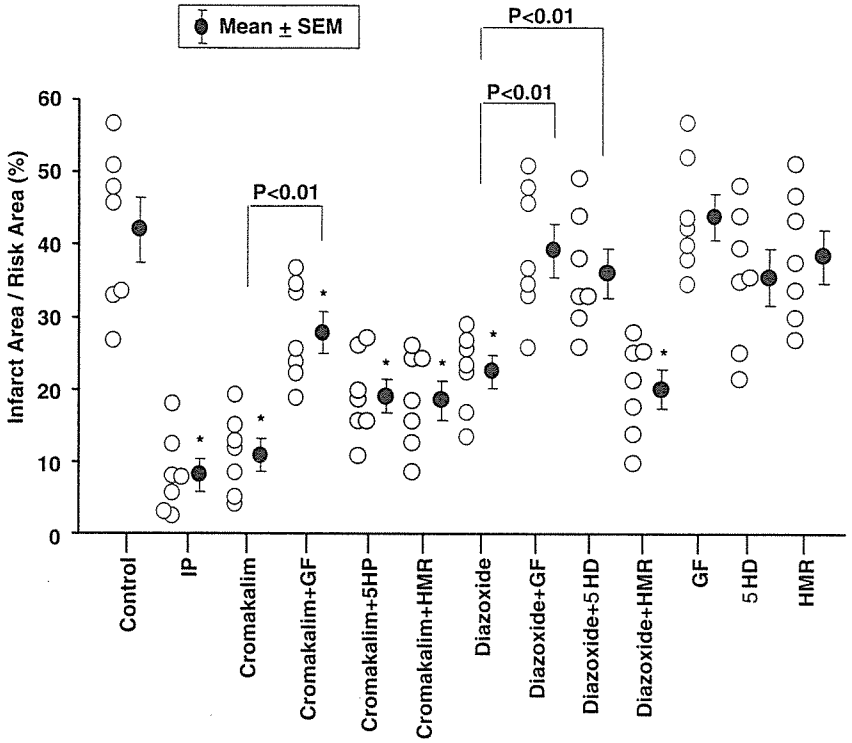

Fig. 1. Infarct size in the control group, the IP group, the cromakalim group, the cromakalim + GF group, the cromakalim + 5HD group, the cromakalim + HMR group, the diazoxide group, the diazoxide + GF groups, the diazoxide + 5HD groups, the diazoxide + HMR groups, the GF group, the 5HD group, and the HMR group. GF, 5HD, and HMR indicate GF109203X, 5-hydroxydecanoate, and HMR-1098, respectively. Infarct size was markedly decreased in IP, cromakalim, or diazoxide group. The infarct size-limiting effect of either cromakalim or diazoxide was attenuated by either GF109203X or 5HD, respectively. $^{*}P < 0.01$ vs. control group.

Table 3
The equation of the linear regression line in each group: Y (infarct size, %) = aX (the collateral blood flow, mL/100 g/min) + b

	a slope	b intercept
Control	−2.87	62.7
IP	−1.28	17.1
Cromakalim	−1.26	20.6
Crornakalim + GF	−1.78	43.0
Cromakalim + 5HD	−1.55	34.5
Crornakalim + HMR	−2.65	41.9
Diazoxide	−1.31	31.7
Diazoxide + GF	−2.19	56.1
Diazoxide + 5HD	−3.97	73.6
Diazoxide + HMR	−1.63	34.1
GF	−2.09	59.0
5HD	−3.14	64.6
HMR	−2.40	62.2

ANOVA indicates $P < 0.01$ between Control group vs. IP, Crornakalim, GF + Cromakalim, Diazoxide group, $P < 0.01$ between Cromakalim group vs. GF + Cromakalim group, and between Diazoxide group vs. GF + Diazoxide group. IP indicates ischemic preconditioning. GF, 5HD, and HMR indicate GF109203X, 5-hydroxydecanoate, and HMR-1098, respectively.

kinase via the production of oxidative stress. The present study also added the evidence that activation of mitoK$_{ATP}$ channels activates PKC [7,25,26], which merits cardioprotection. Indeed, we found that diazoxide activates PKC, and that 5HD blunts either cromakalim- or diazoxide-induced PKC activation, but HMR-1098 does not. Since IP procedure activates PKC, the positive forward and backward feedback loop between PKC-mitoK$_{ATP}$ channels following the IP procedure may contribute to the potent cardioprotection against ischemia and reperfusion injury. However, we should recognize that diazoxide does not necessarily induce PKC translocation and that PKC inhibitors did not necessarily inhibit infarct size-limiting effects of diazoxide and nicorandil in the earlier studies [27,28]. This may be attributable to the differences in the species; these two earlier studies used rabbit hearts, and our study used canine hearts.

There are two types of K$_{ATP}$ channels, i.e., sarcolemmal (sarcoK$_{ATP}$) and mitoK$_{ATP}$ channels [29–31]. Cromakalim opens both sarcoK$_{ATP}$ and mitoK$_{ATP}$ channels, and diazoxide opens only mitoK$_{ATP}$ channels. Since the infarct size-limiting effect of cromakalim was blunted partially by either GF109203 or 5HD and the infarct size-limiting effect of diazoxide was completely blunted by GF109203 in the present study, we suggest that cardioprotection due to the opening of mitoK$_{ATP}$ channels is PKC-dependent. Since the other factors such as catecholamine, bradykinin, or adenosine also activate PKC, the involvement of the opening mitoK$_{ATP}$ channels for the activation of PKC was partial and the opening of mitoK$_{ATP}$ channels activated PKC to the lesser extent of IP.

However, there are reports showing that diazoxide opens the sarcoK$_{ATP}$ channels during myocardial ischemia

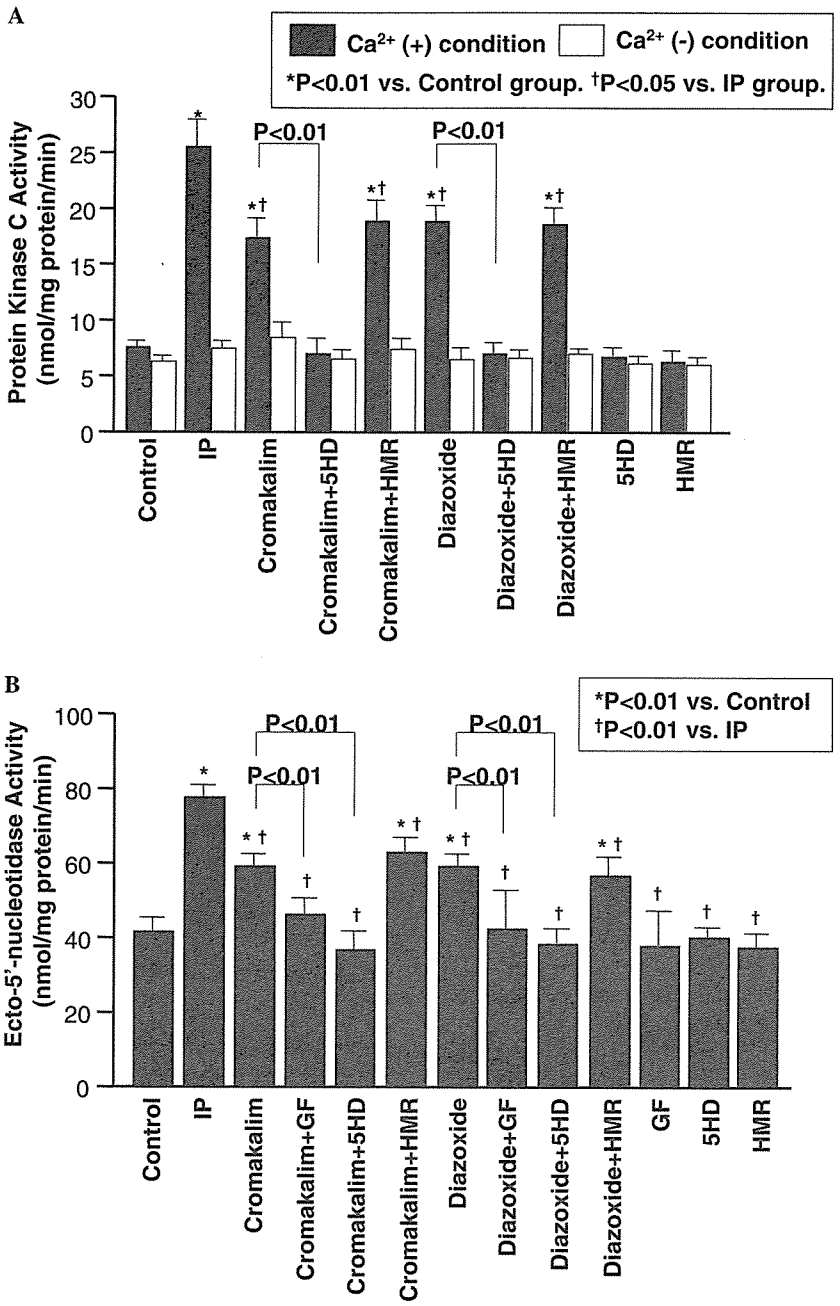

Fig. 2. Comparison of PKC (A) and ecto-5′-nucleotidase (B) activity of canine myocardial samples following the IP procedure or the exposures of cromakalim or diazoxide with and without 5HD, HMR-1098, or GF109203X. GF, 5HD, and HMR indicate GF109203X, 5-hydroxydecanoate, and HMR-1098, respectively. The IP procedure, cromakalim, or diazoxide activated PKC and ecto-5′-nucleotidase. The activation of both PKC and ecto-5′-nucleotidase were prevented by 5HD. Results are presented as means ± SEM.

[32,33]. In fact, the infarct size-limiting effect of diazoxide has been shown to be partially blocked by HMR-1098 [33]. In the present study, the infarct size-limiting effect of diazoxide was blunted by 5HD but not by HMR-1098, suggesting the potential involvement of the opening of mito-K_{ATP} channels, and a lesser role of the opening the sarcoK_{ATP} channels in the infarct size-limiting effect of diazoxide. The difference between the present and previous studies may be attributable to doses of diazoxide and the species differences. Indeed, the higher dose of diazoxide opens not only mitoK_{ATP} channels, but also sarcoK_{ATP} channels. Intriguingly, the infarct size-limiting effect of cromakalim that opens both mitoK_{ATP} and sarcoK_{ATP} channels was partially blunted by HMR-1098, which agrees to our previous finding [17].

The opening of mitoK_{ATP} channels, in turn, activates p38MAP kinase, which also contributes to cardioprotection [11]. Indeed, we have previously reported that p38MAP kinase is involved in cardioprotection of IP [8]. Since PKC activates p38MAP kinase via the route of the opening of mitoK_{ATP} channels, the present and previous studies suggest that PKC-p38MAP kinase linkages follow-

ing the opening of mitoK$_{ATP}$ channels is responsible for cardioprotection.

How is PKC activated by the opening of mitoK$_{ATP}$ channels? There are several reports stating that PKC is activated by free radicals [34–36]. Since the opening of mitoK$_{ATP}$ channels produces oxygen-derived free radicals, the oxygen-derived free radicals produced by the opening of mitoK$_{ATP}$ channels may be involved in the activation of PKC in the present study. Since the opening of sarcoK$_{ATP}$ decreases the membrane potentials and inhibits Ca^{2+} inward, Ca^{2+}-dependent PKC may not be activated by the opening of sarcoK$_{ATP}$.

The role of ecto-5′-nucleotidase in cardioprotection of IP

We have previously reported that ecto-5′-nucleotidase is phosphorylated and activated by the IP procedure [7,8], and the inhibitor of ecto-5′-nucleotidase blunted the infarct size-limiting effect of IP [13]. We also showed that the PKC activation increases ecto-5′-nucleotidase activity and mediates the infarct size-limiting effects [7]. We thought that α-adrenoceptor stimulation is responsible for the activation of PKC [37,38], and others showed the involvements of bradykinin, adenosine, or histamine [39]. We also added the new evidence that the opening of mitoK$_{ATP}$ channels is another factor to activate PKC.

We have proposed the importance of ecto-5′-nucleotidase activation in the infarct size-limitation. We showed that enhanced adenosine exposures due to ecto-5′-nucleoitdase activation following IP procedure triggers the IP-induced cardioprotection, and enhanced production of adenosine during reperfusion period following lethal duration of myocardial ischemia also contributed to the cardioprotection. The latter may explain the cardioprotection of post-conditioning [40]. It is true that adenosine is involved in the cardioprotection of post-conditioning [41].

However, we should also notice the studies disputing the crucial role of PKC in canine hearts and swine hearts [42,43]. Furthermore, there are also studies arguing against contribution of ecto-5′-nucleotidase to IP [44–46], and it remains unclear how activated ecto-5′-nucleotidase protects the myocardium from ischemic necrosis. Although the activation of PKC seems to play the substantial role for cardioprotection, another route such as either P38MAPK or tyrosine kinase aside from PKC may play an alternative role for cardioprotection, which may decrease the relative importance of PKC. This situation may be also the same in ecto-5′-nucleotidase. Although our studies revealed that activation of ecto-5′-nucleotidase plays an important role in the cardioprotection due to IP, if the other pathway to activate adenosine-related process may take the role of ecto-5′-nucleotidase, the activation of ecto-5′-nucleotidase becomes less important for cardioprotection. These differences may be attributable to species differences or the differences in the strength or duration of ischemia–reperfusion episode.

Limitations of the present study

This study did not provide the direct evidence that mitoK$_{ATP}$ and sarcoK$_{ATP}$ channels are opened due to the technical limitation in the present canine study. To obtain such data, we need to perform the in vitro experiments using rat cardiomyocytes with the equipments. There is ample evidence to suggest that the current doses of either cromakalim or diazoxide open both mitoK$_{ATP}$ and sarcoK$_{ATP}$ channels and only mitoK$_{ATP}$ channels, respectively.

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