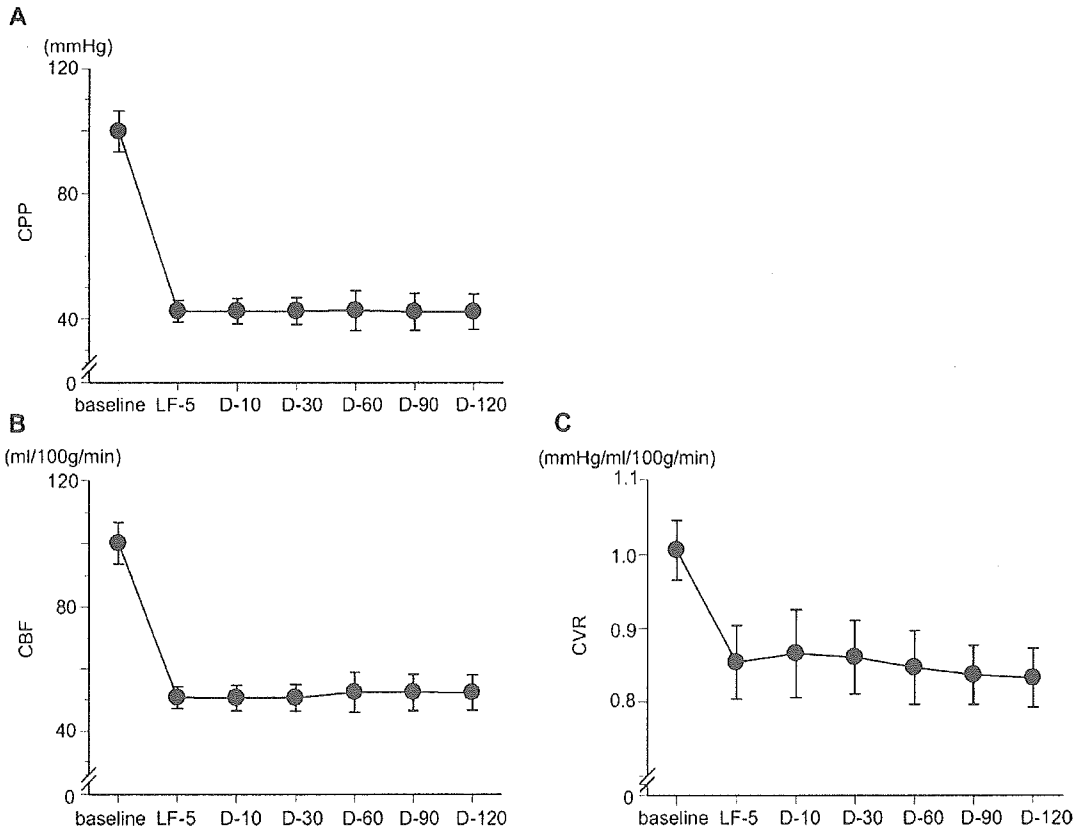


Fig. 5. Effects of amlodipine on CPP (A), CBF (B) and CVR (C) in the presence of SOD in ischemic myocardium. Abbreviations are same as in Fig. 1.

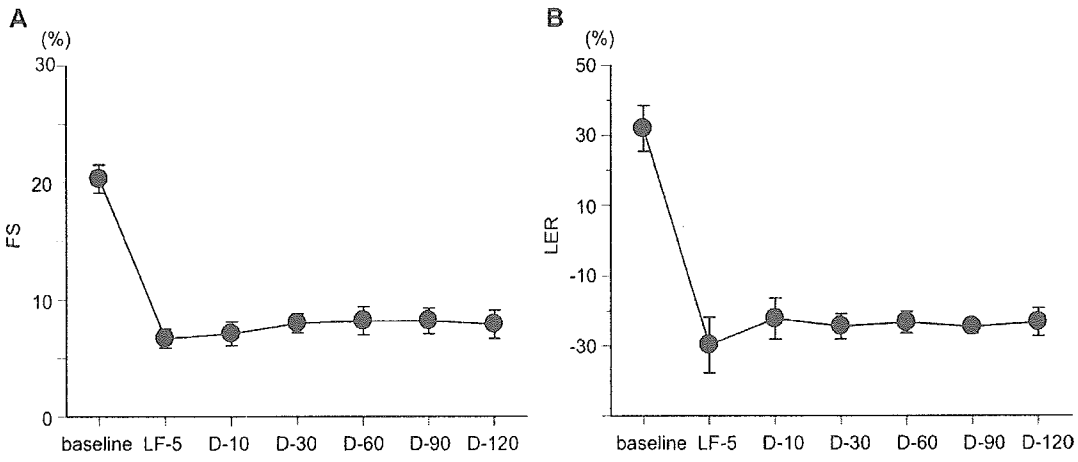


Fig. 6. Effects of amlodipine on FS (A) and LER (B) in the presence of SOD in ischemic myocardium. Abbreviations are same as in Fig. 1.

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 $K^+$ (mito$K_{ATP}$) 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 mito$K_{ATP}$ 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 mito$K_{ATP}$ 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 mito$K_{ATP}$ 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 $K_{ATP}$ (mito$K_{ATP}$) 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 mito$K_{ATP}$ channels activates PKC and contributes to the infarct size-limitation of IP. To test this idea, since cromakalim opens both mito$K_{ATP}$ and surface sarcolenmal $K_{ATP}$ (sarco$K_{ATP}$) channels, and diazoxide opens mito$K_{ATP}$ channels, we administered either cromakalim or diazoxide with and without a selective blocker of mito$K_{ATP}$ channels (5-hydroxydecanoate, 5HD), a selective blocker of sarco$K_{ATP}$ 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.

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 ($89 \pm 2$ to $327 \pm 11$ ml/100 g/min for IP; $88 \pm 2$ to $357 \pm 6$ ml/100 g/min for cromakalim; $87 \pm 2$ to $243 \pm 10$ ml/100 g/min for or diazoxide, $P < 0.001$ each). Infusions of GF109203X, 5HD, and HMR-1098 did not affect either basal CBF ($88 \pm 2$ to $89 \pm 3$, $87 \pm 2$, and $89 \pm 2$ ml/100 g/min) or hyperemic CBF (cromakalim-induced hyperemia: $357 \pm 6$ to $348 \pm 5$, $353 \pm 4$, and $366 \pm 6$ ml/100 g/min; diazoxide-induced hyperemia: $243 \pm 10$ to $231 \pm 6$, $234 \pm 7$, and $251 \pm 4$ 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.

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 | $40 \pm 2$ | $7.2 \pm 1.5$ |
| IP | $42 \pm 3$ | $7.0 \pm 1.7$ |
| Cromakalim | $41 \pm 4$ | $7.3 \pm 1.8$ |
| Cromakalim + GF | $42 \pm 3$ | $8.5 \pm 1.4$ |
| Cromakalim + 5HD | $39 \pm 6$ | $8.0 \pm 1.7$ |
| Cromakalim + HMR | $41 \pm 5$ | $7.8 \pm 1.0$ |
| Diazoxide | $43 \pm 3$ | $7.1 \pm 1.7$ |
| Diazoxide + GF | $41 \pm 3$ | $7.7 \pm 1.7$ |
| Diazoxide + 5HD | $43 \pm 5$ | $8.1 \pm 0.9$ |
| Diazoxide + HMR | $43 \pm 3$ | $6.8 \pm 1.9$ |
| GF | $40 \pm 7$ | $7.3 \pm 1.4$ |
| 5HD | $40 \pm 5$ | $7.5 \pm 1.4$ |
| HMR | $41 \pm 4$ | $7.4 \pm 1.7$ |

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.

*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

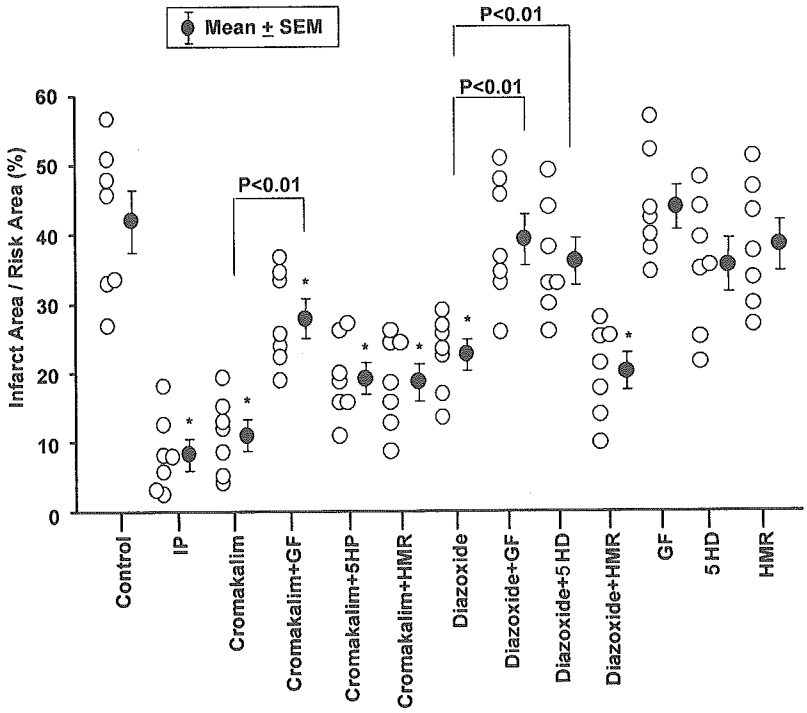


Fig. 1. Infarct size in the control group, the IP group, the cromakalim group, the cromakalim + GF group, the cromakalim + 5HD group, the cromakalim + HMR group, the diazoxide group, the diazoxide + GF groups, the diazoxide + 5HD groups, the diazoxide + HMR groups, the GF group, the 5HD group, and the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

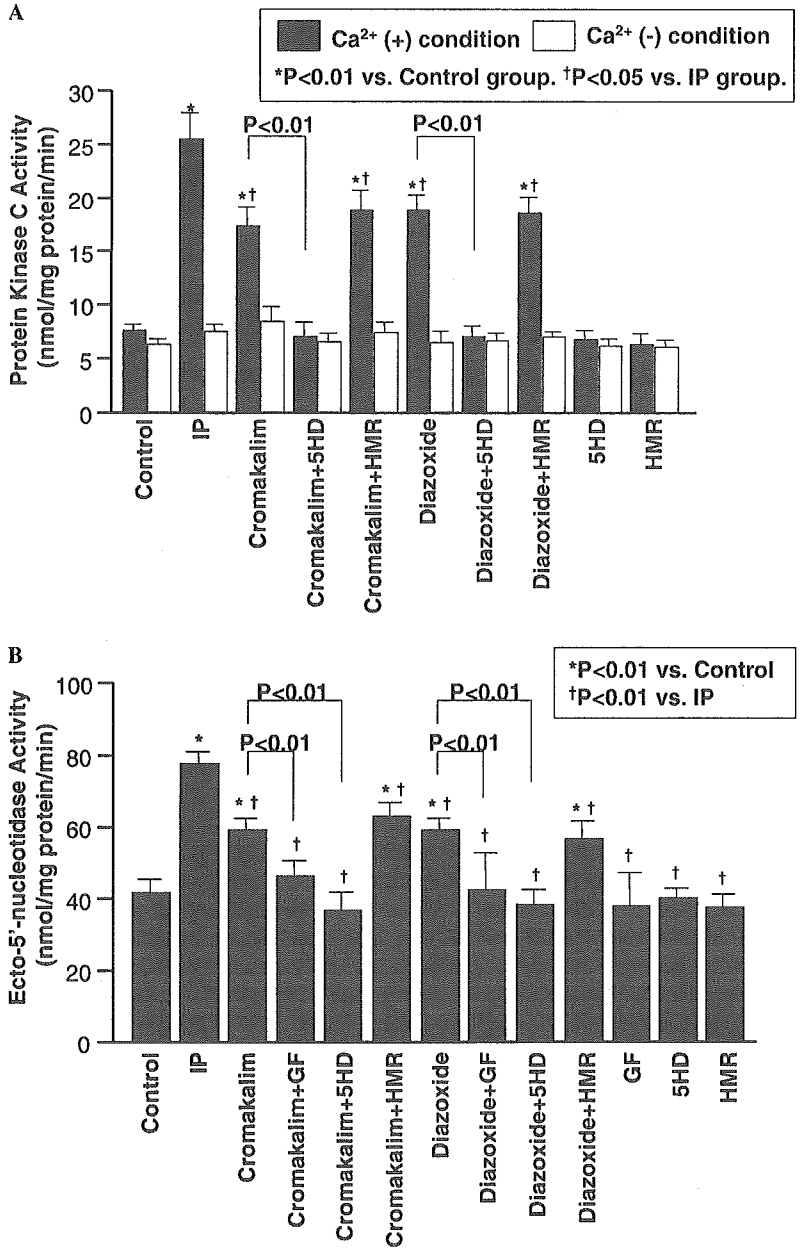


Fig. 2. Comparison of PKC (A) and ecto-5′-nucleotidase (B) activity of canine myocardial samples following the IP procedure or the exposures of cromakalim or diazoxide with and without 5HD, HMR-1098, or GF109203X. GF, 5HD, and HMR indicate GF109203X, 5-hydroxydecanoate, and HMR-1098, respectively. The 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 sarco$K_{ATP}$ 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 mito$K_{ATP}$ channels, but also sarco$K_{ATP}$ channels. Intriguingly, the infarct size-limiting effect of cromakalim that opens both mito$K_{ATP}$ and sarco$K_{ATP}$ channels was partially blunted by HMR-1098, which agrees to our previous finding [17].

The opening of mito$K_{ATP}$ 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 mito$K_{ATP}$ 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

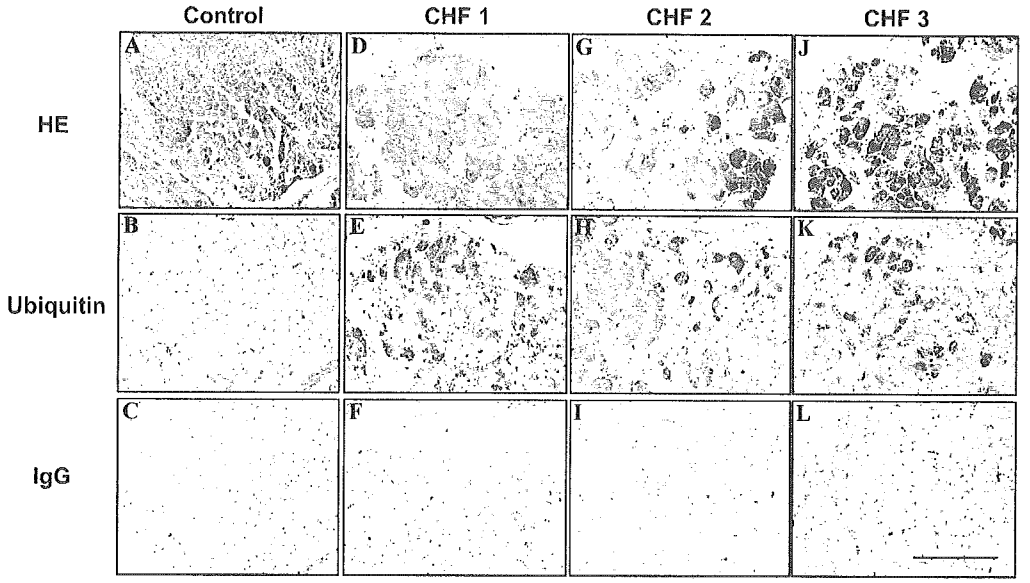


Fig. 1. Accumulation of ubiquitinated proteins in failing human hearts immunohistochemical analysis of ubiquitinated proteins in failing human hearts. (A–C) show the results using the myocardial samples obtained from a patient with normal cardiac function at autopsy. (D–L) show the results using surgical 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).

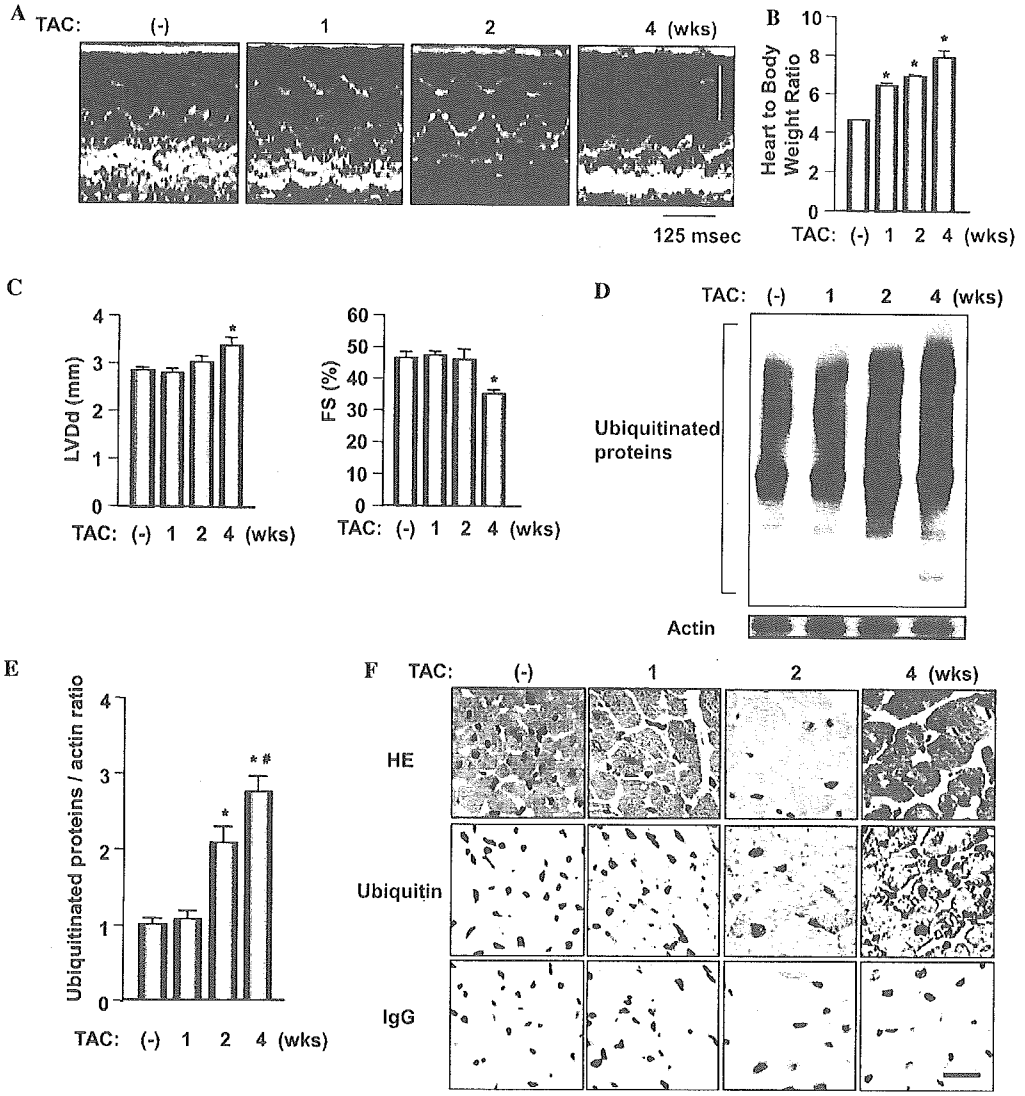


Fig. 2. Time-course changes in cardiac dysfunction and accumulation of ubiquitinated proteins in mice hearts after TAC. (A) Representative echocardiograms of the LV in sham and TAC mice. (B) Heart weight to body weight ratio. (C) Quantitative analysis of cardiac parameters by echocardiography. LVDd indicates 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

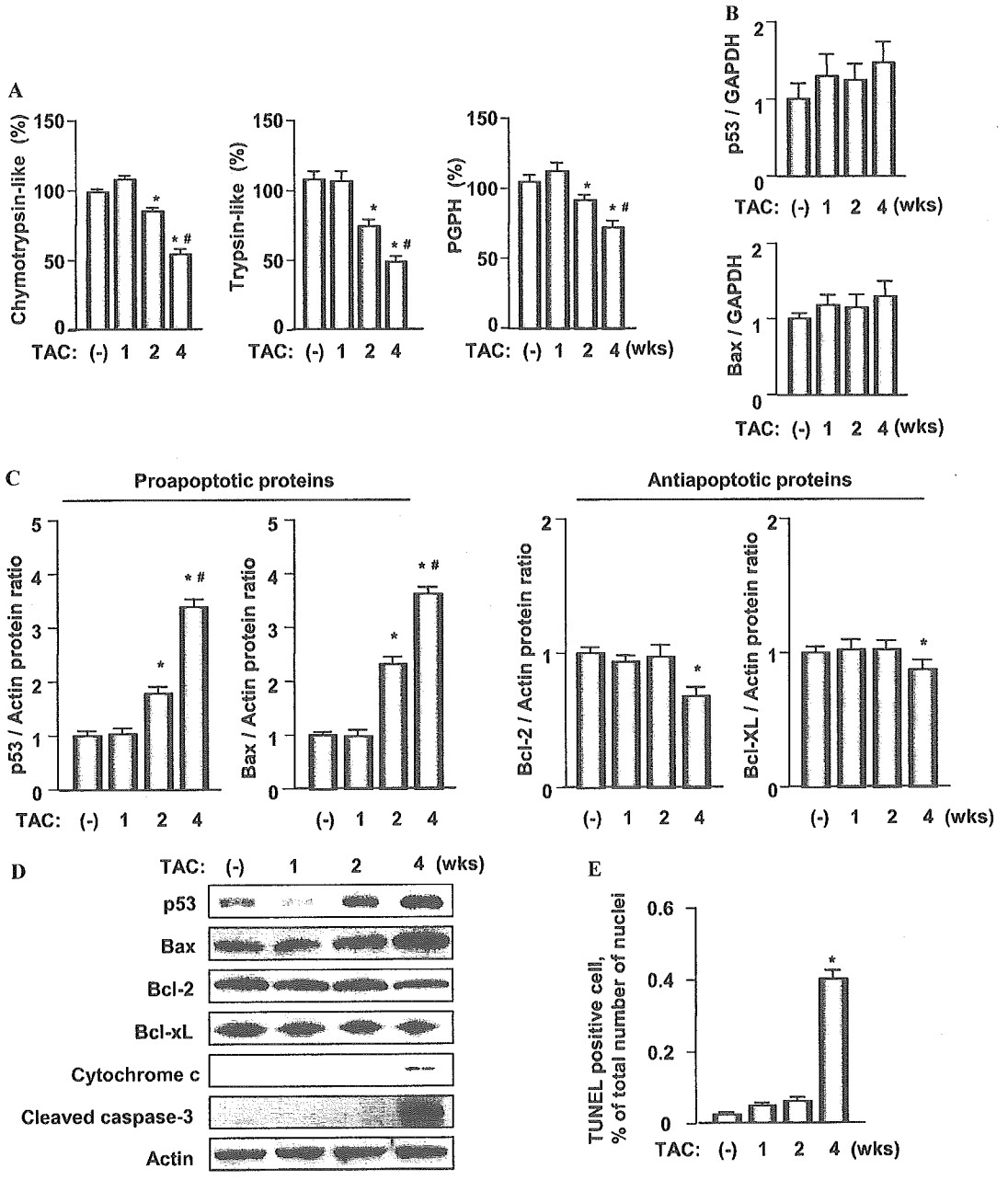


Fig. 3. Imbalance of apoptosis-regulatory proteins and cardiomyocyte apoptosis along with depression of proteasome activities in mice hearts after TAC. (A) Chymotrypsin-like, trypsin-like, and peptidylglutamyl-peptide hydrolyzing (PGPH) activities of the proteasome in the cytosolic fraction of cardiomyocytes from LV tissue. Values are normalized to controls (sham). $n = 6$ in each group. (B) mRNA levels of proapoptotic proteins (p53 and Bax) determined by quantitative real-time PCR in mice hearts. (C) Quantitative analysis of apoptosis-regulatory proteins by densitometry. Values are normalized to controls (sham). (D) Representative immunoblot analysis of apoptosis-regulatory proteins in mice hearts. (E) Quantitative analysis of TUNEL-positive cardiomyocytes in mice hearts. $^{*}P < 0.05$ versus sham operation. $^{\#}P < 0.05$ versus 2 weeks after TAC.

the increase of respective proteins induced by the inhibition of proteasome to the control level, which attenuated the imbalance between the levels of proapoptotic and antiapoptotic proteins (Figs. 4B and C). We also found that siRNA targeting either p53 or Bax partially but significantly (1) decreased both the release of cytochrome *c* and the level of cleaved caspase-3 (Fig. 4B), (2) reduced the number of TUNEL-positive cardiomyocytes (Figs. 4D and E), and (3) prevented the reduction of cell viability induced by the inhibition of proteasome (Fig. 4F). These findings suggest that the deactivation of proteasome causes cardiomyocyte apoptosis partially via imbalance of apoptosis-regulatory proteins due to impaired degradation.

## Discussion

Several experimental studies demonstrated the contribution of proteasome dysfunction to the pathogenesis of

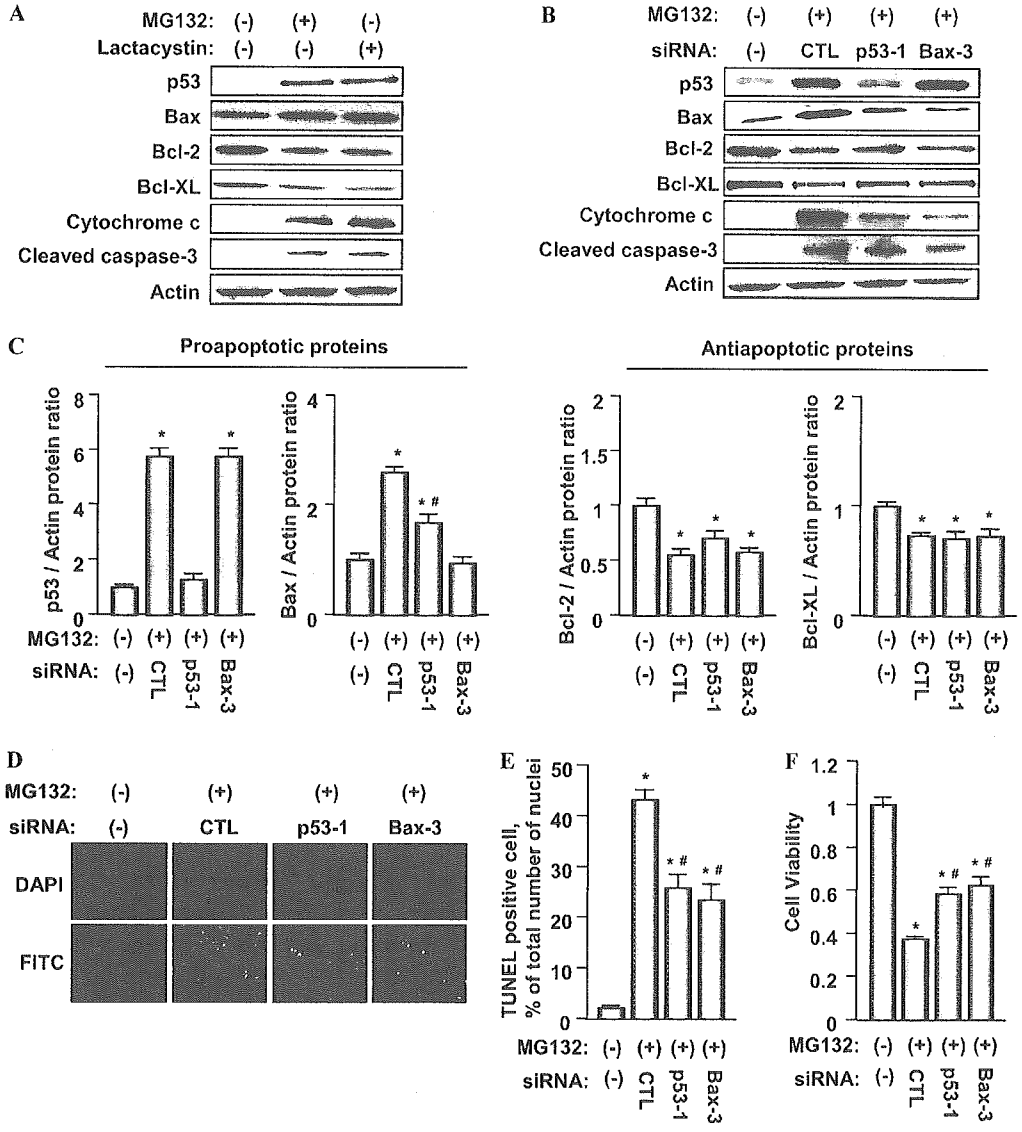


Fig. 4. Imbalance of apoptosis-regulatory proteins and apoptosis by the pharmacological inhibition of proteasome in cultured neonatal rat cardiomyocytes (A) Immunoblot analysis of apoptosis-related proteins in cardiomyocytes treated with either 0.5 μmol/L MG132 or 10 μmol/L lactacystin. (B) Effect of siRNAs targeting proapoptotic proteins on the levels of apoptosis-regulatory proteins determined by immunoblot analysis. (C) Quantitative analysis of apoptosis-regulatory proteins. (D) Representative TUNEL staining of cardiomyocytes. (E) Quantitative analysis of TUNEL-positive cardiomyocytes. (F) Cell viability of cardiomyocytes determined by MTT assay. Results represent analysis of three independent experiments. $^{*}P < 0.05$ versus no treatment. $^{\#}P < 0.05$ versus CLT (siControl).

heart disease, such as ischemia/reperfusion injury [24], doxorubicin cardiotoxicity [25], and transgenic mice model of mutant αB-crystallin[26]. However, there is no report investigating the role of ubiquitin–proteasome system in the pathogenesis of heart failure induced by pressure-overload, one of the most common causes of human heart failure [27]. The present study is the first to demonstrate that the impairment of ubiquitin–proteasome system such as the depression of proteasome activities precedes cardiac dysfunction in the development of heart failure in pressure-overloaded heart of mice. Cardiac remodeling and dysfunction developed as the depression of proteasome activities progressed, suggesting that the impairment of proteasome function contributes to the pathogenesis of heart failure induced by pressure-overload.

Recently, Schaper and co-workers [28] demonstrated that the number of ubiquitin-positive cardiomyocytes increased in patients with CHF. Consistent with their report, we also found an increase of ubiquitin-positive cardiomyocytes in patients with heart failure. These findings led us to speculate that the impairment of ubiquitin–proteasome system contributes to the pathophysiology of CHF. Thus, to investigate the potential role of ubiquitin–proteasome system in the development of heart failure, we examined the changes in the time-course of cardiac function, accumulation of ubiquitinated proteins, and pro-

teasome activities in pressure-overloaded heart of mice following TAC surgery or sham-operation. Importantly, since the presence of cardiac dysfunction was preceded by both the depression of proteasome activities and accumulation of ubiquitinated proteins, it is likely that the depression of proteasome activities was not a consequence of heart failure but one of the causes for heart failure. Since it has been also reported that three different proteasome activities may be regulated independently [23], multiple mechanisms may be involved in the depression of proteasome activities in pressure-overloaded hearts such as (1) altered gene expression, (2) modifications of the proteasome subunits (oxidation, glycation, glycoxidation, and conjugation with lipid peroxidation products), and (3) presence of inhibitory damaged proteins that inhibit proteasome function [24,29]. We need to further investigate the cellular mechanisms of the depression of proteasome activities during the development of heart failure in future study.

We next investigated apoptosis-regulating proteins, the target substrates of proteasome, in pressure-overloaded mice hearts. We demonstrated that the levels of proapoptotic proteins (p53 and Bax) increased 2 weeks and further intensified 4 weeks after TAC despite unchanged levels of mRNA, suggesting that the reduced protein degradation due to the deactivated proteasome contributes to the increased levels of the proapoptotic proteins. On the other hand, antiapoptotic proteins (Bcl-2 and Bcl-XL) decreased 4 weeks after TAC, which exacerbated the imbalance of apoptosis-regulatory proteins (an elevation of proapoptotic/antiapoptotic protein ratio). In addition, both release of cytochrome *c* into the cytosol and presence of cleaved caspase-3 were detected and the number of TUNEL-positive cells increased along with the elevation of proapoptotic/antiapoptotic proteins ratio. Since apoptosis of cardiomyocytes contributes to transition from compensated cardiac hypertrophy to decompensated cardiac failure in pressure-overloaded heart of mice [18,19], an imbalance between proapoptotic and antiapoptotic proteins plays an important role in the development of heart failure. Since both p53 and Bax are strictly regulated by the ubiquitin–proteasome system [6,30], the depression of proteasome activities could modulate the ratio of proapoptotic/antiapoptotic proteins and result in the apoptotic death of cardiomyocytes or enhanced susceptibility of cardiomyocytes to various apoptotic stimuli [31]. However, we must recognize that other factors such as oxidative stress could also contribute to the induction of imbalance between proapoptotic and antiapoptotic proteins [32].

To test the direct effects of proteasome inhibition on apoptosis of cardiomyocytes, we treated cultured neonatal cardiomyocytes with 2 different proteasome inhibitors, MG132 and lactacystin, and obtained identical results. It would not be surprising if the inhibition of proteasome promotes the apoptosis of cardiomyocytes, because various regulatory molecules for programmed cell death have been identified as substrates of proteasome, including p53, Bax, and Smac (second mitochondrial-derived activator of caspases)/DIABLO, Bid, and NF-κB [30,33,34]. In the present study, we focused on two proapoptotic proteins (p53 and Bax) and two antiapoptotic proteins (Bcl-2 and Bcl-XL), because these apoptosis-regulating proteins are known to be involved in cardiomyocyte apoptosis[18]. Pharmacological inhibition of proteasome accumulated proapoptotic proteins, decreased antiapoptotic proteins, and elevated the ratio of proapoptotic/antiapoptotic protein, and resulted in apoptosis in cultured cardiomyocytes. To examine the effects of accumulation of proapoptotic proteins on cardiomyocyte apoptosis induced by proteasome inhibition, we applied RNA interference method to knock down the proapoptotic proteins. Silencing of either p53 or Bax gene effectively prevented the proteasome inhibition-induced accumulation of target proteins to the baseline levels, and partially but significantly prevented cardiomyocyte apoptosis. In contrast, the depression of proteasome activity decreased protein levels of Bcl-2 and Bcl-XL in the present in vivo and in vitro studies. This finding is consistent with the previous reports that the inhibition of proteasome decreases protein levels of Bcl-2 in lung cancer cells [35] and human glioblastoma multiform cell lines [36], although these anti-apoptotic proteins are also the target of proteasome. Further investigation will be needed to clarify how target protein of proteasome is specified in cardiomyocytes. Although we must recognize the difference between it in vivo and in vitro studies such as the nature of the stimulus, the issue of adult myocytes versus neonatal myocytes, and the issue of chronic versus acute inhibition of proteasome [25], these findings suggested that the depression of proteasome activities in pressure-overloaded heart causes cardiomyocyte apoptosis partially via accumulation of proapoptotic proteins and contributes to the development of heart failure [10,18].

We need to consider other mechanisms by which the depression of proteasome activities contributes to the progression or transition to heart failure. It has been reported that proteasome inhibition dramatically alters mitochondrial homeostasis, resulting in impairments of mitochondrial electron transport system, increased formation of mitochondrial reactive oxygen species, and decreased energy production [37–39]. Several studies suggest that the ability of proteasome to degrade oxidized proteins serves as a secondary cellular antioxidant defense system [40,41]. Recently, ubiquitin-related autophagic cell death of cardiomyocytes has been described in hypertrophied and failing myocardium [42,43]. However, autophagy functions as a death process remain controversial [44,45].

In conclusion, we demonstrated a potential role of impairment of ubiquitin–proteasome system in the development of heart failure. We propose that the depression of proteasome activities is a novel factor of the progression of heart failure. The depression of proteasome activities may shift the balance between antiapoptotic and proapoptotic proteins in favor of the latter due to impaired degradation, resulting in cardiomyocyte apoptosis in the development of heart failure. Prevention of the depression

of proteasome activities could be one of potential therapies of heart failure.

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CARDIOVASCULAR MEDICINE

# Heart-type fatty acid binding protein is a novel prognostic marker in patients with non-ischaemic dilated cardiomyopathy

**K Komamura, T Sasaki, A Hanatani, J Kim, K Hashimura, Y Ishida, Y Ohkaru, K Asayama, T Tanaka, A Ogai, T Nakatani, S Kitamura, K Kangawa, K Miyatake, M Kitakaze**

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**Objective:** To determine whether concentrations of heart-type fatty acid binding protein (H-FABP) measured before hospital discharge predict critical cardiac events in patients with idiopathic dilated cardiomyopathy (DCM).
**Patients:** 92 consecutive patients with DCM were enrolled and followed up for four years.
**Main outcome measures:** Serum concentrations of H-FABP, brain natriuretic peptide (BNP), cardiac troponin T before hospital discharge and survival rate.
**Results:** 23 patients died of cardiac causes, received a left ventricular assist device or underwent heart transplantation during the four-year follow up. Univariate analyses showed that New York Heart Association functional class, heart rate, ejection fraction, serum H-FABP and plasma BNP were significant variables. According to multivariate analysis, serum H-FABP and plasma BNP concentrations were independent predictors of critical cardiac events. Cardiac troponin T before hospital discharge was not a predictor. The area under the receiver operating characteristic curve for death from critical cardiac events was similar between H-FABP and BNP. Patients with an H-FABP concentration at or above the median (≥ 5.4 ng/ml) had a significantly lower survival rate than those below the median, according to analysis by log rank test ($p < 0.0001$). When combined with BNP concentration at or above the median (≥ 138 pg/ml), H-FABP below the median predicted the worst prognosis among the combinations.
**Conclusions:** The concentration of serum H-FABP before discharge from hospital may be an independent predictor for critical cardiac events in DCM.

Idiopathic non-ischaemic dilated cardiomyopathy (DCM) has wide range of phenotypes and variable clinical outcomes.[1] Identification of patients with DCM at higher risk for adverse outcomes in its earlier stage may optimise the use of limited health care resources. Brain natriuretic peptide (BNP) is now widely recognised as the most powerful prognostic marker for heart failure,[2] and BNP-guided tailored treatment[3] is advocated. Raised BNP in heart failure is mainly attributable to its gene expression in stretched cardiomyocytes undergoing raised ventricular pressure.[4] The regulation of BNP secretion, however, is complex. BNP is also raised in patients with cardiac hypertrophy,[5] renal failure[6] or acute coronary syndrome.[7] β blocker directly enhances expression and release of BNP from cardiomyocytes.[8]

Fatty acid binding protein is extremely abundant in cytoplasm, has low molecular weight and is considered to be one of the key fatty acid carrier proteins.[9] Thus, fatty acid binding proteins are rapidly released into the circulation shortly after cell damage. Heart-type fatty acid binding protein (H-FABP) is immunologically specific to cardiomyocytes and is used as an early diagnostic marker for acute myocardial infarction.[10] In the present study, we tested whether the concentrations of H-FABP measured before discharge predict critical cardiac events for patients with DCM.

## METHODS

Of 97 consecutive patients with DCM admitted to our institute for diagnosis or treatment between January 1997 and December 2000, five patients were excluded from this study because of renal dysfunction (serum creatinine concentration ≥ 177 μmol/l). The remaining 92 patients (66 men and 26 women; mean age 49 years, range 16–76 years) were enrolled in the study. The study procedures were in accordance with the guidelines of our institute, and informed consent was obtained from each patient. The diagnosis of DCM was based on the definition of the World Health Organization/International Society and Federation of Cardiology Task Force.[11] No patient had a history of myocardial infarction, infective myocarditis, metabolic disease or systemic illness. All patients underwent coronary angiography and endomyocardial biopsy for differential diagnosis of DCM. No significant coronary stenosis was found in any patient. Myocarditis was excluded on the basis of the Dallas criteria[12] as well as the method of Edwards *et al*[13] and Katsuragi *et al*.[14] Immunohistochemical analysis with CD45RO was conducted to clarify T lymphocyte infiltration. In a quiescent condition with optimal medical treatment, patients underwent electrocardiography, echocardiography and blood sampling for standard laboratory chemical analysis, myocardial markers and complete blood count just before discharge from hospital. Myocardial markers measured in the present study were as follows: plasma BNP (Shionogi Co, Osaka, Japan), serum H-FABP (MARKIT-M, Dainippon Pharmaceutical Company, Osaka, Japan) and cardiac troponin T (cTnT) (Boehringer Mannheim,

**Abbreviations:** BNP, brain natriuretic peptide; cTnT, cardiac troponin T; DCM, dilated cardiomyopathy; H-FABP, heart-type fatty acid binding protein