responsible for adenosine production, because it is located on the cell membrane and may be targeted by oxygen-derived free radicals. These lines of evidence support the hypothesis that amlodipine increases coronary blood flow (CBF) in ischemic myocardium via both adenosine- or NO-dependent mechanisms.

The aim of this study was to determine whether 1) amlodipine increases either adenosine or NO release from ischemic myocardium in canine hearts, and 2) whether coronary vasodilation mediated by amlodipine is attenuated by either 8-sulfophenyltheophylline (8SPT) or L$^{\omega}$-nitro arginine methyl ester (L-NAME). To approach the goal, we examined the difference in the concentrations of adenosine (VAD (Ado)) and nitrate + nitrite (VAD (NOx)) between coronary venous blood and coronary arterial blood during infusion of amlodipine with 8SPT or L-NAME treatment. Furthermore, we tested whether amlodipine could increase VAD (Ado), VAD (NOx), and CBF in the presence of superoxide dismutase (SOD). We showed that amlodipine could increase both adenosine and NO release in ischemic canine myocardium, thus contributing to coronary vasodilation. We also detected a role of amlodipine-induced attenuation of oxygen-derived free radical generation in these actions.

2. Materials and methods

2.1. Instrumentation [13]

The hybrid dogs (HBD) mated with the Beagle, the American Fox Hound and the Labrador Retriever for the laboratory use (body weight: 15–21 kg) were anesthetized by an intravenous injection of sodium pentobarbital (30 mg/kg), intubated, and ventilated using room air mixed with oxygen (100% O_2 at a flow rate of 1.0–1.5 l/min). The chest was opened through the left fifth intercostal space, and the heart was suspended a pericardial cradle. After intravenous administration of heparin (500 U/kg), the left anterior descending (LAD) coronary artery was cannulated and perfused with blood from the left carotid artery through an extracorporeal bypass tube. CBF in the perfused region was measured with an electromagnetic flow probe attached to the bypass tube, and coronary perfusion pressure (CPP) was monitored at the tip of the coronary artery cannula. We obtained coronary vascular resistance (CVR) by calculation of CPP/CBP.

A thin (1 mm) and short (70 mm) cannula connected to a thin tube was inserted into a small coronary vein near the center of the perfused region to sample coronary venous blood. The draining venous blood was collected in a reservoir at the level of the left atrium and then was returned to the jugular vein. Hydration was maintained by slow infusion of normal saline. The pH, pO_2, and pCO_2 of systemic arterial blood before the protocol was 7.39 ± 0.02, 106 ± 3, and 38.0 ± 2.0 mmHg, respectively. A pair of ultrasonic crystals was placed at the inner one-third of the myocardium about 10 mm apart in order to measure myocardial segment length with an ultrasonic dimension gauge (5 MHz, 2 mm in diameter; Schuessler, Cardiff-by-the Sea, CA). Hemodynamic parameters were recorded on a multi-channel recorder (Rm-6000; Nihon Kohden, Tokyo, Japan). End-diastolic length (EDL) was measured at the R wave of the electrocardiogram and end-systolic length (ESL) was measured at the minimal value of the first derivative of left ventricular pressure. Then fractional shortening (FS) was calculated as [(EDL − ESL)/EDL] × 100%.

Agents were administered into the LAD via the bypass tube. We purchased sodium pentobarbital, heparin, 8SPT, L-NAME, adenosine, bradykinin, and SOD from Sigma (St. Louis, MO, USA). Amlodipine was kindly provided by Pfizer Pharmaceutical Inc. (Sandwich, UK). The investigation conforms with the *Guide for the Care and Use Laboratory Animals* published by the US National Institutes of Health (NIH Publication NO. 85-23, revised 1996).

2.2. Experimental protocols

2.2.1. Protocol I

After hemodynamic stabilization, coronary arterial and venous blood were sampled for blood gas analysis and for measurement of the lactate, nitrate + nitrite [14], and adenosine [15] concentrations, allowing the calculation of VAD (NOx), VAD (Ado), myocardial oxygen consumption. Myocardial oxygen consumption (ml/100 g/min) is calculated by CBF (ml/100 g/min) × the oxygen difference between coronary arterial and venous blood (ml/dl).

Lactate concentrations were measured by an enzymatic assay, and lactate extraction ratio (LER) was calculated as the coronary arteriovenous difference of the lactate concentration multiplied by 100 and divided by the arterial lactate concentration.

We used 29 dogs for Protocol I. Hemodynamic parameters (systolic and diastolic aortic blood pressure, and heart rate) were monitored. To examine whether either adenosine- or NO-dependent mechanisms were involved in amlodipine-induced coronary vasodilation in ischemic heart, we infused saline (n = 9), 8SPT (25 μg/kg per min at an infusion rate of 0.0167 ml/kg per min and a concentration of 1.5 mg/ml, n = 5), L-NAME (10 μg/kg per min at an infusion rate of 0.0167 ml/kg per min and a concentration of 0.6 mg/ml, n = 5), or L-NAME + 8SPT (n = 5) into the bypass. We dissolved amlodipine in 0.15% DMSO with saline and either L-NAME or 8SPT in saline. After we confirmed that either systemic or coronary hemodynamics did not change after 5 min of the infusion, we reduced CPP so that CBF decreased to 50% of the baseline value for 5 min. Then amlodipine (2 μg/kg per min at an infusion rate of 0.0167 ml/kg per min and a concentration of 0.12 mg/ml) was infused and continued for 120 min while CPP was maintained at the set low constant value. As for the control of the amlodipine treatment, we infused the solvent of amlodipine (at an infusion rate of 0.0167 ml/kg per min and a concentration of 0.12 mg/ml, n = 5). The times for the measurements of coro-

nary and systemic hemodynamics, and adenosine and NO concentrations after the onset of coronary hypoperfusion were 10, 30, 60, 90 and 120 min after the onset of coronary hypoperfusion. As a marker of oxidative stress, the 8-iso-prostaglandin $F_{2\alpha}$ (8-Iso-$F_{2\alpha}$) concentration was measured in coronary arterial and venous blood in nine dogs in the amlodipine-treated dogs without either 8SPT or L-NAME, and we calculated the arteriovenous difference of 8-Iso-$F_{2\alpha}$ (VAD(8-Iso-$F_{2\alpha}$)).

In a preliminary study, we confirmed that the doses of 8SPT and L-NAME were the minimum doses that prevented coronary vasodilation induced by intracoronary administration of adenosine (2 μg/kg per min) and bradykinin (20 ng/kg per min), respectively. The dose of amlodipine (2 μg/kg per min) for an intracoronary infusion was the lowest dose that caused maximal coronary vasodilation.

2.2.2. Protocol II

To examine whether elimination of oxidative stress was involved in amlodipine-induced adenosine- or NO-dependent coronary vasodilation in ischemic hearts, we infused recombinant human SOD (5340 IU/mg, > 99% purity) (25 μg/kg per min at an infusion rate of 0.0167 ml/kg per min and a concentration of 1.5 mg/ml, $n = 10$) into the bypass tube at a rate of 1.5 ml/kg per min after hemodynamic stability was achieved. Then we reduced CPP so that CBF decreased to 50% of baseline. At 5 min after the onset of ischemia, infusion of amlodipine was initiated and continued for 120 min while keeping CPP at the 50% value. We previously confirmed that this dose of SOD did not affect systemic and coronary hemodynamic parameters [11]. Adenosine, NO and 8-Iso-$F_{2\alpha}$ concentrations were measured in coronary arterial and venous blood. In the preliminary study, we tested the coronary effects of the solvent of amlodipine (at an infusion rate of 0.0167 ml/kg per min and a concentration of 0.12 mg/ml, $n = 5$) as the control of the amlodipine treatment.

2.2.3. Protocol III

This protocol tested the effects of amlodipine in the constant flow model because the increases in CBF due to amlodipine may secondarily alter the adenosine or NO release in ischemic hearts. We used five dogs in Protocol III. Hemodynamic parameters (systolic and diastolic aortic blood pressure, and heart rate) were monitored. After the hemodynamic stabilization, we reduced CPP so that CBF decreased to 50% of the baseline value for 5 min, and we kept CBF constant at this low level thereafter. Then an infusion of amlodipine (2 μg/kg per min at an infusion rate of 0.0167 ml/kg per min and a concentration of 0.12 mg/ml) was initiated and continued for 120 min while CBF was maintained at the 50% value. The times for the measurements of coronary and systemic hemodynamics, and adenosine and NO concentrations were same as in Protocol I.

2.2.4. Protocol IV

Adenosine and NO were produced via ecto-5′-nucleotidase and NOS, respectively. To test the cellular mechanisms of amlodipine-induced production of either adenosine or NO, we tested whether amlodipine restores the deactivation of ecto-5′-nucleotidase and NOS caused by the oxygen-derived free radicals in rat cardiomyocytes. In rat cardiomyocytes with xanthine (1×10^{-4} M) and xanthine oxidase (1.6×10^{-3} U/ml), that produces oxygen-derived free radicals, the time courses of the changes in both ecto-5′-nucleotidase and NOS activities were observed with and without the concomitant administration of amlodipine of 1×10^{-7} and 1×10^{-8} M for 60 min in rat cardiomyocytes ($n = 6$ each). We obtained the rat cardiomyocytes following the previously reported method [16]. The methods to measure both myocardial ecto-5′-nucleotidase [17] and NOS [18] activities have been reported previously.

Since the calculated amlodipine levels in the hearts in Protocols I–III correspond to 1×10^{-6} M of amlodipine, we employed 1×10^{-7} and 1×10^{-8} M of amlodipine. This is because in in vivo experiments, amlodipine may be conjugated by protein in the blood, and amlodipine concentrations at the site of small coronary smooth muscles may be decreased by the barrier of endothelial cells, which suggest that we need to use a smaller dose of amlodipine to exert the comparable effects of amlodipine in vivo.

2.3. Statistical analysis

Statistical analysis was performed using two-way ANOVA [19,20] to compare data among the groups. When ANOVA reached significance, paired data were compared using Bonferroni's test. Changes of the hemodynamic and metabolic parameters over time were compared by ANOVA for repeated measures. Values were expressed as the mean ± S.E.M., with $P < 0.05$ indicating significance.

3. Results

Either mean systemic blood pressure (101 ± 2 mmHg) and heart rate (134 ± 2 per min) did not differ significantly among all of the groups in Protocols I–III. Before and during coronary hypoperfusion with or without pharmacological intervention, these systemic hemodynamic parameters did not change significantly. Before coronary hypoperfusion, CPP was not different among the groups (104 ± 6 mmHg in the amlodipine group, 99 ± 5 mmHg in the solvent group, 102 ± 4 mmHg in the amlodipine + 8SPT group, 103 ± 4 mmHg in the amlodipine + L-NAME group, 99 ± 6 mmHg in the amlodipine + 8SPT + L-NAME group, 100 ± 6 mmHg in the SOD with or without amlodipine groups and 101 ± 6 mmHg in the constant CBF group), and the additional intracoronary infusion of amlodipine, the solvent of amlodipine, 8SPT, L-NAME or 8SPT + L-NAME did not significantly alter CPP. In the 8SPT, L-NAME, and 8SPT + L-NAME groups, CPP (57 ± 2 mmHg, 58 ± 3 mmHg, and 61 ± 3 mmHg, respectively) was higher (all $P < 0.05$) than in the solvent group (51 ± 3 mmHg) during coronary hypoperfusion, while CPP was lower ($P < 0.05$) in the SOD groups (42 ± 1 mmHg).

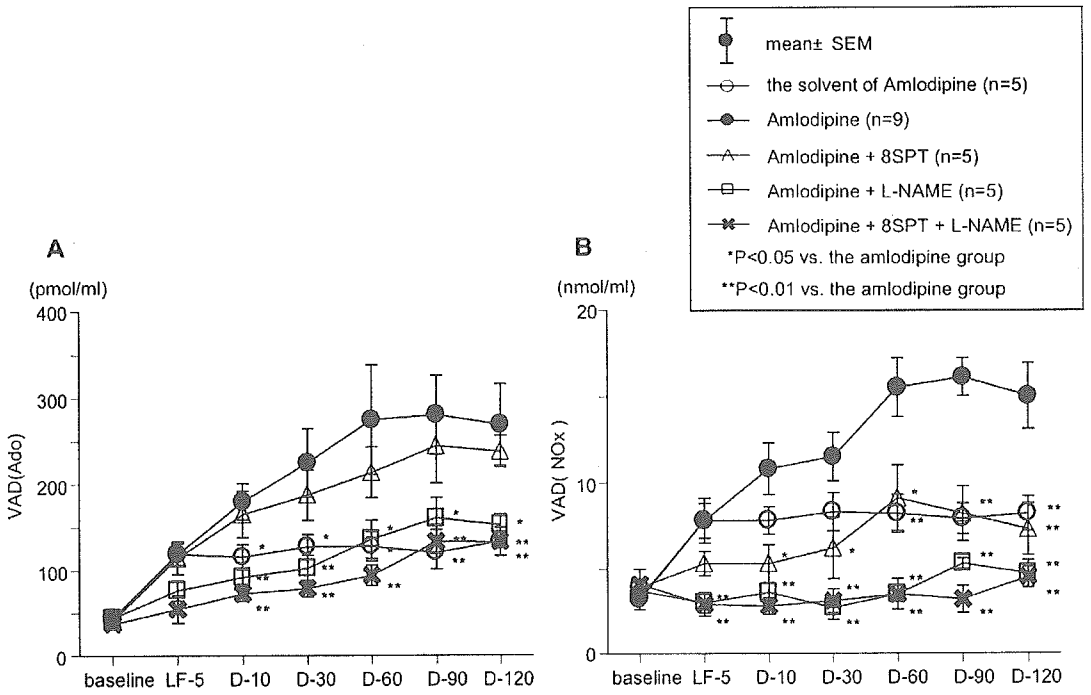

Fig. 1. Effects of either amlodipine or solvent of amlodipine on VAD (Ado) (A) and VAD (NOx) (B) in canine ischemic myocardium. VAD (Ado) and VAD (NOx) = the differences of adenosine and nitrate + nitrite concentrations between coronary venous and arterial blood, respectively, LF-5 = at 5 min of low flow, D-10, 30, 60, 90 and 120 = at 10, 30, 60, 90 and 120 min after the onset of either amlodipine or solvent of amlodipine infusion, respectively. Data are mean ± S.E.M. * $P < 0.05$, ** $P < 0.01$ vs. the amlodipine group.

Fig. 1 shows VAD (Ado) (A) and VAD (NOx) (B) in the amlodipine, the solvent, amlodipine + 8SPT, amlodipine + L-NAME, and amlodipine + 8SPT + L-NAME groups. Both VAD (Ado) and VAD (NOx) increased ($P < 0.05$) in response to decreases of both CBF and CPP, and the amlodipine infusion further increased both VAD (Ado) and VAD (NOx) (both $P < 0.01$). The increase of VAD (Ado) was attenuated by L-NAME ($P < 0.05$), and that of VAD (NOx) was attenuated by both L-NAME ($P < 0.01$) and 8SPT ($P < 0.01$). The solvent for amlodipine did not alter either of these parameters. Fig. 2 shows that amlodipine increased CBF ($P < 0.01$) and decreased CVR ($P < 0.05$) in ischemic hearts, while either 8SPT or L-NAME attenuated the increase of CBF (both $P < 0.05$), and 8SPT + L-NAME almost completely blocked the changes of both CBF ($P < 0.01$) and CVR ($P < 0.05$). The solvent for amlodipine did not alter either CBF or CVR. Fig. 3 shows that FS (A), LER (B) and myocardial oxygen consumption (C) decreased after the onset of coronary hypoperfusion, while all increased (both $P < 0.01$) along with the amlodipine infusion. The infusion of the solvent for amlodipine changed none of these parameters during coronary hypoperfusion. These results indicate that amlodipine increased CBF and ameliorated myocardial ischemia via either adenosine- or NO-dependent mechanisms.

We also found that ischemia increases VAD (8-Iso-$F_{2\alpha}$) from 5.0 ± 0.71 to 10.8 ± 1.1 pg/ml ($P < 0.01$), and amlodipine decreases ($P < 0.01$) VAD (8-Iso-$F_{2\alpha}$) (7.4 ± 0.9, 6.8 ± 1.2, 5.8 ± 1.0 5.4 ± 0.9, 5.0 ± 0.5 pg/ml at 10, 30, 60 90 and 120 min after the onset of amlodipine administration, respectively). Figs. 4 and 5 show the effect of SOD on the amlodipine-induced increase of VAD (Ado) (Fig. 4A), VAD (NOx) (Fig. 4B) and VAD (8-Iso-$F_{2\alpha}$) (Fig. 4C), CPP (Fig. 5A), CBF (Fig. 5B), and CVR (Fig. 5C). Treatment with SOD increased both VAD (Ado) ($P < 0.01$) and VAD (NOx) ($P < 0.05$) and reduced VAD (8-Iso-$F_{2\alpha}$) ($P < 0.01$), while amlodipine did not further increase VAD (Ado), VAD (NOx), or CBF, or decrease either CVR or VAD (8-Iso-$F_{2\alpha}$). Fig. 6 shows that amlodipine did not further increase either FS (A) or LER (B) in the presence of SOD during coronary hypoperfusion.

We examined the effects of the solvent for amlodipine in ischemic myocardium. We observed no additional effects of the solvent for amlodipine on VAD (Ado) (125 ± 17 and 123 ± 14 pmol/ml at LF-5 and D-60, ns), VAD (NOx) (7.9 ± 1.7 and 8.1 ± 1.2 nmol/ml at LF-5 and D-60, ns), VAD (8-Iso-$F_{2\alpha}$) (11.4 ± 1.2 and 12.0 ± 1.5 pg/ml at LF-5 and D-60, ns), CPP (51 ± 2 and 51 ± 2 mmHg at LF-5 and D-60, ns), CBF (51 ± 5 and 50 ± 5 ml/100 g/min at LF-5 and D-60, ns), CVR (1.0 ± 0.1 and 1.0 ± 0.1 mmHg/ml/100 g/min at LF-5 and D-60, ns), FS (7.7 ± 1.2 and 7.7 ± 1.1% at LF-5 and D-60, ns), and LER (−29 ± 11 and −25 ± 5% at LF-5 and D-60, ns) during coronary hypoperfusion.

In Protocol III, we kept CBF constant at the 50% of the control levels to keep the severity of myocardial ischemia constant. We observed that amlodipine decreased CPP, and increased the ischemia-induced increase of VAD (Ado) and VAD (NOx) despite unchanged CBF and myocardial oxygen consumption (Table 1).

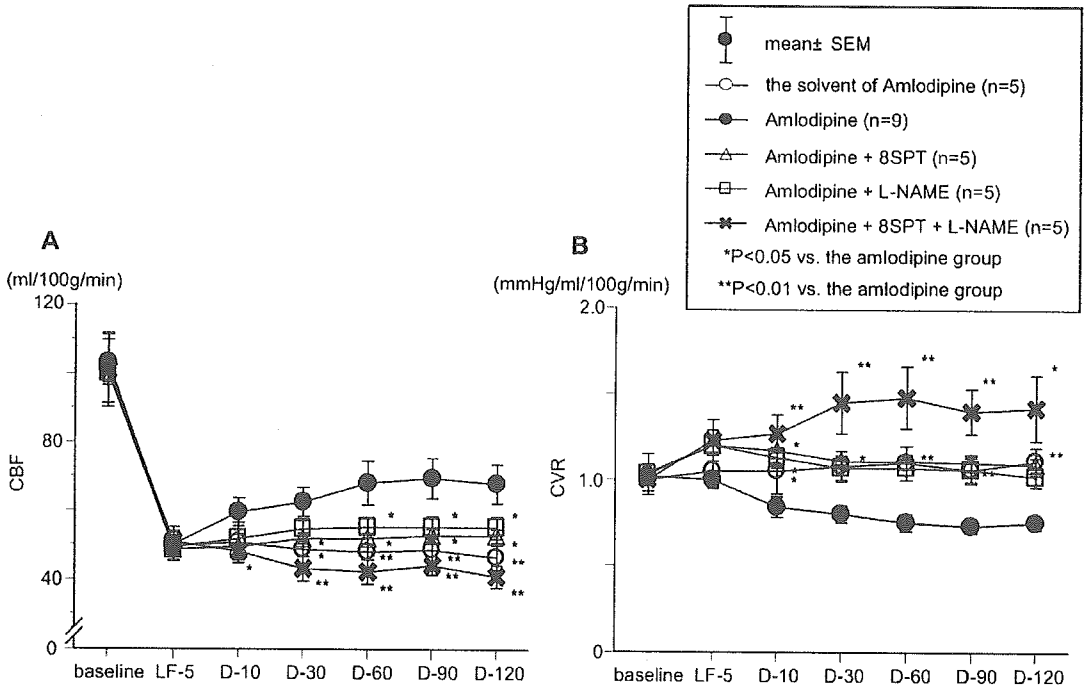

Fig. 2. Effects of either amlodipine or a solvent of amlodipine in CBF and CVR in ischemic myocardium. Either 8SPT or L-NAME attenuated the increases in CBF, and both 8SPT + L-NAME almost completely attenuated the increases in CBF. Abbreviations are same as in Fig. 1. Data are mean ± S.E.M. * $P < 0.05$, ** $P < 0.01$ vs. the amlodipine + saline group.

In Protocol IV, we observed the effects of amlodipine on the activities of ecto-5′-nucleotidase and NOS. Xanthine + xanthine oxidase deactivated ecto-5′-nucloetidase (control; 24.2 ± 2.4, 15 min; 16.8 ± 2.7 ($P < 0.01$ vs. control), 60 min; 14.5 ± 1.1 ($P < 0.01$ vs. control) nmol/mg protein per min, $n = 6$ each) and NOS (control; 100%, 15 min; 78 ± 3% ($P < 0.01$ vs. control), 60 min; 73 ± 3% ($P < 0.01$ vs. control), $n = 6$ each). We confirmed that these decreases are stable for 2 h. The exposure to xanthine + xanthine oxidase with 1×10^{-7}amlodipine prevented the deactivation of ecto-5′-nucleotidase (control; 23.6 ± 1.9, 30 min; 25.3 ± 1.1 ($P < 0.01$ vs. the condition without amlodipine), 60 min 25.9 ± 1.8 ($P < 0.01$ vs. the condition without amlodipine) nmol/mg protein/min, $n = 6$ each) and NOS (control; 100%, 30 min; 92 ± 5% ($P < 0.01$ vs. the condition without amlodipine), 60 min; 97 ± 4% ($P < 0.01$ vs. the condition without amlodipine), $n = 6$ each) of rat cardiomyocytes. These effects were also the case when we used 1×10^{-8} M amlodipine (ecto-5′-nucleotidase activity (control; 25.2 ± 2.3, 30 min; 22.5 ± 1.7 ($P < 0.01$ vs. the condition without amlodipine), 60 min 22.5 ± 1.3 ($P < 0.01$ vs. the condition without amlodipine) nmol/mg protein per min, $n = 6$ each) and NOS activity (control; 100%, 30 min; 92 ± 3% ($P < 0.01$ vs. the condition without amlodipine), 60 min; 92 ± 3% ($P < 0.01$ vs. the condition without amlodipine), $n = 6$ each)).

4. Discussion

The present study revealed that amlodipine, a long-acting Ca channel blocker, could increase CBF and ameliorate myocardial ischemia by increasing adenosine or NO release in ischemic hearts. Additionally, we found that elimination of oxygen-derived free radicals by amlodipine plays an important role in its induction of coronary vasodilation and in the increase of both VAD (Ado) and VAD (NOx), since these effects of amlodipine were blunted by treatment with SOD. Furthermore, we showed amlodipine attenuated the deactivation of ecto-5′-nucleotidase and NOS due to oxygen-derived free radicals in rat cardiomyocytes.

4.1. Adenosine, NO, and amlodipine in ischemic hearts

Under ischemic conditions, both alpha-adrenoceptor stimulation and H^+ increase the activity of ecto-5′-nucleotidase [21,22] and NOS [23,24]. Since oxygen-derived free radicals attenuate the ischemia-induced activation of ecto-5′-nucleotidase and enhance the degradation of NO, elimination of oxidative stress may increase adenosine or NO release in the myocardium. Since amlodipine has been reported to reduce oxidative stress, amelioration of oxidative stress by this drug may explain the present findings. Indeed, in the present study, we observed that 8-Iso-$F_{2\alpha}$ concentration, an index of oxidative stress, is attenuated by amlodipine, and amlodipine restores the decreased activities of ecto-5′-nucleotidase and NO synthase due to oxidative stress in rat cardiomyocytes.

Nevertheless, we need to consider other possibilities to explain the present findings. First of all, if amlodipine caused an increase of catecholamine or H^+ production, adenosine and NO release could be enhanced, but this is unlikely because

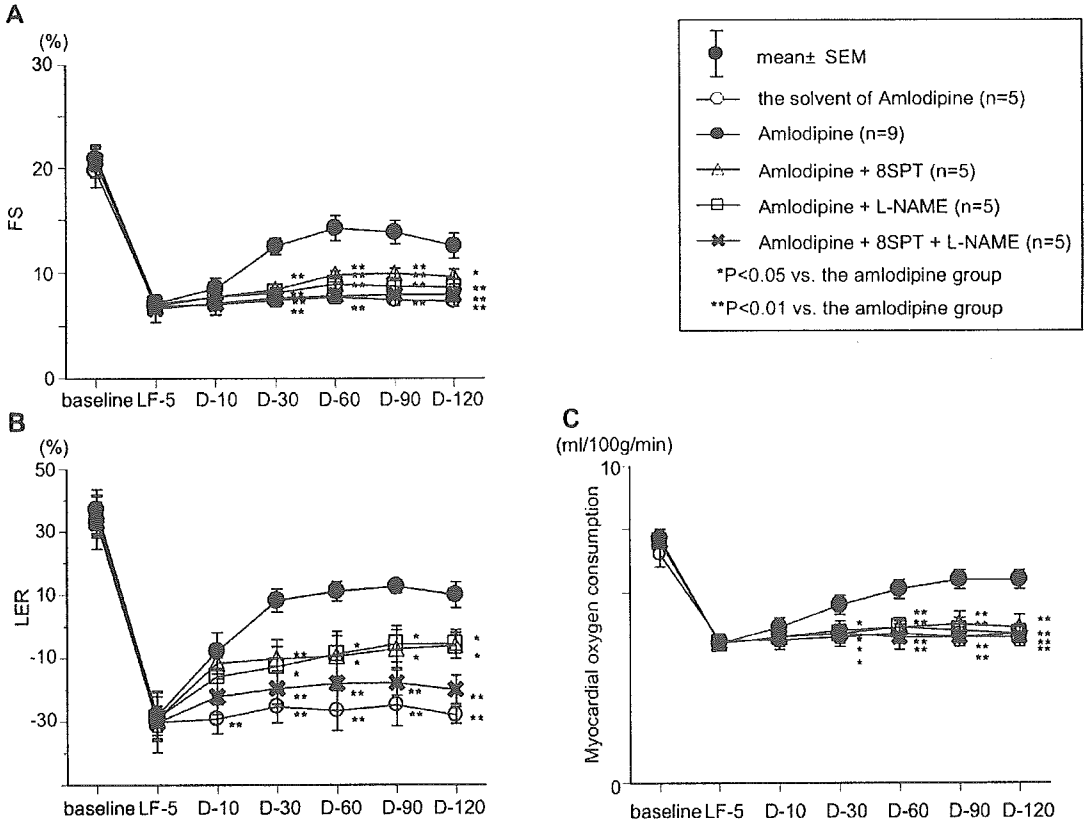

Fig. 3. Effects of either amlodipine or a solvent of amlodipine on FS (A), LER (B) and myocardial oxygen consumption (C) in ischemic myocardium. Abbreviations are same as in Fig. 1. Data are mean ± S.E.M. * $P < 0.05$, ** $P < 0.01$ vs. the amlodipine + saline group.

there have been no reports that amlodipine can modulate these parameters. Secondly, amlodipine may affect myocardial oxygen consumption, and secondarily increase CBF. We observed that amlodipine mediates no changes in myocardial oxygen consumption during constant low levels of CBF in Protocol III.

Although amlodipine increased both adenosine and NO production, thus improving CBF in ischemic hearts, the cellular signal transduction pathways that lead to coronary vasodilation by adenosine or NO are completely different. Adenosine and NO are reported to elevate cyclic AMP and GMP concentrations, respectively, which independently cause coronary vasodilation. The former mainly causes detachment of actin from myosin [25], while the latter mainly promotes re-uptake of Ca^{2+} into the SR [26]. Therefore, the increases of both adenosine and NO release independently leads to coronary vasodilation, as revealed by the fact that the attenuation of increased CBF was greater in the 8SPT + L-NAME group than in the 8SPT or L-NAME groups.

It was intriguing to find that L-NAME reduces adenosine release and 8SPT reduces NO release, because this observation implies that NO contributes to adenosine release, and that adenosine contributes to NO release. Interactions between adenosine and NO have been reported previously [27], and we have found that blockade of adenosine receptors activates NO synthase (unpublished data). Indeed, adenosine increases

Table 1
Changes in CPP, VAD (Ado), VAD (NOx), and myocardial oxygen consumption in CBF constant model

Baseline	LF-5	D-10 D-30	D-60	D-90	D-120
CPP (mmHg)					
101 ± 6**	51 ± 3	43 ± 2** 41 ± 2**	38 ± 2**	36 ± 2**	36 ± 3**
VAD (Ado) (pmol/ml)					
41 ± 7**	115 ± 14	161 ± 17* 174 ± 23**	256 ± 26**	262 ± 28**	255 ± 25**
VAD (NOx) (nmol/ml)					
3.4 ± 0.7**	8.0 ± 1.2	9.5 ± 0.8 10.8 ± 1.6**	13.2 ± 1.4**	13.9 ± 1.6**	13.4 ± 1.3**
Myocardial oxygen consumption (ml/100 g/min)					
7.1 ± 0.3**	4.4 ± 0.2	4.5 ± 0.3 5.0 ± 0.5	5.5 ± 0.6	5.5 ± 0.7	5.5 ± 0.6

Abbreviations: CPP = coronary perfusion pressure, VAD (Ado) and VAD (NOx) = the differences of adenosine and nitrate + nitrite concentrations between coronary venous and arterial blood, respectively, LF-5 = at 5 min of low flow, D-10, 30, 60, 90 and 120 = at 10, 30, 60, 90 and 120 min after the onset of the amlodipine infusion, respectively. Data are mean ± S.E.M. *$P < 0.05$, **$P < 0.01$ vs. the value of LF-5.

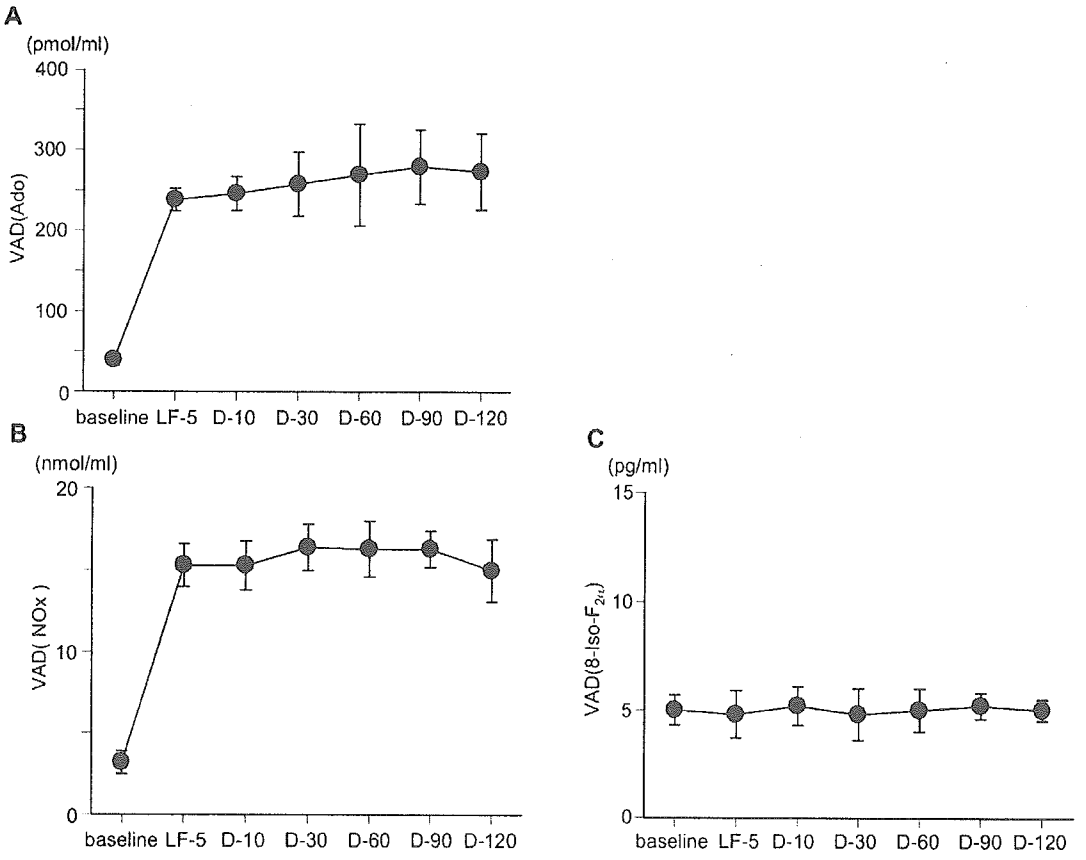

Fig. 4. Effects of amlodipine on VAD (Ado) (A), VAD (NOx) (B) and VAD(8-Iso-$F_{2\alpha}$) (C) in the presence of SOD in ischemic myocardium. Abbreviations are same as in Fig. 1.

the cyclic GMP concentration in vascular smooth muscle cells [28]. We also found that inhibition of NO synthase activates ecto-5′-nucleotidase in endothelial cells [29], suggesting that NO may reduce adenosine production. This may not agree to the present finding, and this difference may be attributable to the variations in experimental conditions (in vitro vs. in vivo, non-ischemic vs. ischemic hearts, and endothelial cells vs. whole hearts). We conclude that at least in ischemic hearts, both endogenous adenosine and NO mutually enhance their production in the in vivo hearts.

Our observation that both concentrations of adenosine and NO increase during an infusion of amlodipine meets the therapeutic requirements of ischemic hearts. Both adenosine and NO are believed to ameliorate myocardial ischemia [27] because both adenosine and NO 1) increase CBF, 2) reduce anaerobic metabolism, 3) inhibit platelet and leukocyte activation, and 4) reduce sympathetic activity in ischemic hearts. Indeed, the present study indicated that amlodipine could decrease the severity of myocardial ischemia (shown by FS and LER), with its effect being blunted by either 8SPT or L-NAME.

The present study suggested that elimination of oxygen derived-free radicals in ischemic hearts by amlodipine contributes to elevation of cardiac adenosine and NO concentrations, coronary vasodilation, and amelioration of myocardial ischemia because SOD blunted the beneficial effects of amlodipine. However, this result of SOD suggests two logical possibilities. One is that amlodipine increases oxygen-derived free radicals and that oxygen-derived free radicals induces coronary vasodilation. Indeed, SOD seems to decrease CBF, and thus, FS and LER in Figs. 5 and 6. However, in this protocol, we treated SOD prior to the time to decrease CPP, and in such a condition, oxygen-derived free radials are already eliminated in non-ischemic or ischemic myocardium. Since amlodipine has no more additional effects on ischemic hearts, the effects of amlodipine may be explained by the scavenging effects of oxygen-derived free radicals. In fact, another possibility that amlodipine attenuates oxygen-derived free radicals and oxygen-derived free radicals induced coronary vasoconstriction is more likely than the former possibility. We found that amlodipine attenuates the production of 8-Iso-F2α, supporting the latter hypothesis.

Since ecto-5′-nucleotidase is susceptible to oxygen derived-free radicals and oxygen derived-free radicals deactivate NO, it is likely that the beneficial effect of amlodipine on ischemic hearts in the present study was attributable to a combination of its anti-oxidant effect, the actions of adenosine and NO, and the Ca channel-blocking effects of amlodipine. However, if amlodipine works as a free radical scavenger, we need to consider the reason that almost 30 min is

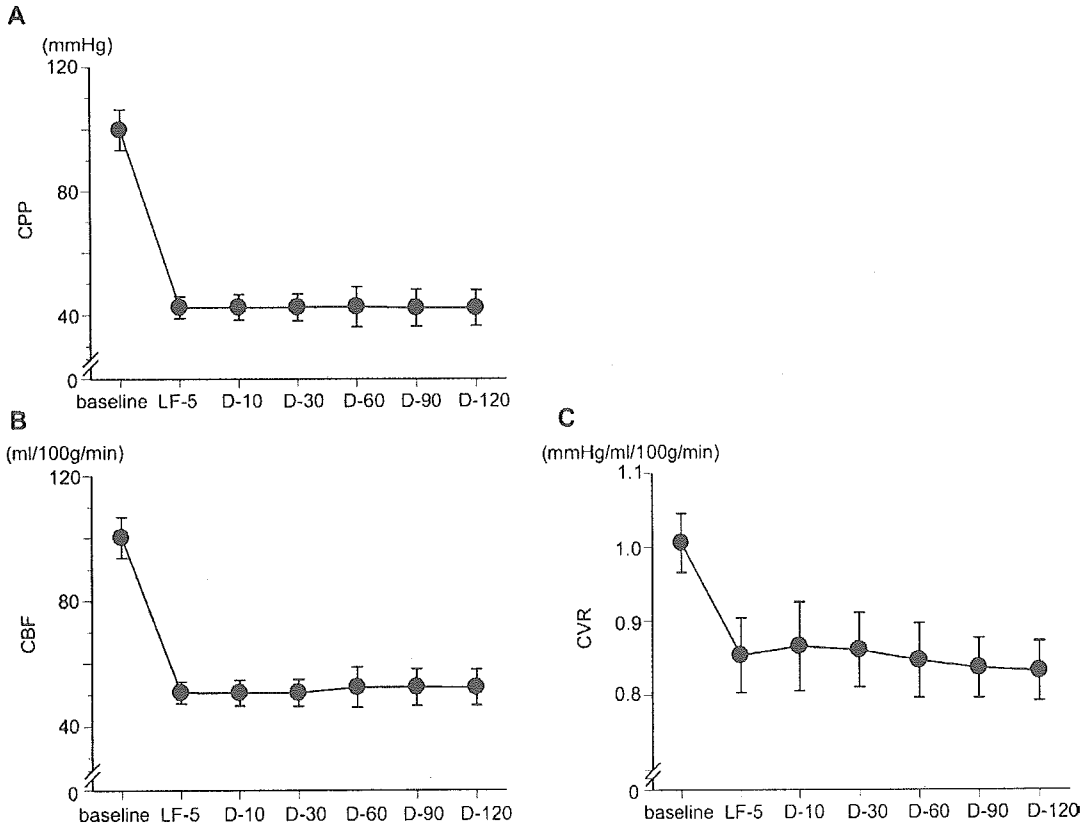

Fig. 5. Effects of amlodipine on CPP (A), CBF (B) and CVR (C) in the presence of SOD in ischemic myocardium. Abbreviations are same as in Fig. 1.

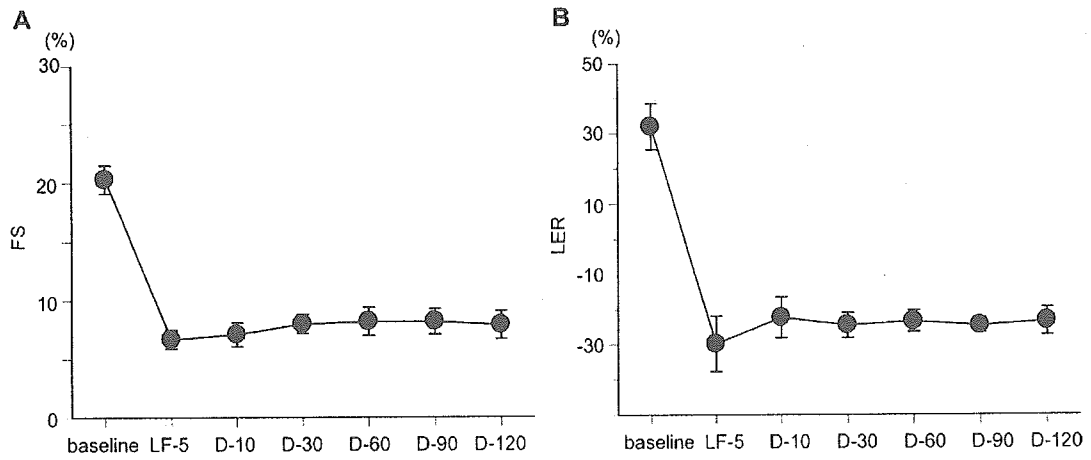

Fig. 6. Effects of amlodipine on FS (A) and LER (B) in the presence of SOD in ischemic myocardium. Abbreviations are same as in Fig. 1.

necessary to normalize VAD (8-Iso-F2α), and increase VAD (Ado) and VAD (NOx) following the amlodipine infusion. Since amlodipine is hydrophobic and amlodipine takes time to enter to the cellular membrane, VAD (8-Iso-$F_{2\alpha}$) began to decrease in 10 min of an infusion of amlodipine (Fig. 4) and returned to the level of the amlodipine + SOD group in 30-60 min, which is linked to the changes in VAD (Ado) and VAD (NOx). Indeed, in Fig. 1, VAD (Ado) and VAD (NOx) began to increase in 10 min and reached to the high steady levels in 30-60 min.

4.2. Physiological and clinical relevance

To extent the results of the present study to the cardiac physiology, we need to be careful to discuss the venous adenosine, NO and lactate concentrations at the limited myocardial regions, because the area of the perfusion by artery is not completely same as the area for the drainage by vessels [30–32]. Especially, collateral vessels may be modulated by amlodipine. However since 1) the areas of the perfusion and the drainage are 80–90% identical [30–32], and 2) an intrac-

oronary selective administration may not affect collateral vessels so much, the venous adenosine or NO concentrations reflects the production of either adenosine or NO in the regional myocardium.

It has been reported that amlodipine increases NO production by canine coronary endothelial cells [6], and that benidipine and nifedipine can increase NO release in ischemic hearts in vivo [7,8]. These results suggest that long-acting Ca channel blockers have the potential to increase tissue NO concentrations, or that common molecular characteristics may lead to linkage to the mechanisms of NO production. Interestingly, the present study revealed that amlodipine increases adenosine release as well as NO release, which is a novel therapeutic effect of the drug.

Although amlodipine increases both adenosine and NO concentrations near the Ca channels to which it binds, the concentrations of these mediators and their sites of action (e.g. endothelial cells, smooth muscle cells, and cardiomyocytes) may be different.

Intriguingly, we have previously reported that either benidipine or nifedipine increases CBF via NO-dependent mechanisms. Therefore, we believe that production of NO by amlodipine is the common feature of dihydropyridine Ca channel blockers [7,8], however, an adenosine receptor antagonist could not blunt the coronary hyperemia induced by benidipine [7]. Therefore, amlodipine-induced adenosine production is the unique property of amlodipine. We could not clarify the effects of different Ca channel blockers on adenosine or NO release. One possibility is as follows. Although NOS and ecto-5′-nucleotidase are sensitive for free radicals, the species of free radicals to reduce NOS and ecto-5′-nucleotidase may be different, and amlodipine may affect the species of free radicals to reduce NOS and ecto-5′-nucleotidase, however, other Ca channel blockers do not. Another possibility is the involvement of P38MAP kinase; Asano et al. showed that amlodipine activates P38 MAP kinase and increases the activities of NOS and ecto-5′-nucleotidase [33], which may overlap the present results.

In the PRAISE II study [34], amlodipine did not improve the mortality and morbidity of patients with chronic heart failure, suggesting that its beneficial effects may not be sufficient for this purpose, or that deleterious effects of Ca channel blockade may counteract the beneficial effects of adenosine and/or NO on the failing heart. In contrast, the CAMELOT study revealed that amlodipine decreased the incidence of cardiovascular events in patients with coronary artery disease than the angiotensin converting enzyme inhibitor (ACEI) did [35]. Since ACEI also increases NO release, the beneficial effects of amlodipine in this study may be related to adenosine.

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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

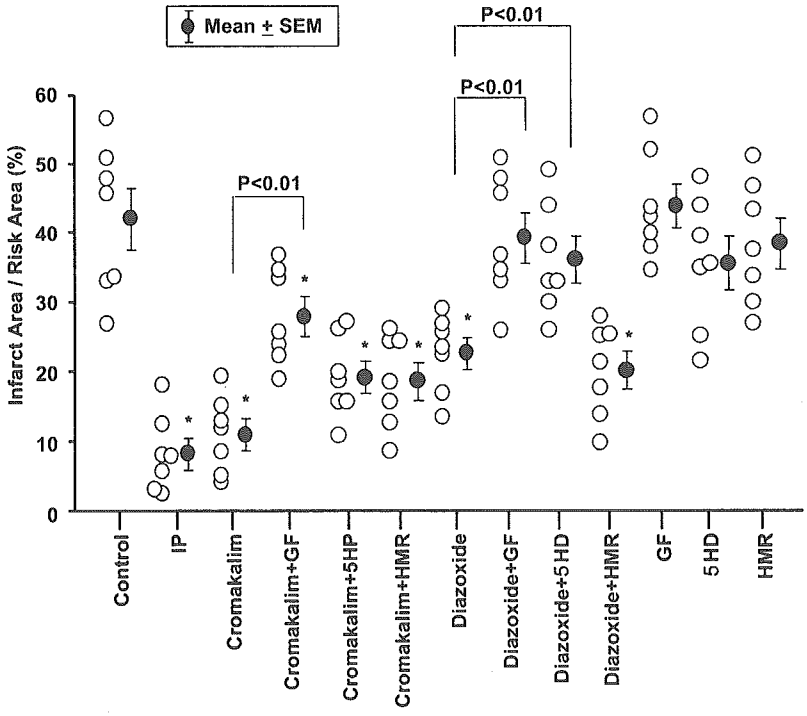

Fig. 1. Infarct size in the control group, the IP group, the cromakalim group, the cromakalim + GF group, the cromakalim + 5HD group, the cromakalim + HMR group, the diazoxide group, the diazoxide + GF groups, the diazoxide + 5HD groups, the diazoxide + HMR groups, the GF group, the 5HD group, and the 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

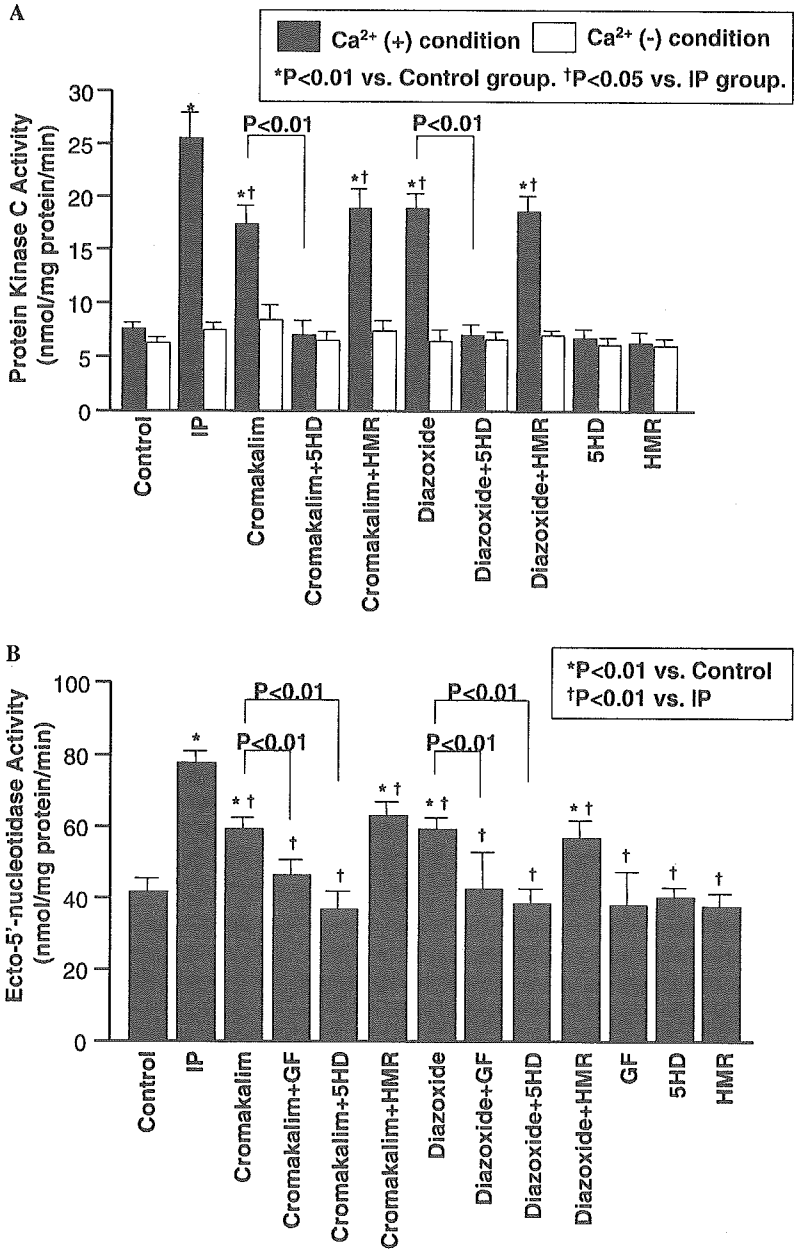

Fig. 2. Comparison of PKC (A) and ecto-5′-nucleotidase (B) activity of canine myocardial samples following the IP procedure or the exposures of cromakalim or diazoxide with and without 5HD, HMR-1098, or GF109203X. GF, 5HD, and HMR indicate GF109203X, 5-hydroxydecanoate, and HMR-1098, respectively. The 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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Depression of proteasome activities during the progression of cardiac dysfunction in pressure-overloaded heart of mice ☆

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Abstract

The ubiquitin–proteasome system contributes to regulation of apoptosis degrading apoptosis-regulatory proteins. Marked accumulation of ubiquitinated proteins in cardiomyocytes of human failing hearts suggested impaired ubiquitin–proteasome system in heart failure. Since cardiomyocyte apoptosis contributes to the progression of cardiac dysfunction in pressure-overloaded hearts, we investigated the role of ubiquitin–proteasome system in such conditions. We found that proteasome activities already depressed before the onset of cardiac dysfunction in pressure-overloaded hearts of mice. Cardiomyocyte apoptosis was observed along with depression of proteasome activities and elevation of proapoptotic/antiapoptotic protein ratio in failing hearts. In cultured cardiomyocytes, pharmacological inhibition of proteasome accumulated proapoptotic proteins such as p53 and Bax. Gene silencing of these proapoptotic proteins by RNA interference prevented the accumulation of respective proteins and attenuated cardiomyocyte apoptosis induced by proteasome inhibition. We conclude that depression of proteasome activities contributes to cardiac dysfunction resulting from cardiomyocyte apoptosis through accumulation of proapoptotic proteins by impaired degradation.

Keywords: Ubiquitin–proteasome; Apoptosis; Heart failure; Protein degradation

The ubiquitin–proteasome system plays an important role in the elimination of damaged and misfolded proteins, as well as being involved in the rapid proteolysis of intracellular proteins [1–3]. Degradation of proteins by this system involves two distinct processes, which are covalent binding of multiple ubiquitin molecules to the target protein and subsequent degradation of this protein by proteasome[4,5]. Recent studies have shown that the ubiquitin–proteasome system plays a central role in various key cellular processes, including proliferation, differentiation, and apoptosis [6–9]. Inhibition of the ubiquitin–proteasome system induces an imbalance of various apoptosis-regulating proteins [10]. Indeed, pharmacological inhibition of proteasome has been found to induce apoptosis of neoplastic and rapidly growing mammalian cells of hematopoietic [11], neuronal [12], mesenchymal [13], and epithelial origin [14]. Despite advances in medical therapy, congestive heart failure (CHF) causes a high morbidity and high mortality with high prevalence [15], suggesting a great impact of investigation of the mechanism(s) and innovation for the prevention in the field of CHF. Pressure-overload plays an important role in left ventricular remodeling and subsequent development of heart failure [16,17]. An

☆ We declare that all authors listed in this paper have no conflict of interest.

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essential event that is associated with and suspected to be causally related to the transition from compensatory cardiac hypertrophy to heart failure is the apoptotic death of cardiomyocytes [18,19]. Thus, we investigated the role of the ubiquitin–proteasome system in the development of heart failure and its potential role on cardiomyocyte apoptosis using pressure-overloaded heart model of mice.

Methods

Antibodies and reagents. Proteasome inhibitors (MG132 and clasto-lactacystin β-lactone) were purchased from Calbiochem. Antibodies directed against p53 (Ab-1) were from Oncogene Research Products, cytochrome *c* from BD Biosciences, cleaved caspase-3 (Asp175) from Cell Signaling, Bcl-2 (C-2), Bax (B-9), and actin (C-11) from Santa Cruz Biotechnology, ubiquitin from DAKO for immunohistochemistry, and ubiquitinated proteins (clone FK2) from BIOMOL for immunoblotting. Clone FK2 recognizes both mono- and polyubiquitinated proteins but not free ubiquitin, thus the degree of protein ubiquitination may be determined.

Preparation of human heart samples. Human heart samples were studied according to the protocol approved by the Institutional Review Boards of Ethical Committee in National Cardiovascular Center (Nos. 14–18). The heart samples for immunohistological analysis were obtained as surgical specimens from three patients (male/female = 1/2; mean age: 46 years; NYHA functional class III or IV) with end-stage heart failure due to dilated cardiomyopathy (DCM) and at autopsy from one patient with acute leukemia (as a control). Tissue samples were fixed and embedded in paraffin.

Immunohistological analysis. Immunohistological analysis was performed as described previously [20,21].

Mouse model of pressure-overload heart. C57BL/6 male mice (8 weeks old) were subjected to either thoracic aortic constriction (TAC) or sham operation as described previously. [22] The 28 mice survived for 6 h after TAC surgery and seven mice died in the TAC group, but there were no dead mice in the sham-operation group. In sham-operation group, since we confirmed that cardiac function, the level of ubiquitinated proteins, and proteasome activities were compatible among 1, 2, and 4 weeks after sham operation (data not shown), we obtained myocardial samples taken 4 weeks after sham operation. Echocardiogram was performed as described previously. [22] All procedures were done in accordance with the guiding principles of Osaka University School of Medicine with regard to animal care and the "Position of the American Heart Association on Research Animal Use".

Preparation of rat cardiomyocytes. Primary cultures of neonatal cardiomyocytes were prepared from Wistar-Kyoto rats as described previously [20]. Cardiomyocytes were cultured in DMEM (Sigma) supplemented with 10% FBS (Equitech-Bio). The myocytes were seeded into 6-well plates coated with human fibronectin (Biocoat, BD-Falcon) at 80% confluence.

Proteasome activity assay. The peptidase activities of proteasome in the cytosolic fraction from left ventricular (LV) free walls or cultured neonatal rat cardiomyocytes were measured according to the method reported previously [12]. Proteasome peptidase substrates, i.e., Suc-Leu-Leu-Val-Tyr-7-amino-4-methylcoumarin (LLVY-AMC), benzonyl-Val-Gly-Arg-4-methylcoumarin (Bz-VGR-AMC), and benzyloxycarbonyl-Leu-Leu-Glu-methylcoumarylamide (Z-LLE-AMC), were purchased from BIOMOL. Chymotrypsin-like, trypsin-like, and peptidylglutamyl-peptide hydrolyzing activities of proteasome were assayed using the fluorogenic peptides LLVY-AMC, LSTR-AMC, and Z-LLE-AMC, respectively. Cultured rat neonatal cardiomyocytes were harvested, lysed in proteasome buffer [10 mmol/L Tris–HCl, pH 7.5, 1 mmol/L EDTA, 2 mmol/L ATP, 20% glycerol, and 4 mmol/L dithiothreitol (DTT)], sonicated, and then centrifuged at 13,000*g* at 4 °C for 10 min. Then the supernatant (20 μg of protein) was incubated with proteasome activity assay buffer [0.05 mol/L Tris–HCl, pH 8.0, 0.5 mmol/L EDTA, 40 μmol/L LLVY-AMC, LSTR-AMC, or Z-LLE-AMC] for 1 h at 37 °C. The reaction was stopped adding 0.9 mL of cold water and placing the reaction mixture on ice at least for 10 min. Subsequently, the fluorescence of each solution was measured by spectrophotometry (HitachF-2000; Hitachi Instruments, Tokyo, Japan) with excitation at 380 nm (Ex) and emission at 440 nm (Em). All readings were standardized relative to the fluorescence intensity of an equal volume of free 7-amino-4-methylcoumarin (Sigma) solution (40 μmol/L). The peptidase activities of proteasome from the LV free wall were measured using 10 μg of cytosolic protein according to the method reported previously [23].

Apoptosis assay. The terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) reaction was performed as reported previously[19].

MTT assay. Cell viability was assessed by the MTT assay (Cell Counting Kit 8) according to the method reported previously [19]. Cell viability was expressed as a percentage of the control.

Gene silencing via RNA interference. Cardiomyocytes were seeded into 6-well plates coated with human fibronectin at 80% confluence. After 6 h, the cells were transfected with a pool of four short interfering RNAs (siRNAs; 100 nmol/L) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. siRNA duplexes that targeted either the rat p53 [(1) sense: 5′-GAG AAU AUU UCA CCC UUA AUU-3′, antisense: 5′-PUU AAG GGU GAA AUA UUC UCU U-3′, (2) sense: 5′-GCG ACA GGG UCA CCU AAU UUU-3′, antisense: 5′-PAA UUA GGU GAC CCU GUC GCU U-3′, and (3) sense: 5′-GUA CUC AAU UUC CCU CAA UUU -3′, antisense: 5′-PAU UGA GGG AAA UUG AGU ACU U-3′] or rat Bax [(1) sense: 5′-GCU CUG AAC AGU UCA UGA ATT-3′, antisense: 5′-UUC CAU GAA CUG UUC AGA GCT T-3′, (2) sense: CCG GCG AAU UGG AGA UGA ATT-3′, antisense: 5′-UUC AUC UCC AAU UCG CCG GTT-5′, and (3) sense: GGG AAG GCC UCC UCU CCU ATT-5′, antisense: UAG GAG AGG AGG CCU UCC CTT-3′] were purchased from Dharmacon. As a negative control, cells were transfected with siControl Non-Targeting siRNA#1 (Dharmacon). Experiments were performed at 48 h after the transfection.

Quantitative real-time RT-PCR. Total RNA from either left ventricles or cultured rat neonatal cardiomyocytes was extracted using RNA-Bee-RNA Isolation Reagent (Tel-Test). Then 200 ng of total RNA was reverse transcribed and amplified using an Omniscript RT Kit (Qiagen) according to the manufacturer's protocol. Oligonucleotide primers and TaqMan probes for mouse p53, mouse Bax, rat p53, rat Bax, and rodent GAPDH were purchased from Applied Biosystems. Quantitative real-time PCR was performed with an ABI PRISM7700 Sequence Detection System (Applied Biosystems) by the relative standard curve method. The amount of target RNA was determined from the relative standard curves constructed with serial dilutions of control total RNA.

Immunoblotting. Immunoblotting was performed as described previously [20] and immunoreactive bands were quantified by densitometry (Molecular Dynamics).

Statistical analysis. Data were expressed as means ± SEM. Results were compared by one-way ANOVA, followed by Bonferroni's test. Comparison of categorical variables was done by Fisher's exact test. In all analyses, $P < 0.05$ was accepted as statistically significant.

Results

Accumulation of ubiquitinated protein in cardiomyocytes of failing human hearts

To examine an involvement of the ubiquitin–proteasome system in human heart failure, we stained samples of failing human myocardium with either hematoxylin–eosin (HE) or a rabbit antibody for ubiquitin. Fig. 1

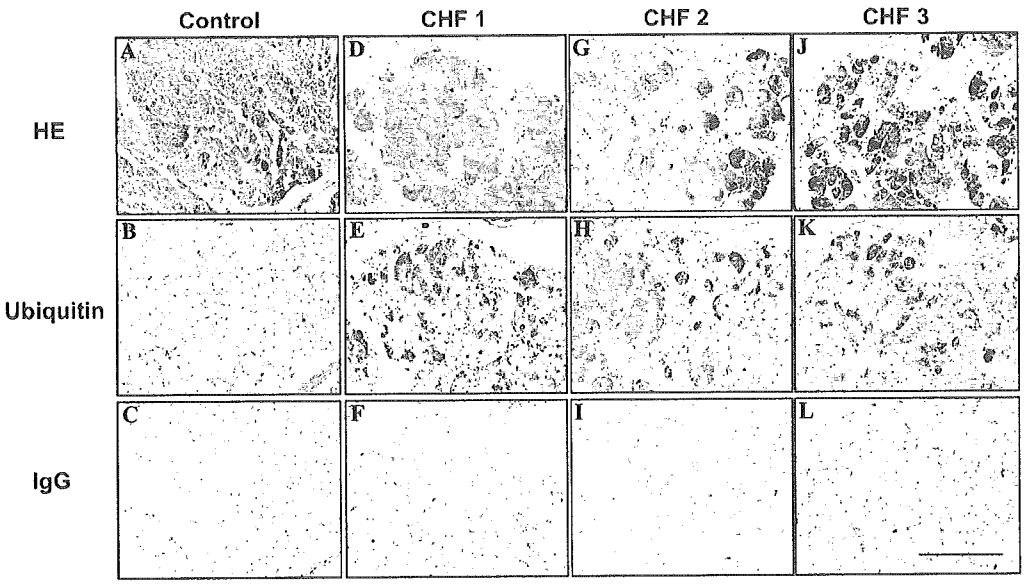

Fig. 1. Accumulation of ubiquitinated proteins in failing human hearts immunohistochemical analysis of ubiquitinated proteins in failing human hearts. (A–C) show the results using the myocardial samples obtained from a patient with normal cardiac function at autopsy. (D–L) show the results using surgical specimens obtained from patients with end-stage congestive heart failure. Top, middle, and bottom panels show staining with hematoxylin–eosin (HE) and with antibodies for ubiquitin, and IgG, respectively. Bar indicates 100 μm.

indicates a marked cardiomyocyte hypertrophy and fibrosis in the failing hearts; the cardiomyocytes in failing hearts showed prominent accumulation of ubiquitinated proteins while no accumulation in control heart. This result suggests that the impairment of ubiquitin–proteasome system is involved in pathophysiology of heart failure.

Time-course changes in cardiac dysfunction and accumulation of ubiquitinated proteins in mice hearts after TAC

To investigate a potential role of ubiquitin–proteasome system in the development of heart failure, we produced mice model of pressure-overloaded heart by TAC surgery and checked the time-course relationship between cardiac function and accumulation of ubiquitinated protein. Echocardiographic analysis revealed marked LV dilatation and impairment of contractility in the TAC group 4 weeks, but not 1and 2 weeks, after TAC (Figs. 2A and C). Heart to body weight ratio following TAC increased compared with sham-operated mice (Fig. 2B). Thus, compensated cardiac hypertrophy (hypertrophic hearts) and decompensated cardiac failure (failing hearts) were produced 1 week and 4 weeks after TAC, respectively, as previously reported. [19] Immunoblot analysis revealed initiation and progression of accumulation of ubiquitinated proteins 2 and 4 weeks after TAC, respectively (Figs. 2D and E). Immunohistological analysis also revealed identical results obtained using immunoblot analysis (Fig. 2F). These results suggest the accumulation of ubiquitinated proteins prior to the onset of cardiac dysfunction.

Imbalance of apoptosis-regulatory proteins and cardiomyocyte apoptosis along with depression of proteasome activities in mice hearts after TAC

Since ubiquitinated proteins are thought to be accumulated when proteasome is deactivated, we examined the changes in the time-course in three different proteasome activities, i.e., chymotrypsin-like, trypsin-like, and post-glutamyl peptide hydrolyzing (PGPH) activities in hearts after TAC. All three different proteasome activities began to decrease 2 weeks after TAC and became substantially lower 4 weeks after TAC (Fig. 3A). Since echocardiographic analysis revealed that cardiac function was preserved 2 weeks after TAC, proteasome activities already decreased before the onset of cardiac dysfunction. Furthermore, we examined the levels of apoptosis-regulating proteins that are degraded by ubiquitin–proteasome system in this model (Figs. 3C and D). Despite the unchanged mRNA levels of proapoptotic proteins (such as p53 and Bax) throughout the study (Fig. 3B), the protein levels of such proapoptotic proteins began to increase 2 weeks after TAC and further increased 4 weeks after TAC (Figs. 3C and D), resulting in an imbalance of apoptosis-regulatory proteins (an elevation of proapoptotic/antiapoptotic protein ratio). By contrast, the levels of antiapoptotic proteins (Bcl-2 and Bcl-XL) decreased 4 weeks after TAC (Figs. 3C and D), resulting in further exacerbation of imbalance of apoptosis-regulatory protein. Along with the imbalance of apoptosis-regulatory proteins, both released cytochrome *c* into the cytosol from mitochondria and presence of cleaved caspase-3 were detected, and the number of TUNEL-positive cells increased in failing hearts 4 weeks after TAC, but not hypertrophic hearts 1 and 2 weeks after TAC (Figs. 3C–E).

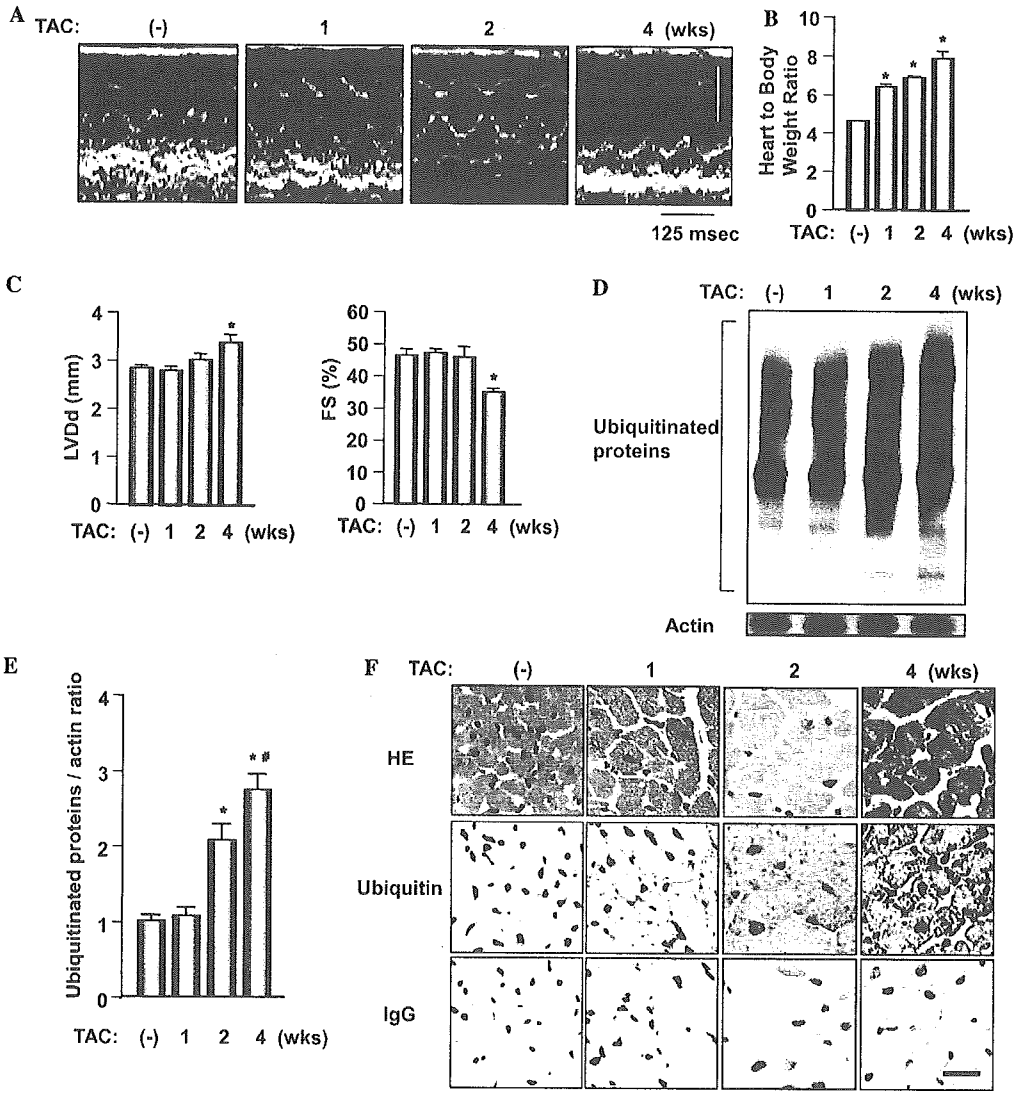

Fig. 2. Time-course changes in cardiac dysfunction and accumulation of ubiquitinated proteins in mice hearts after TAC. (A) Representative echocardiograms of the LV in sham and TAC mice. (B) Heart weight to body weight ratio. (C) Quantitative analysis of cardiac parameters by echocardiography. LVDd indicates LV end-diastolic dimension, LVFS; LV fractional shortening. (D) Representative immunoblot analysis of ubiquitinated proteins in mice hearts. For immunoblot analysis, 20 μg of proteins was separated on 5–20% gradient SDS–PAGE gel. (E) Quantitative analysis of ubiquitinated proteins by densitometry. Values are normalized to controls (sham). $^{*}P < 0.05$ versus sham operation. $^{\#}P < 0.05$ versus 2 weeks after TAC. $n = 7$ in each group. (F) Immunohistological analysis of ubiquitinated proteins in mice hearts. Top, middle, and bottom panels show staining with hematoxylin–eosin (HE) and with antibodies for ubiquitin, and IgG, respectively. Bar indicates 20 μm.

Imbalance of apoptosis-regulatory proteins and apoptosis induced by the pharmacological inhibition of proteasome in cultured neonatal rat cardiomyocytes

To examine the effects of a decrease in proteasome activity on the expression of apoptosis-regulating proteins, we treated neonatal rat cardiomyocytes with either 0.5 μmol/L of MG132 for 24 h and then determined the levels of apoptosis-regulatory proteins by immunoblotting. The treatment with MG132 suppressed chymotrypsin-like proteasome activity by 30.2 ± 0.1%. Furthermore, MG132 increased the expression level of proapoptotic proteins such as p53 and Bax, and decreased those of antiapoptotic proteins such as Bcl-2 and Bcl-XL (Fig. 4A). This imbalance between proapoptotic and antiapoptotic proteins induced by the inhibition of proteasome resulted in the release of cytochrome *c* into the cytosol and the presence of cleaved caspase-3 (Fig. 4A). Identical results were obtained using 10 μmol/L of lactacystin (Fig. 4A). To examine the effect of an increase of proapoptotic proteins on cardiomyocyte apoptosis induced by proteasome inhibition, we employed specific siRNAs targeting proapoptotic proteins such as p53 and Bax. These siRNAs effectively suppressed the levels of mRNA by more than 80% for the respective targets determined by quantitative real-time PCR (data not shown) and we chose the most effective siRNAs for further examination (siRNA p53-1 and siRNA Bax-3). Importantly, siRNA targeting either p53 or Bax effectively prevented