in fatty acid oxidation is not fully compensated for by an increase in glucose oxidation (29). Thus, the failing heart suffers from chronic energy starvation (30). Insulin resistance also is common in patients with heart failure (31). Adiponectin improves both glucose metabolism and insulin resistance via the AMPK signaling pathway (32). Therefore, we believe that the administration of recombinant natriuretic peptide has beneficial effects on cardiac energy metabolism via adiponectin in patients with CHF.

Interestingly, the plasma adiponectin level was reported to be decreased in patients with risk factors for heart failure (9,33–35) and increased along with BNP after the onset of heart failure (14). Although approximately 10% increase in adiponectin levels in the ANP group seems relatively small, this would not be the case because there was about a 20% reduction in plasma adiponectin levels in patients with coronary artery disease compared with those in control subjects (35), which leads us to believe that the 10% increase in adiponectin is important from the viewpoint of pathophysiology of heart diseases. Therefore, we hypothesized that ANP and/or BNP regulates the plasma level of adiponectin in patients with CHF and conducted this study.

Conclusions

We demonstrated that natriuretic peptides increase the production of adiponectin by human adipocytes, as well as in patients with CHF. These findings may help to shed more light on the pathophysiology of heart failure.

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PRE-CLINICAL RESEARCH

Prolonged Targeting of Ischemic/Reperfused Myocardium by Liposomal Adenosine Augments Cardioprotection in Rats

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Objectives The purpose of this study was to investigate whether liposomal adenosine has stronger cardioprotective effects and fewer side effects than free adenosine.

Background Liposomes are nanoparticles that can deliver various agents to target tissues and delay degradation of these agents. Liposomes coated with polyethylene glycol (PEG) prolong the residence time of drugs in the blood. Although adenosine reduces the myocardial infarct (MI) size in clinical trials, it also causes hypotension and bradycardia.

Methods We prepared PEGylated liposomal adenosine (mean diameter 134 ± 21 nm) by the hydration method. In rats, we evaluated the myocardial accumulation of liposomes and MI size at 3 h after 30 min of ischemia followed by reperfusion.

Results The electron microscopy and ex vivo bioluminescence imaging showed the specific accumulation of liposomes in ischemic/reperfused myocardium. Investigation of radioisotope-labeled adenosine encapsulated in PEGylated liposomes revealed a prolonged blood residence time. An intravenous infusion of PEGylated liposomal adenosine (450 μg/kg/min) had a weaker effect on blood pressure and heart rate than the corresponding dose of free adenosine. An intravenous infusion of PEGylated liposomal adenosine (450 μg/kg/min) for 10 min from 5 min before the onset of reperfusion significantly reduced MI size (29.5 ± 6.5%) compared with an infusion of saline (53.2 ± 3.5%, $p < 0.05$). The antagonist of adenosine A_1, A_{2a}, A_{2b}, or A_3 subtype receptor blocked cardioprotection observed in the PEGylated liposomal adenosine-treated group.

Conclusions An infusion as PEGylated liposomes augmented the cardioprotective effects of adenosine against ischemia/reperfusion injury and reduced its unfavorable hemodynamic effects. Liposomes are promising for developing new treatments for acute MI. (J Am Coll Cardiol 2009;53:709–17) © 2009 by the American College of Cardiology Foundation

Liposomes are now widely used for drug delivery in cancer treatment to target specific organs actively or passively and to prevent the degradation of chemotherapy agents (1). However, the application of liposomes for cardiovascular diseases is still limited. In ischemic/reperfused myocardium, cellular permeability is enhanced and vascular endothelial integrity is disrupted (2,3), suggesting that nanoparticles

See page 718

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JACC Vol. 53, No. 8, 2009
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Abbreviations and Acronyms

8-SPT = 8-(p-sulfophenyl) theophylline
EM = electron microscopy
MI = myocardial infarction
PEG = polyethylene glycol
RI = radioisotope
TTC = triphenyltetrazolium chloride

such as liposomes may be a promising drug delivery system for targeting damaged myocardium with cardioprotective agents. Additionally, coating liposomes with polyethylene glycol (PEG) prolongs their residence time in the circulation (1). Because enhanced microvascular permeability persists for at least 48 h after the occurrence of myocardial infarction (MI) (2), drugs delivered in PEGylated liposomes should be able to display their maximum beneficial effects on myocardial damage after MI.

Adenosine has multiple physiological functions that are mediated via the adenosine A_1, A_{2a}, A_{2b}, and A_3 receptors (4,5). Although large-scale clinical trials suggested the potential value of adenosine therapy for patients with acute MI (6,7), this agent has an extremely short half-life (1 to 2 s) and causes hypotension and bradycardia because of vasodilatory and negative chronotropic effects (4). Because a high dose of adenosine is required to exert cardioprotective effects, it is difficult to use clinically because of the associated hemodynamic consequences. Therefore, we hypothesized that adenosine encapsulated in PEGylated liposomes would cause less hemodynamic disturbance and might also specifically accumulate in ischemic/reperfused myocardium, leading to augmented cardioprotective effects. To test this hypothesis, we created PEGylated liposomal adenosine by the hydration method and investigated: 1) whether liposomal adenosine accumulated in ischemic/reperfused myocardium and prolonged blood residence time; 2) whether liposomal adenosine caused less severe hypotension and bradycardia than free adenosine; and 3) which adenosine receptor subtype was involved in mediating the cardioprotective effects of liposomal adenosine against ischemia/reperfusion injury.

Methods

Materials. The materials for preparing PEGylated liposomes, including hydrogenated soy phosphatidyl choline (HSPC), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-n-[methoxy (polyethylene glycol)-2000] (DSPE-PEG2000), and cholesterol were obtained from Nissei Oil Co., Ltd. (Tokyo, Japan) and Wako Pure Chemical Co., Ltd. (Osaka, Japan). [^{3}H]-adenosine was purchased from Daiichi Pure Chemicals Co., Ltd. (Tokyo, Japan). Other materials were obtained from Sigma (St. Louis, Missouri), including 8-(*p*-sulfophenyl)theophylline (8-SPT; a nonselective adenosine receptor antagonist), 1,3-diethyl-8-phenylxanthine (DPCPX; a selective adenosine A_1 receptor antagonist), 5-amino-7-(phenylethyl)-2-(2-furyl)-pyrazolo[4,3-e]-1,2,4-triazolo[1,5-c] pyrimidine (SCH58261; a selective adenosine A_{2a} receptor antagonist), 8-[4-[((4-cyanophenyl)carbamoylmethyl) oxy]phenyl]-1, 3-di(n-propyl)xanthine (MRS1754; a selective adenosine A_{2b} receptor antagonist), and 5-propyl-2-ethyl-4-propyl-3-(ethylsulfanylcarbonyl)-6-phenylpyridine-5-carboxylate (MRS1523, a selective adenosine A_3 receptor antagonist).

Animals. Male Wistar rats (9 weeks old and weighing 250 to 310 g, Japan Animals, Osaka, Japan) were used. The animal experiments were approved by the National Cardiovascular Center Research Committee and were performed according to institutional guidelines.

Preparation of PEGylated liposomes. The PEGylated liposomes were prepared by the hydration method. Briefly, adenosine was added to the lipid solution. After mixture of lipid and adenosine, DSPE-PEG2000 was added and incubated. The final composition of PEGylated liposomes was HSPC:cholesterol:DSPE-PEG2000 = 6.0:4.0:0.3 (molar ratio). After ultracentrifugation several times, the pellet of liposomal adenosine was resuspended in sodium lactate at each required concentration for use in the experimental protocols. Some samples of final liposomal adenosine were disrupted by dilution with 50% methanol (1.5 ml per 30-μl of liposomes). After 10 min of ultracentrifugation, the concentration of adenosine in the supernatant was measured by high-performance liquid chromatography.

To prepare fluorescent-labeled liposomes, 0.5 mol% tetramethylrhodamine isothiocyanate (rhodamine) was added to the lipid mixture. To prepare radioisotope (RI)-labeled adenosine encapsulated in liposomes, [^{3}H]-radiolabeled adenosine (Daiichi Pure Chemicals, Tokyo, Japan) was diluted with free adenosine and was encapsulated in liposomes as described above.

Characterization of PEGylated liposomal adenosine. The characterization of the liposomes was performed by the dynamic scatter analysis (Zetasizer Nano ZS, Malvern, Worcestershire, United Kingdom). The analyses were performed 10 times per sample, and results represented analyses of 4 independent experiments.

Experimental protocols. PROTOCOL 1: EFFECTS OF PEGYLATED LIPOSOMAL ADENOSINE ON HEMODYNAMICS IN RATS. Rats were anesthetized with intraperitoneal sodium pentobarbital (50 mg/kg). Catheters were advanced into a femoral artery and vein for the measurement of systemic blood pressure and infusion of drugs, respectively. Both blood pressure and heart rate were monitored continuously during the study using a Power Lab (AD Instruments, Castle Hill, Australia). After hemodynamics became stable, we intravenously administered empty PEGylated liposomes (n = 8), free adenosine (n = 8), or PEGylated liposomal adenosine (n = 8) for 10 min. Either PEGylated liposomal or free adenosine was infused at an initial dose of 225 μg/kg/min (0.1 ml/min) for 10 min. After a 5-min interval, either PEGylated liposomal adenosine or free adenosine was infused at 450 μg/kg/min (0.1 ml/min) for 10 min. In the same manner, PEGylated liposomal adenosine or free adenosine was then infused at 900 μg/kg/min (0.1 ml/min).

PROTOCOL 2: EFFECTS OF PEGYLATED LIPOSOMAL ADENOSINE ON INFARCT SIZE IN RATS. The MI was induced by transient ligation of the left coronary artery as described previously (8). In the first series of experiments, to examine the dose-dependent effects of liposomal adenosine on MI size, PEGylated liposomal adenosine was infused intravenously at 50, 150, or 450 μg/kg/min for a 10-min period starting from 5 min before the onset of reperfusion. In the second series of experiments, to determine the adenosine receptor subtype involved in cardioprotective effects by the liposomal adenosine, the antagonist of adenosine subtype receptor was intravenously injected as a bolus followed by the infusion of liposomal adenosine for 10 min. The MI size was evaluated at 3 h after the start of reperfusion. The doses of adenosine receptor subtype antagonists were determined according to the previous reports (9–11).

Measurement of infarct size. At 3 h after the onset of reperfusion, the area at risk and the infarcted area were determined by Evans blue and triphenyltetrazolium chloride (TTC) staining, respectively, as previously described (8). Infarct size was calculated as [infarcted area/area at risk] × 100(%) in a blind manner. The area at risk was composed of border (TTC staining) and infarcted (TTC nonstaining) areas.

Electron microscopy (EM). Myocardial samples for EM were obtained from the central and peripheral areas in ischemic/reperfused myocardium, which roughly corresponded to the infarcted and border areas, respectively, after the left coronary artery was occluded for 30 min of ischemia followed by 3 h of reperfusion. Samples were prepared as previously reported (12). Liposomes, whose major membrane component is unsaturated phospholipids, were visualized as homogenous dark dots with a diameter of 100 to 150 nm (13).

Accumulation of fluorescent-labeled PEGylated liposomes in ischemic/reperfused myocardium. Unlabeled or fluorescent-labeled PEGylated liposomes were infused intravenously at a dose of 0.1 ml/min as liposomal adenosine was infused in protocol 2. At 3 h after reperfusion, hearts were quickly removed and cut into 4 sections parallel to the axis from base to apex. Then ex vivo bioluminescence imaging was performed with an Olympus OV 100 imaging system (Olympus, Tokyo, Japan) and signals were quantified using WASABI quantitative software (Hamamatsu Photonics K.K., Shizuoka, Japan). Fluorescent intensity in the region of interest was measured as previously reported (14). Control intensity indicated the fluorescent intensity in the nonischemic area of the individual rat.

Time-course changes of free and PEGylated liposomal RI-labeled adenosine in plasma and myocardium. Free or PEGylated liposomal [^{3}H]-adenosine (83 kBq per rat) was infused intravenously at a dose of 0.1 ml/min as liposomal adenosine was infused in protocol 2. At the time indicated, rat hearts were harvested for counting of radioactivity (LSC-3100, Aloka Co., Tokyo, Japan). Results are expressed as a percentage of the injected dose per 1 ml of blood or 1 g of wet tissue weight.

Statistical analysis. The parameters of the liposomes were expressed as the average ± SD, whereas other data were expressed as the average ± SEM. Comparison of time-course changes in hemodynamic parameters between groups was performed by 2-way repeated-measures analysis of variance (ANOVA) followed by a post-hoc Bonferroni test. For comparison of RI activity between groups, statistical analysis was done with the Mann-Whitney *U* test. To address the differences in infarct size among groups, we performed a nonparametric (Kruskal-Wallis) test followed by evaluation with the Mann-Whitney *U* test. Resulting p values were corrected according to the Bonferroni method. To compare parameters of liposomes, an unpaired *t* test was performed. In all analyses, $p < 0.05$ was considered to indicate statistical significance.

Results

Characterization of liposomes by dynamic light scatter analysis. The dynamic light scatter analysis showed no significant difference in mean diameter, polydispersity index, or zeta-potential distribution between empty and adenosine-loaded PEGylated liposomes (Table 1).

Liposomes in ischemic/reperfused myocardium. The EM revealed the intact vascular endothelial cells and cardiomyocytes in the nonischemic myocardium (Figs. 1A and 1B). There were no homogenous dark dots indicating liposomes in the nonischemic myocardium of rats that received either saline (Fig. 1A) or liposomes (Fig. 1B). In the border area, many homogenous dark dots indicating liposomes were accumulated in rats that received liposomes, but not saline (Figs. 1C and 1D). In this area, significant structural damage was not observed in endothelium, but slight swelling of mitochondria was often observed. In the infarcted area, numerous liposomes were detected in rats that received liposomes, but not saline (Figs. 1E and 1F). In this area, the disrupted endothelial integrity and marked swelling of mitochondria were often observed.

Table 1 Characterization of Liposomes by Dynamic Light Scatter Analysis

	Mean Diameter (nm)	Polydispersity Index	Zeta Potential (mV)
PEGylated liposomes (empty liposomes)	126 ± 12	0.035 ± 0.003	−1.7 ± 0.4
PEGylated liposomal adenosine	134 ± 21	0.094 ± 0.002	−2.3 ± 1.1

Results represented analysis of 4 independent experiments. Values are expressed as mean ± SD.
PEG = polyethylene glycol.

JACC Vol. 53, No. 8, 2009
February 24, 2009:709–17

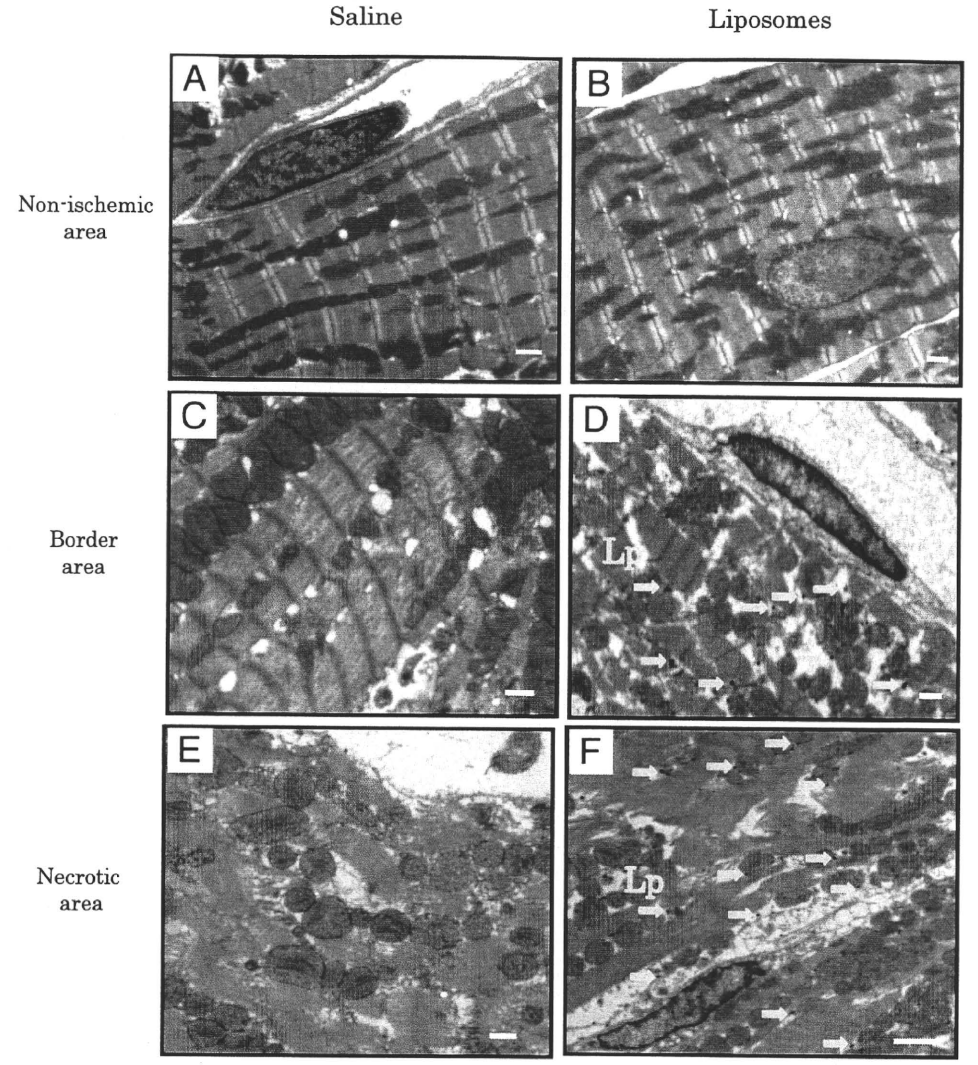

Figure 1 **Liposomes in Ischemic/Reperfused Myocardium**

(A, B) Representative electron micrographs of the nonischemic area in rats that received saline **(A)** or liposomes (Lp) **(B)**. **(C, D)** Representative electron micrographs of border area at 3 h after myocardial infarction (MI). Many **dark dots** accumulated in this area in the rat that received liposomes but not saline. **(E, F)** Representative electron micrographs of infarcted areas at 3 h after MI. Numerous **dark dots** accumulated in this area in the rat that received liposomes but not saline. Scale bars represent 1 μm.

Fluorescent-labeled PEGylated liposomes in ischemic/reperfused myocardium. Quantitative analysis by bioluminescence ex vivo bioluminescene imaging revealed that the target to control fluorescent intensity ratio was higher in the border (noninfarcted area at risk) as well as infarcted areas compared with a nonischemic one, suggesting that fluorescent-labeled liposomes were accumulated in the border as well as infarcted areas. Since there was no high-intensity area when unlabeled liposomes were infused, it was suggested that this was not a nonspecific phenomenon to MI by the ex vivo bioluminescence imaging system (Fig. 2). The Evans blue staining was unrelated to the fluorescence intensity (data not shown).

Plasma radioactivity of RI-labeled adenosine was markedly higher in the PEGylated liposomal adenosine group at 10 min and 3 h after the intravenous infusion than in the free adenosine group (Fig. 3A). Encapsulation within PEGylated liposomes also augmented the accumulation of adenosine in ischemic/reperfused myocardium compared with that of free adenosine (Fig. 3B).

Hemodynamic effects of PEGylated liposomal adenosine. Baseline hemodynamic parameters did not differ among the groups. An intravenous infusion of free adenosine at doses of 225, 450, and 900 μg/kg/min decreased the mean blood pressure by 14.8%, 25.4%, and 33.7%, respectively, compared with the effect of empty PEGylated lipo-

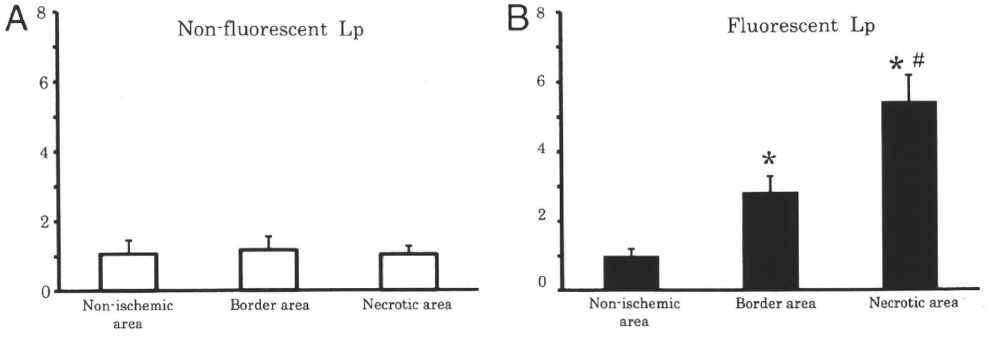

Figure 2 **Detection of Fluorescence-Labeled PEGylated Liposomes in Ischemic/Reperfused Myocardium**

Quantitative analysis of target-to-control fluorescent intensity ratio for each area in rats (n = 3 each group) that received nonfluorescent **(A)** or fluorescent **(B)** liposomes. The values of bioluminescence signals in the border and infarcted areas were expressed as the fold to that of the each nonischemic area. Values are expressed as the mean ± SEM (error bars). *p < 0.05 versus nonischemic areas. #p < 0.05 versus border areas.

somes. In contrast, the intravenous infusion of PEGylated liposomal adenosine at a dose of either 225 or 450 μg/kg/min did not significantly alter mean blood pressure (Fig. 4). Changes of the heart rate after infusion of PEGylated liposomal adenosine or free adenosine were similar to those observed for mean blood pressure (Fig. 4).

Effects of PEGylated liposomal adenosine on MI size. Baseline hemodynamic parameters were similar among all of the groups (Table 2). Intravenous infusion of free adenosine for 10 min reduced both the blood pressure and the heart rate, although these parameters returned to baseline within 5 min of ceasing infusion (Table 2). In contrast, hemodynamic parameters of the other groups were not altered (Table 2). The area at risk in the control group (61 ± 3%) did not differ compared with those of other groups that received liposomal adenosine. Intravenous infusion of PEGylated liposomal adenosine caused a dose-dependent decrease of MI size compared with that in the control group, whereas intravenous infusion of empty PEGylated liposomes or free adenosine did not (Fig. 5B).

The bolus injection of adenosine receptor antagonist did not alter the hemodynamic parameters (Table 3). The area at risk in the liposomal adenosine group (58 ± 3%) did not differ compared with those of other groups that received adenosine receptor antagonist. Infusion of 8-SPT, a nonspecific adenosine receptor antagonist, blunted the cardioprotective effect of liposomal adenosine (Fig. 6B). Furthermore, the infusion of the adenosine A_1, A_{2a}, A_{2b}, or A_3 receptor antagonist also blunted cardioprotective effects of liposomal adenosine (Fig. 6B). Infusion of 8-SPT alone did not significantly affect myocardial infarct size compared with the control (52 ± 5%, n = 4).

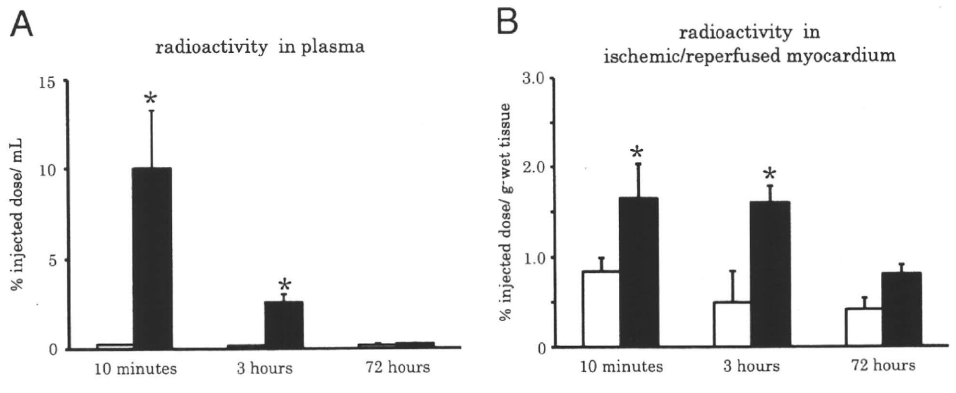

Figure 3 **Radioisotope-Labeled Adenosine in Plasma and Ischemic/Reperfused Myocardium**

(A) Changes in plasma radioactivity after infusion of radioisotope-labeled adenosine. **Solid and open bars** indicate the PEGylated liposomal adenosine and free adenosine groups, respectively (n = 4 each). In the PEGylated liposomal adenosine group, plasma radioactivity was markedly higher than in the free adenosine group. **(B)** Changes in radioactivity in ischemic/reperfused myocardium. **Solid and open bars** indicate the PEGylated liposomal adenosine and free adenosine groups, respectively (n = 4 each). In the PEGylated liposomal adenosine group, myocardial radioactivity was markedly higher than in the free adenosine group. Values are expressed as the mean ± SEM (error bars). *p < 0.05 versus the free adenosine group at the corresponding time.

JACC Vol. 53, No. 8, 2009
February 24, 2009:709–17

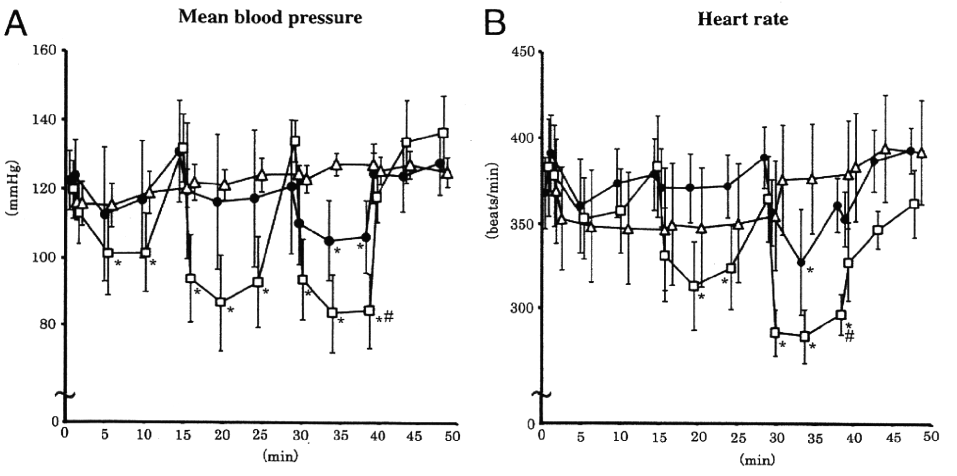

Figure 4 **Hemodynamic Effects of PEGylated Liposomal Adenosine**

Changes in the mean blood pressure **(A)** and heart rate **(B)** after intravenous infusion of various doses of empty PEGylated liposomes **(triangles)**, PEGylated liposomal adenosine **(circles)**, or free adenosine **(squares)** (n = 8 each). Values are expressed as the mean ± SEM. *p < 0.05 versus baseline at the corresponding group. #p < 0.05 versus PEGylated liposomes.

Discussion

In the present study, EM, bioluminescence ex vivo imaging, and fluorescent analysis revealed the accumulation of liposomes in the border (noninfarcted areas at risk) as well as infarcted ones, but not nonischemic myocardium, at 3 h after MI. These findings suggested that liposomes could specifically accumulate in ischemic/reperfused myocardium. Interestingly, EM revealed the existence of liposomes at sites where endothelial integrity was still morphologically maintained. Endothelial dysfunction such as enhanced permeability is induced by ischemic insult without morphological endothelial disruption (3,15). Enhanced permeability might lead to the accumulation of liposomes in the border as well as infarcted area, which will contribute to salvage the ischemic/reperfused myocardium. However, further investigation will be needed to determine the precise mechanism by which liposomes accumulate in ischemic/reperfused myocardium.

Analysis using RI-labeled adenosine encapsulated in liposomes revealed that plasma radioactivity was markedly higher in the PEGylated liposomal adenosine group compared with the free adenosine group. This indicates that encapsulation of adenosine by PEGylated liposomes considerably prolonged its residence time in the circulation and delayed its degradation. Consistent with the histological data, RI-labeled adenosine also showed preferential accumulation in ischemic/reperfused myocardium.

Table 2 **Effects of Liposomal Adenosine on Hemodynamic Parameters**

		Ischemia				Reperfusion	
	Baseline	0 min	15 min	25 min	30 min	5 min	10 min
Mean blood pressure (mm Hg)							
Saline	122 ± 5	102 ± 10	108 ± 7	107 ± 9	108 ± 7	105 ± 9	104 ± 9
Vehicle	127 ± 4	109 ± 8	108 ± 7	111 ± 9	111 ± 5	105 ± 5	103 ± 5
Free-Ado	124 ± 8	115 ± 8	111 ± 5	109 ± 4	66 ± 4*	62 ± 4*	112 ± 6
Lp-Ado 50 μg/kg/min	121 ± 5	106 ± 6	105 ± 6	110 ± 10	102 ± 6	101 ± 6	104 ± 4
Lp-Ado 150 μg/kg/min	122 ± 3	107 ± 6	107 ± 6	109 ± 11	105 ± 6	100 ± 6	103 ± 4
Lp-Ado 450 μg/kg/min	124 ± 3	104 ± 6	105 ± 6	107 ± 5	102 ± 6	99 ± 6	104 ± 4
Heart rate (beats/min)							
Saline	363 ± 22	366 ± 19	369 ± 14	413 ± 22	372 ± 12	372 ± 16	371 ± 14
Vehicle	363 ± 32	363 ± 6	383 ± 6	396 ± 25	367 ± 6	374 ± 7	372 ± 7
Free-Ado	360 ± 18	361 ± 17	384 ± 13	379 ± 18	305 ± 11*	293 ± 13*	356 ± 14
Lp-Ado 50 μg/kg/min	378 ± 19	386 ± 21	366 ± 12	376 ± 12	367 ± 19	369 ± 9	377 ± 17
Lp-Ado 150 μg/kg/min	388 ± 27	376 ± 20	371 ± 14	377 ± 13	378 ± 16	373 ± 16	369 ± 17
Lp-Ado 450 μg/kg/min	368 ± 17	376 ± 21	361 ± 13	386 ± 15	368 ± 15	363 ± 6	367 ± 7

Values are expressed as mean ± SEM. *p < 0.05 versus baseline.
Free-Ado = free adenosine; Lp-Ado = PEGylated liposomal adenosine; PEG = polyethylene glycol; vehicle = PEGylated liposomes.

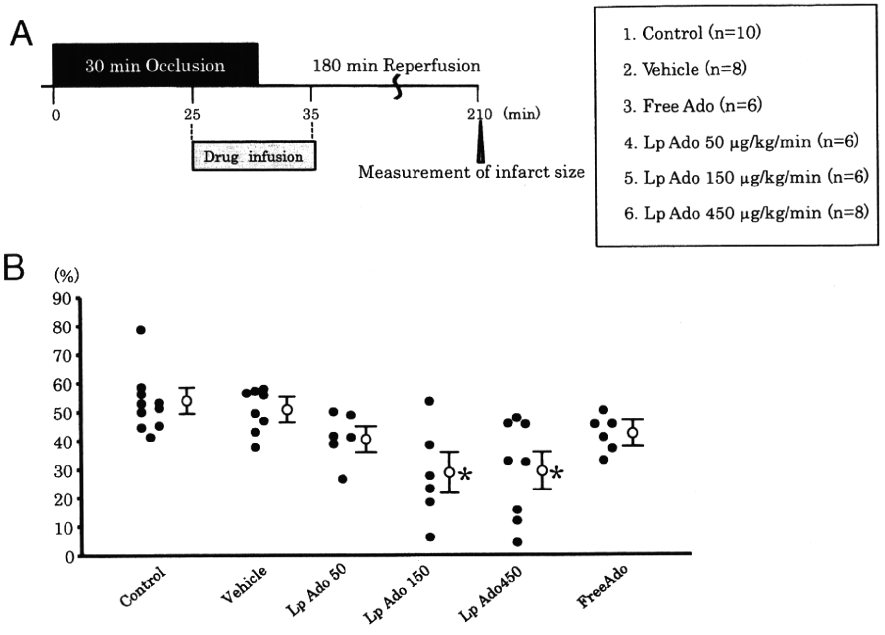

Figure 5 **Dose-Dependent Effects of PEGylated Liposomal Adenosine on Myocardial Infarct Size**

(A) Protocol showing the timing of infusion of PEGylated liposomal adenosine and of infarct size measurement. **(B)** Myocardial infarct size. Values are expressed as mean ± SEM (error bars). *$p < 0.05$ versus the control group. Free-Ado = free adenosine; Lp-Ado = PEGylated liposomal adenosine; Vehicle = PEGylated liposomes.

Furthermore, this study showed that PEGylated liposomal adenosine had a weaker effect on the blood pressure and heart rate than free adenosine. Thus, encapsulating adenosine in PEGylated liposomes attenuated its vasodilatory and negative chronotropic effects, presumably by reducing the amount of circulating free adenosine. However, the changes of hemodynamic parameters in this in vivo model suggested that significant release of adenosine from PEGylated liposomes would still occur if a large dose of liposomal adenosine (e.g., 900 μg/kg/min) were administered. Thus, further investigation of the in vivo pharmacodynamics of PEGylated liposomal adenosine is needed.

An intravenous infusion of PEGylated liposomal adenosine at the maximum dose that did not disturb hemodynamic parameters for 10 min before reperfusion reduced MI size in a dose-dependent manner, and this improvement was blocked by 8-SPT, a nonselective adenosine receptor antagonist. These findings suggest that adenosine released from liposomes acts via an adenosine receptor-dependent pathway. One possible mechanism by which PEGylated lipo-

Table 3 **Effects of Adenosine Receptor Antagonist on Hemodynamic Parameters**

		Ischemia				Reperfusion	
	Baseline	0 min	15 min	25 min	30 min	5 min	10 min
Mean blood pressure (mm Hg)							
Lp-Ado + 8SPT	120 ± 6	113 ± 4	112 ± 6	112 ± 5	107 ± 6	102 ± 8	109 ± 7
Lp-Ado + DPCPX	130 ± 6	105 ± 4	121 ± 4	100 ± 10	122 ± 6	120 ± 6	111 ± 4
Lp-Ado + SCH58261	132 ± 2	98 ± 12	99 ± 8	110 ± 8	118 ± 10	113 ± 10	109 ± 6
Lp-Ado + MRS1754	130 ± 3	95 ± 12	106 ± 8	105 ± 10	100 ± 10	96 ± 10	99 ± 7
Lp-Ado + MRS1523	130 ± 2	109 ± 8	104 ± 8	105 ± 9	100 ± 9	101 ± 10	104 ± 6
Heart rate (beats/min)							
Lp-Ado + 8SPT	404 ± 17	385 ± 10	374 ± 8	396 ± 8	389 ± 9	383 ± 8	385 ± 9
Lp-Ado + DPCPX	396 ± 24	380 ± 11	399 ± 9	398 ± 12	385 ± 9	382 ± 9	380 ± 7
Lp-Ado + SCH58261	393 ± 14	399 ± 15	381 ± 9	395 ± 15	376 ± 9	373 ± 9	385 ± 7
Lp-Ado + MRS1754	398 ± 14	392 ± 11	401 ± 9	379 ± 15	378 ± 9	374 ± 9	377 ± 7
Lp-Ado + MRS1523	396 ± 9	390 ± 11	390 ± 11	392 ± 10	373 ± 9	391 ± 7	388 ± 11

Values were expressed as mean ± SEM. *$p < 0.05$ versus baseline.
Lp-Ado = PEGylated liposomal adenosine; PEG = polyethylene glycol; Vehicle = PEGylated liposomes.

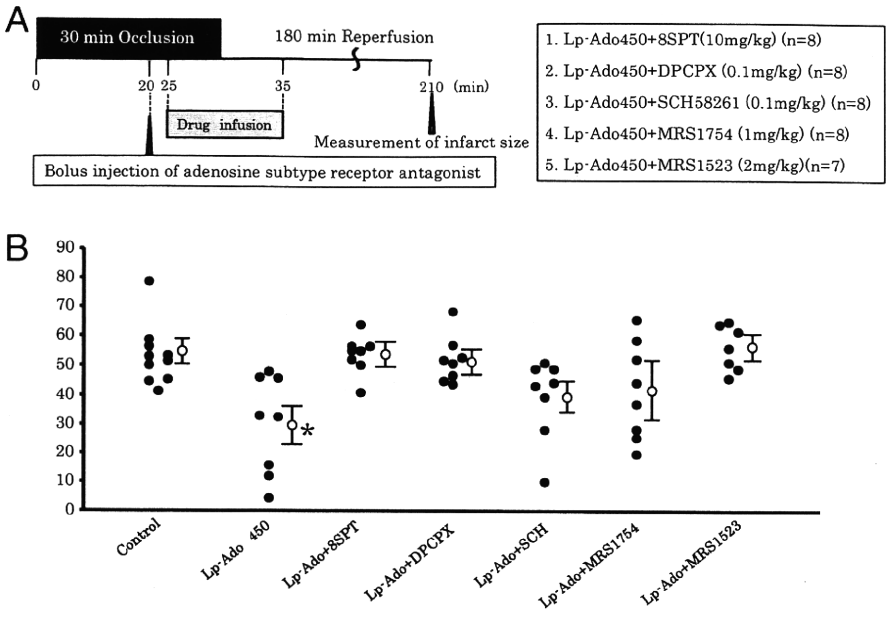

Figure 6 **Effects of Adenosine Receptor Antagonists on Myocardial Infarct Size**

(A) Protocol showing the timing of infusion of PEGylated liposomal adenosine and bolus injection adenosine receptor antagonists. **(B)** Myocardial infarct size. Values are expressed as the mean ± SEM (error bars). *$p < 0.05$ versus the control group. The abbreviations for adenosine antagonists were described in the text. Abbreviations as in Figure 5.

somes could augment cardioprotective effects of liposomal adenosine with minimum effects on hemodynamic parameters is the enhanced accumulation of PEGylated liposomal adenosine in ischemic/reperfused myocardium, which could augment various beneficial actions such as preventing calcium overload in the myocardium (5). The prolonged persistence of PEGylated liposomal adenosine would also increase its beneficial effect on ischemic/reperfused myocardium. Although continuous high-dose, long-term infusion of free adenosine was reported to reduce infarct size in rats (16), the present study did not confirm such a cardioprotective effect, probably because the total dose of free adenosine that we used was not high enough.

We found that myocardial infarct size in the group that received PEGylated liposomal adenosine with the antagonist of adenosine A_1, A_{2a}, A_{2b}, or A_3 subtype receptor was no different from the control group, indicating that every adenosine subtype receptor could possibly play a role in mediating cardioprotection by liposomal adenosine and that it was difficult to identify one particular subtype in the present study. Numerous studies reported that A_1, A_{2a}, A_{2b}, and A_3 receptors have been involved in cardioprotection against ischemia/reperfusion injury, and it remains controversial which adenosine subtype receptor is most responsible for cardioprotection (17–20). Furthermore, because the adenosine receptor antagonists used in the present study had some nonspecific effects, future investigation will be needed to examine the precise role of each adenosine receptor subtype using genetically engineered mice.

Because liposomal adenosine infused during reperfusion could reduce MI size, this agent could be a candidate for the adjunctive therapy of patients with acute MI. Importantly, adenosine is currently used for the diagnosis of ischemic heart disease and PEGylated liposomes are used to deliver anticancer agents (21). Thus, it should not be difficult to introduce PEGylated liposomal adenosine into clinical practice. Finally, PEGylated liposomes may provide a useful drug delivery system for targeting ischemic/reperfused myocardium with other agents.

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Overexpression of endoplasmic reticulum-resident chaperone attenuates cardiomyocyte death induced by proteasome inhibition

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KEYWORDS
ER stress;
CHOP;
GRP78;
Proteasome inhibition;
Cardiomyocyte

Aims Proteasome inhibitors are a novel class of anticancer agents that induce tumour cell death via endoplasmic reticulum (ER) stress. Since ER stress is involved in the development of heart failure, we investigated the role of ER-initiated cardiomyocyte death by proteasome inhibition.

Methods and results Rat neonatal cardiomyocytes were used in this study. Proteasome activity was assayed using proteasome peptidase substrates. Cell viability and apoptosis were measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenol tetrazolium bromide and flow cytometry, respectively. Western blot analysis, real-time polymerase chain reaction (PCR) and reverse transcriptional PCR were used to detect the expression of protein and messenger ribonucleic acid (RNA). The location of overexpressed glucose-regulated protein (GRP) 78 was observed by confocal fluorescence microscopy. Proteasome inhibition induced cardiomyocyte death and activated ER stress-induced transcriptional factor ATF6, but not XBP1 (X-box binding protein 1), without up-regulating ER chaperones. ER-initiated apoptosis signalling, including cytosine-cytosine-adenine-adenine-thymine enhancer-binding protein (C/EBP) homologous protein (CHOP), c-Jun-N-terminal kinase (JNK), and caspase-12, was activated by proteasome inhibition. Short interference RNA targeting CHOP, but not the blockage of caspase-12 or JNK pathway, attenuated cardiomyocyte death. Overexpression of GRP78 suppressed both CHOP expression and cardiomyocyte death by proteasome inhibition.

Conclusion These findings demonstrate that proteasome inhibition induces ER-initiated cardiomyocyte death via CHOP-dependent pathways without compensatory up-regulation of ER chaperones. Supplement and/or pharmacological induction of GRP78 can attenuate cardiac damage by proteasome inhibition.

1. Introduction

Endoplasmic reticulum (ER) is an organelle that participates in the folding of membrane and secretory proteins. The conditions or stresses that interfere with ER function are named ER stress.[1] There are two ER stress-induced transcriptional factors to up-regulate ER-resident chaperones that promote the folding of accumulated proteins in ER: activating transcription factor 6 (ATF6) and X-box binding protein 1 (XBP1). ATF6 is cleaved in response to ER stress and the cleaved ATF6 traffics to nuclei to induce the expression of ER-resident chaperone.[2] In addition, ER stress induces *XBP1* messenger ribonucleic acid (mRNA) splicing, producing a new spliced *XBP1* mRNA.[3] The spliced XBP1 protein and cleaved ATF6 cooperatively up-regulate the expression of ER-resident chaperones that reduce ER stress.[4] Another important pathway to cope with ER stress is the degradation of misfolded proteins by the ubiquitin-proteasome system.[5] It is therefore conceivable that treatment of cells with proteasome inhibitors causes accumulation of misfolded proteins and ER stress. When the overload of misfolded proteins is not resolved, cell apoptosis signals are initiated from ER. This effect is mediated by increased expression of the transcription factor cytosine-cytosine-adenine-adenine-thymine enhancer-binding protein (C/EBP) homologous protein (CHOP) and activation of caspase-12 and c-Jun-N-terminal kinase (JNK).[6–8]

Recently, the ubiquitin-proteasome system is reported to be involved in the growth and survival of cells and

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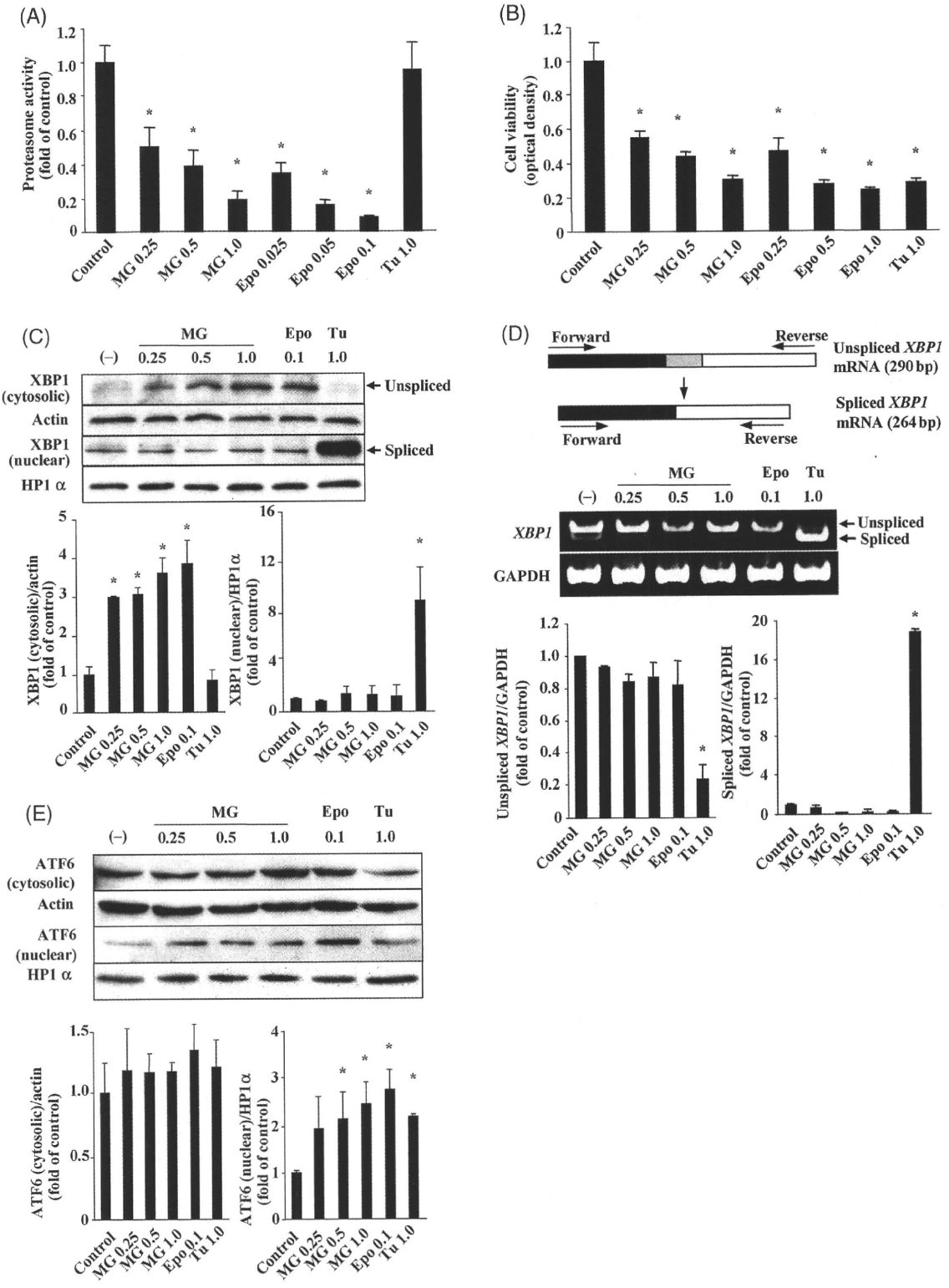

Figure 1 Effects of pharmacological proteasome inhibitors on the proteasome activity, cell death and endoplasmic reticulum stress-induced transcriptional factors in cultured cardiomyocytes. (*A*) Proteasome activity after the treatment with MG132 (MG) (0.25, 0.5, 1.0 μmol/L), epoxomicin (Epo) (0.025, 0.05, 0.1 μmol/L) or tunicamycin (Tu) (1.0 mg/mL) for 30 min. Experiments were repeated independently for three times ($n = 3$ in each experiment). (*B*) Cardiomyocyte viability after the treatment with MG, Epo or Tu for 48 h. Experiments were repeated independently for four times ($n = 6$ in each experiment). (*C*) Western blot analysis of spliced and unspliced X-box binding protein 1 (XBP1) proteins after the treatment with MG (0.25, 0.5, 1.0 μmol/L), Epo (0.1 μmol/L) or Tu (1.0 μg/mL) for 6 h. Actin and HP1α were used as the internal controls of cytosolic and nuclear fractions, respectively. (*D*) The upper panel shows the design of polymerase chain reaction (PCR) primers for *XBP1* messenger ribonucleic acid (mRNA) used in this study. This pair of primers can detect both unspliced and spliced *XBP1* mRNA. The middle and lower panels are representative and quantitative results of reverse transcriptional PCR for spliced and unspliced *XBP1* mRNA after the treatment with MG (0.25, 0.5, 1.0 μmol/L), Epo (0.1 μmol/L) or Tu (1.0 μg/mL) for 6 h. Glyceraldehyde-3-phosphate dehydrogenase was used as the internal control of mRNA expression. (*E*) Western blot analysis of ATF6 (activating transcription factor 6) in cytosolic and nuclear fractions after the treatment with MG (0.25, 0.5, 1.0 μmol/L), Epo (0.1 μmol/L) or Tu (1.0 μg/mL) for 6 h. The quantitative data in *C*, *D*, and *E* were achieved from three independent experiments. (Asterisk) $P < 0.05$ vs. control.

considered as an attractive therapeutic target.[9] Proteasome inhibitors are usually short peptides linked to a pharmacophore that reacts with the active site of proteasome.[10] Based on the pharmacophores, proteasome inhibitors can be divided into several groups: peptide aldehydes (e.g. MG132), peptide boronates (e.g. PS341), and peptide epooxyketones (e.g. epoxomicin).[11] Among these proteasome inhibitors, bortezomib (PS341) has been used as anticancer agent against haematological malignancy and solid tumours.[12] Recently, the treatment with bortezomib was reported to be associated with cardiac failure in patients with lung cancer and multiple myeloma.[13,14] Furthermore, we have found that the accumulation of ubiquitinated proteins in failing heart samples from humans demonstrated the impairment of proteasome function in failing hearts.[15] These findings led us to hypothesize that the proteasome inhibition could cause cardiomyocyte death via an ER-dependent pathway. To test this hypothesis, we checked the role of ER-initiated apoptotic signalling in cardiomyocyte death when proteasome activity was pharmacologically inhibited. Furthermore, we also investigated whether overexpression of ER-resident chaperone could rescue cardiac cell death by proteasome inhibition. In the present study, we used MG132 and epoxomicin, two typical proteasome inhibitors, to investigate the effect of proteasome inhibition on cardiomyocytes. We also used tunicamycin, an inhibitor of N-linked glycosylation, as an ER stress inducer without affecting proteasome activity.

2. Methods

2.1 Materials

MG132, epoxomicin, and tunicamycin were purchased from Sigma Chemical Co. (St Louis, MO, USA). The antibodies for CHOP, XBP1, ATF6, and actin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The antibodies for phospho-JNK and JNK were obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA). The antibodies for caspase-12 and HP1α were obtained from Sigma Chemical Co., while those for Lys-Asp-Glu-Leu (KDEL) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were obtained from Assay Designs, Inc. (Ann Arbor, MI, USA) and Millipore Co. (Billerica, MA, USA). Z-Ala-Thr-Ala-Asp (Z-ATAD) and SP600125 were purchased from BioVision Inc. (Mountain View, CA, USA) and Calbiochem (San Diego, CA, USA), respectively.

2.2 Preparation of neonatal rat cardiomyocytes

Primary cardiomyocyte cultures were prepared from neonatal rat hearts as described previously.[16] All procedures were in accordance with the guiding principles of Osaka University School of Medicine, Position of the American Heart Association on Research Animal Use, and the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health (NIH Publication No. 85-23, revised 1996).

2.3 Proteasome activity assay

Chymotrypsin-like activities of proteasome were assayed using the fluorogenic peptides Suc-Leu-Leu-Val-Tyr-7-amino-4-methylcoumarin (LLVY-AMC) (Biomol, Plymouth Meeting, PA, USA) according to the method reported previously.[15] Briefly, after the treatment with MG132 or epoxomicin for 30 min, cultured rat neonatal cardiomyocytes were harvested, lysed in proteasome buffer (10 mmol/L Tris–HCl, pH 7.5, 1 mmol/L ethylene diamine tetraacetic acid (EDTA), 2 mmol/L adenosine-5'-triphosphate, 20% glycerol, and 4 mmol/L dithiothreitol), and 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) for 1 h at 37°C. The reaction was stopped by adding 0.9 mL of cold water and placing the reaction mixture on ice for at least 10 min. Subsequently, the fluorescence of the solution was measured by Fluorescence Microplate Reader (Gemini XS; Molecular Devices, Sunnyvale, CA, USA) 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).

2.4 Caspase-12 activity assay

Caspase-12 activity was assayed using its substrate ATAD-7-amino-4-trifluoromethyl coumarin. Cell lysate aliquots were assayed by Fluorescence Microplate Reader (Gemini XS; Molecular Devices) with 400 nm excitation and 505 nm emission filter according to the manufacturer's protocol (BioVision).

2.5 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenol tetrazolium bromide assay

Cardiomyocytes were seeded at 3×10^4/well in 96-well plates. After MG132 administration at appropriate conditions, cell numbers were measured with a water-soluble tetrazolium reagent [WST-8; 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt] (Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer's instructions. Cell viability was expressed as a percentage of the control. The wavelengths used in this assay were 450 nm (sample) and 630 nm (reference).

2.6 Western blot analysis

Cardiomyocytes were lysed in the buffer (0.15 mmol/L, NaCl 0.05 mmol/L Tris–HCl, pH 7.2, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS) containing a protease inhibitor cocktail (Nakarai Tesque, Kyoto, Japan). Electrophoresis, immunoblotting, and detection were done as described previously.[15]

2.7 Reverse transcriptional polymerase chain reaction

After rat cardiomyocytes were treated with the drugs for 6 h, *XBP1* mRNA splicing was assessed using reverse transcriptional polymerase chain reaction (PCR) method. The primers that spanned the splice site are designed as followed: forward, ACGAGAGAAAACTCATGG; reverse, ACAGGGTCCAACTTGTCC (*Figure 1D*). This pair of primers can detect both spliced and unspliced *XBP1* at the size of 290 and 264 bp, respectively. The primers for GAPDH are forward, CATCAACGACCCCTTCATTGACCTCAACTA;reverse,TCCACGATGCCAAAGTTGTCATGGATGACC. PCR products were resolved on a 2% agarose gel and viewed by UV illumination.

2.8 Real-time quantitative polymerase chain reaction

We obtained samples after the drug treatment and then they were prepared according to the Omniscript Reverse Transcription Handbook (QIAGEN Inc., Hilden, Germany). The rat primers and probes used for quantification of glucose-regulated protein (GRP) 78, GRP94, CHOP, and GAPDH were all designed according to the manufacturer's protocol (Applied Biosystems, Foster City, CA, USA. https://www.appliedbiosystems.com/). Real-time PCR was performed with an ABI PRISM 7000 Sequence Detection System

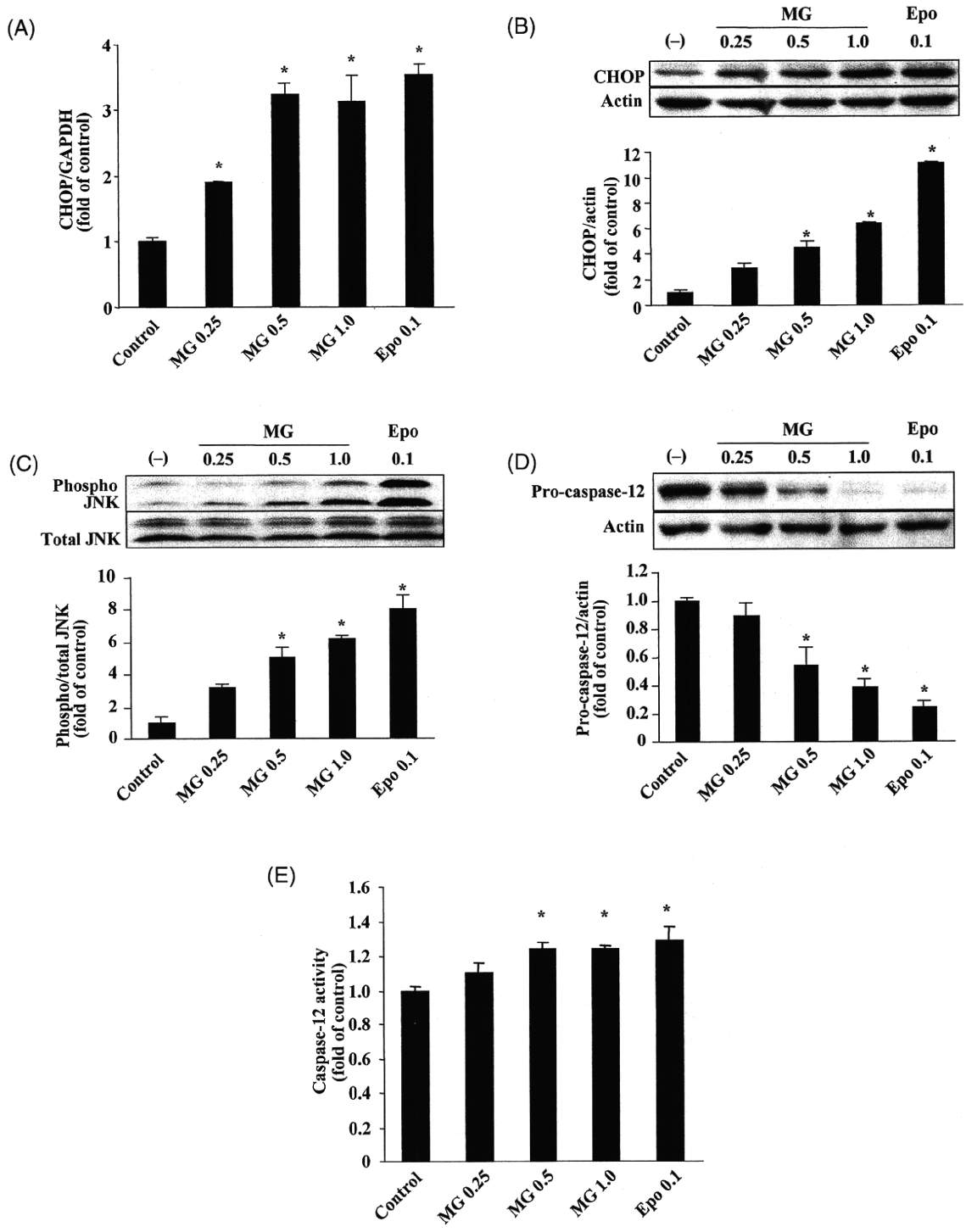

Figure 3 Activation of endoplasmic reticulum-initiated apoptosis signalling by proteasome inhibition in cultured cardiomyocytes. Real-time polymerase chain reaction (*A*) ($n = 3$ in each experiment) and western blot (*B*) analysis of CHOP [cytosine-cytosine-adenine-adenine-thymine (CCAAT) enhancer-binding protein (C/EBP) homologous protein] after the treatment with MG132 (MG) (0.25, 0.5, 1.0 μmol/L) or epoxomicin (Epo) (0.1 μmol/L) for 6 h. Western blot analysis of phospho-c-Jun-N-terminal kinase (JNK) (*C*) and pro-caspase-12 (*D*) after the treatment with MG (0.25, 0.5, 1.0 μmol/L) or Epo (0.1 μmol/L) for 1 and 6 h, respectively. (*E*) Caspase-12 activity after the treatment with MG (0.25, 0.5, 1.0 μmol/L) or Epo (0.1 μmol/L) for 6 h in cultured cardiomyocytes. Experiments were repeated independently for three times ($n = 3$ in each experiment). The quantitative data were achieved from three independent experiments. (Asterisk) $P < 0.05$ vs. control.

2.13 Statistical analysis

Data are expressed as the mean ± SEM. The results of cardiac proteasome activity, caspase-12 activity, cell viability and quantitative analysis of western blot analysis, real-time PCR, reverse transcription-PCR, and flow cytometry were compared by one-way factorial ANOVA followed by Bonferroni's correction. For all analyses, $P < 0.05$ was accepted as statistically significant.

(Applied Biosystems) by the relative standard curve method. The thermal cycle reaction was performed as follows: 50°C for 2 min, 95°C for 10 min followed by 40 cycles at 95°C for 15 s, 60°C for 1 min. The target amount was determined from the relative standard curves constructed with serial dilutions of the control total cDNA.

2.9 Ribonucleic acid interference

We ordered four different short interfering ribonucleic acid (siRNA) from B-Bridge International, Inc. (Mountain View, CA, USA) to knock down CHOP mRNA (CHOP siRNA-1: 5′-CGAAGAGGAAGAAUCAAA-3′, siRNA-2: 5′-GGAAACAGCGACUGAAGGA-3′, siRNA-3: 5′-GGGACUGA GGGUAGACCAA-3′, siRNA-4: cocktail containing equal amounts of the above three types of siRNA). Rat cardiomyocytes were isolated and then incubated in Dulbecco's modified Eagle's medium (Invitrogen Co., Carlsbad, CA, USA). Opti-MEM (Invitrogen Co.), siRNA oligonucleotides (CHOP siRNA 1–4) (60 nmol/L) and Optifect (Invitrogen Co.) were added 4 h after cardiomyocyte isolation. As a negative control, cells were transfected with siRNA against firefly luciferase from Photinus pyralis (GL2 siRNA).

2.10 Flow cytometry

An Annexin V-fluorescein isothiocyanate (FITC) Apoptosis Detection Kit was purchased from Sigma. After the treatment of MG132, cardiomyocytes were washed twice with PBS and resuspended in binding buffer. FITC-Annexin V and propidium iodide were added according to the manufacturer's protocol. The mixture was incubated for 10 min in dark at room temperature and then cellular fluorescence was measured with a FACSscan flow cytometry (Becton, Dickinson and Company, Franklin Lakes, NJ, USA).

2.11 Adenovirus transduction

Recombinant adenovirus harbouring GRP78 gene was constructed as described previously,[17] and adenovirus harbouring LacZ was used as a control. Adenovirus was transfected 24 h after cardiomyocytes were isolated or 20 h after siRNA against CHOP was added. And the experiments were performed another 24 h after adenovirus infection.

2.12 Confocal fluorescence microscopy

Cardiomyocytes were observed by confocal microscopy (Radiance 2100 Laser Scanning System Bio-Rad, Hemei Hempstead, UK) and saved by LaserSharp 2000 (Bio-Rad). Alexa568 (red) (Invitrogen Co.) was scanned by helium/neon laser (wavelength 543 nm laser line) with long path 590 filter (560–700 nm excitation). Alexa488 (green) was captured by Argon laser (wavelength 488 nm laser line) with band path 500–550 IR filter (500–550 nm excitation). DAPI (blue) for nuclei staining of all cells was obtained in range of 400–470 nm excitation.

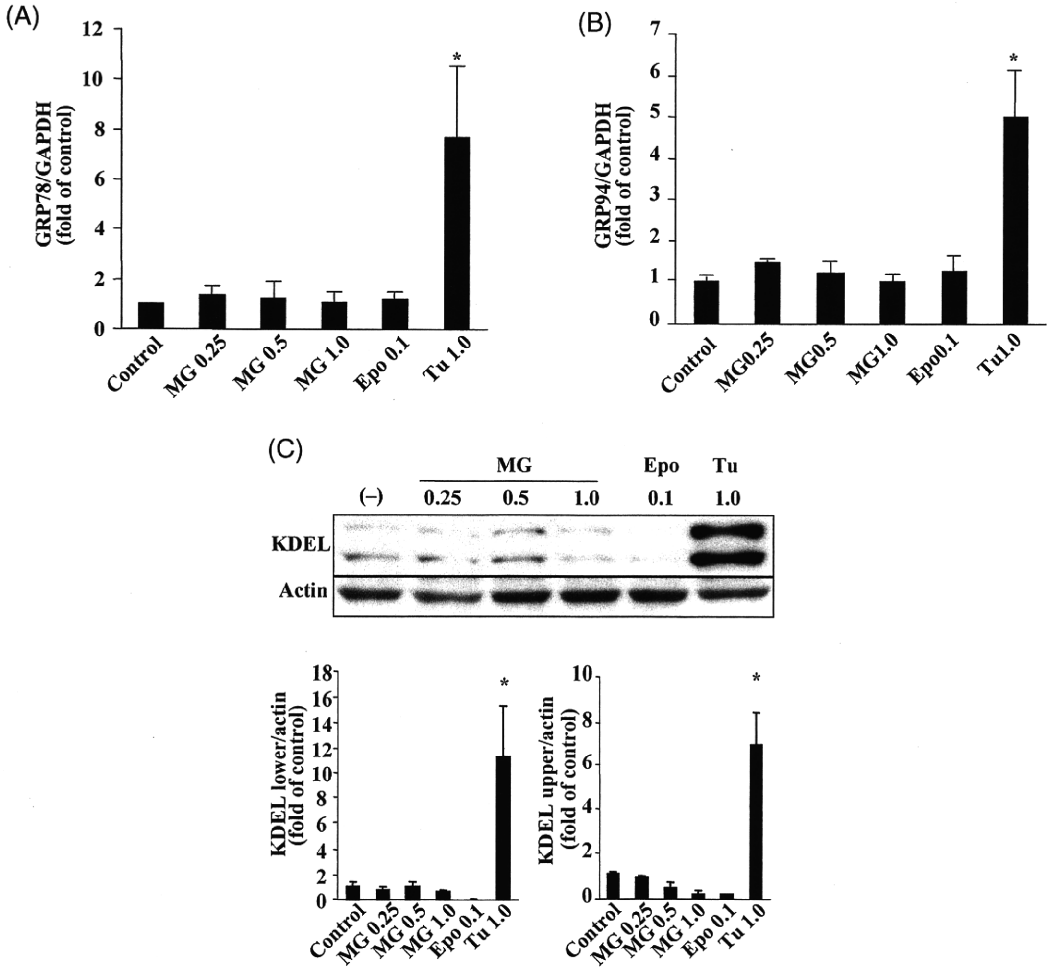

Figure 2 Endoplasmic reticulum chaperon expression by proteasome inhibition in cultured cardiomyocytes. Real-time polymerase chain reaction analysis of glucose-regulated protein (GRP) 78 (*A*) and GRP94 (*B*) ($n = 3$ in each experiment) and western blot analysis of Lys-Asp-Glu-Leu (KDEL) proteins (*C*) (upper and lower bands indicate GRP94 and GRP78, respectively) after the treatment with MG132 (MG) (0.25, 0.5, 1.0 μmol/L), epoxomicin (Epo) (0.1 μmol/L) or tunicamycin (Tu) (1.0 μg/mL) for 6 h. The western blot analysis and real-time PCR experiment were repeated for three times independently. (Asterisk) $P < 0.05$ vs. control.

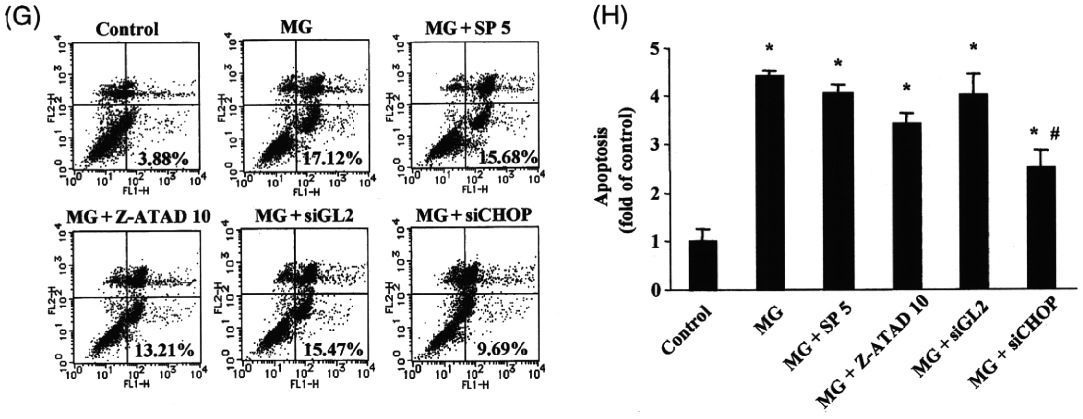

Figure 4 Continued.

3. Results

3.1 Proteasome activity and cell death by proteasome inhibition in cultured cardiomyocytes

Pharmacological proteasome inhibitors such as MG132 or epoxomicin dose-dependently decreased proteasome activity and reduced cell viability in rat-cultured cardiomyocytes. However, tunicamycin, an ER-stress inducer, induced cardiomyocyte death without inhibiting proteasome activity (*Figure 1A* and *B*).

3.2 Activation of endoplasmic reticulum stress-induced transcriptional factors and endoplasmic reticulum chaperone expression by proteasome inhibition in cultured cardiomyocytes

After the addition of MG132 or epoxomicin, protein level of unspliced XBP1 in cytosolic fraction, but not spliced XBP1 in nuclear fraction, was increased in rat-cultured cardiomyocytes (*Figure 1C*). The result of reverse transcriptional PCR demonstrated that either MG132 or epoxomicin did not change mRNA level of unspliced *XBP1* in cardiomyocytes (*Figure 1D*), suggesting that the increase in unspliced XBP1 protein level was due to the inhibition of its degradation by proteasome inhibition. In contrast, pharmacological ER stressor, tunicamycin, decreased unspliced *XBP1* mRNA expression and increased both mRNA and protein levels of spliced *XBP1* (*Figure 1C* and *D*). Proteasome inhibitors increased the protein level of ATF6 in the nuclear fraction in cultured cardiomyocytes (*Figure 1E*) to the similar extent as tunicamycin did. Importantly, proteasome inhibition did not induce the mRNA and protein expressions of either GRP78 or GRP94, although tunicamycin increased both of them (*Figure 2A–C*).

3.3 Activation of endoplasmic reticulum-initiated apoptosis signalling and cell death by proteasome inhibition in cultured cardiomyocytes

Proteasome inhibition by MG132 or epoxomicin increased both mRNA and protein levels of CHOP in rat-cultured cardiomyocytes (*Figure 3A* and *B*). In addition, it also induced JNK phosphorylation (*Figure 3C*) and caspase-12 activation (*Figure 3D* and *E*). CHOP siRNA 1 or 4, but not 2 or 3, significantly attenuated the MG132-induced increase in both mRNA and protein levels (*Figure 4A* and *B*). SP600125, an inhibitor of JNK phosphorylation, prevented the JNK phosphorylation by MG132 at both 5 and 10 μmol/L (*Figure 4C*). Z-ATAD, a caspase-12 inhibitor, attenuated the activation of caspase-12 by MG132 at 10, but not 2, μmol/L (*Figure 4D* and E). Cell viability analysed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenol tetrazolium bromide (MTT) assay showed that siRNA targeting CHOP, but not SP600125 (5 μmol/L) or Z-ATAD (10 μmol/L) compound, prevented cell death induced by proteasome inhibition in rat-cultured cardiomyocytes (*Figure 4F*). Furthermore, consistent with the data of MTT assay, flow cytometry analysis showed that siRNA targeting CHOP, but not SP600125 or Z-ATAD, attenuated the apoptosis of cardiomyocyte induced by proteasome inhibition (*Figure 4G* and *H*).

3.4 Overexpression of glucose-regulated protein 78 attenuated endoplasmic reticulum stress and cell death by proteasome inhibition in cultured cardiomyocytes

Location of GRP78 overexpressed by adenovirus in cultured cardiomyocyte was almost consistent with that of protein disulphide isomerase, an ER-resident oxidoreductase (*Figure 5A*). The increase in GRP78 expression was confirmed by western blot analysis with the specific antibody of KDEL. Interestingly, GRP78 overexpression specifically inhibited the induction of CHOP, but not activation of caspase-12 or JNK (*Figure 5B–F*). Moreover, GRP78 overexpression dose-dependently decreased CHOP induction and increased cardiomyocyte viability (*Figure 5G–J*). Furthermore, the flow cytometry analysis also showed that overexpression of GRP78 attenuated apoptosis induced by proteasome inhibition in rat-cultured cardiomyocytes (*Figure 5K* and *L*). The overexpression of GRP78 combined with CHOP knockdown did not show additional effects on cardiomyocytes viability compared with GRP78 overexpression or CHOP knockdown alone (*Figure 5M*).

4. Discussion

The present study demonstrated that proteasome inhibitors, such as MG132 and epoxomicin, activated the ER stress-induced transcriptional factor ATF6, but not XBP1, without commeasurable expression of ER chaperone upon proteasome inhibition. Furthermore, proteasome inhibition induced cardiac apoptosis via CHOP-, but not JNK- or

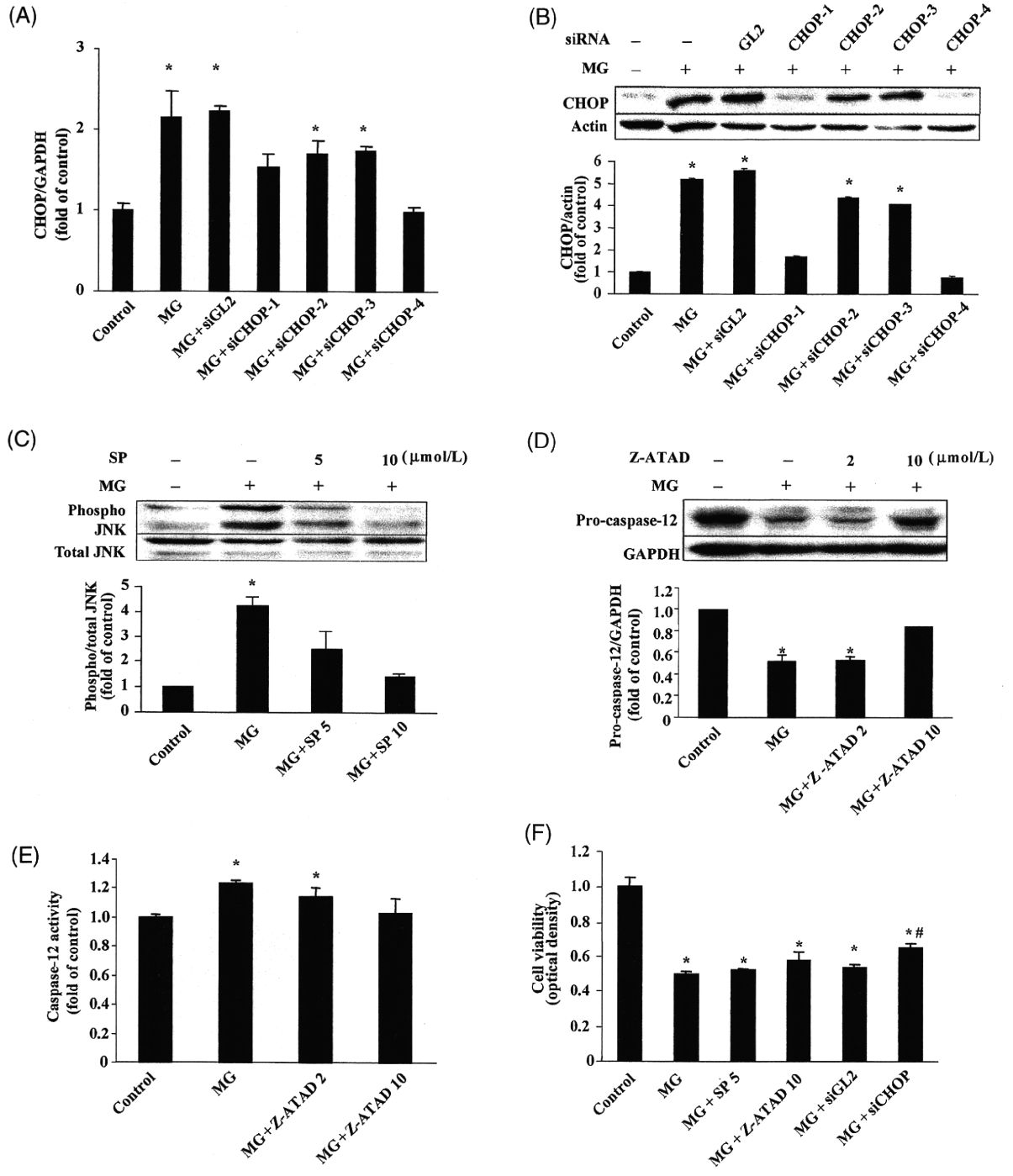

Figure 4 Effects of blockade of endoplasmic reticulum (ER)-initiated apoptosis signalling on apoptosis by proteasome inhibition in cultured cardiomyocytes. Effects of four different types of siRNA (short interfering ribonucleic acid) targeting CHOP [CCAAT enhancer-binding protein (C/EBP) homologous protein] on *CHOP* mRNA (*A*) ($n = 3$ in each experiment) and protein expression (*B*) after the treatment with MG132 (MG) (1.0 μmol/L) for 6 h. (*C*) Effects of SP600125 on JNK (c-Jun-N-terminal kinase) phosphorylation after the treatment with MG (1.0 μmol/L) for 1 h. SP600125 was added 1 h before MG (1.0 μmol/L) administration. (*D*) and (*E*) Effects of Z-Ala-Thr-Ala-Asp (Z-ATAD) on caspase-12 activation after the treatment with MG (1.0 μmol/L) for 6 h. Z-ATAD was added 1 h before MG (1.0 μmol/L) administration ($n = 3$ in each experiment). (*F*) Results of cardiomyocyte viability by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenol tetrazolium bromide] assay after the co-treatment with MG (1.0 μmol/L) and blockers of ER-initiated apoptosis signals ($n = 6$ in each experiment). Representative (*G*) and quantitative (*H*) data of cardiomyocyte apoptosis by flow cytometry ($n = 3$ in each experiment). The population of cells in the lower right quadrant of dot plot indicated apoptotic cardiomyocytes. Results of western blot and flow cytometry analysis represented three independent experiments, while the result of cell viability was from four independent experiments, respectively. (Asterisk) $P < 0.05$ vs. control; (Hash) $P < 0.05$ vs. MG (1.0 μmol/L).

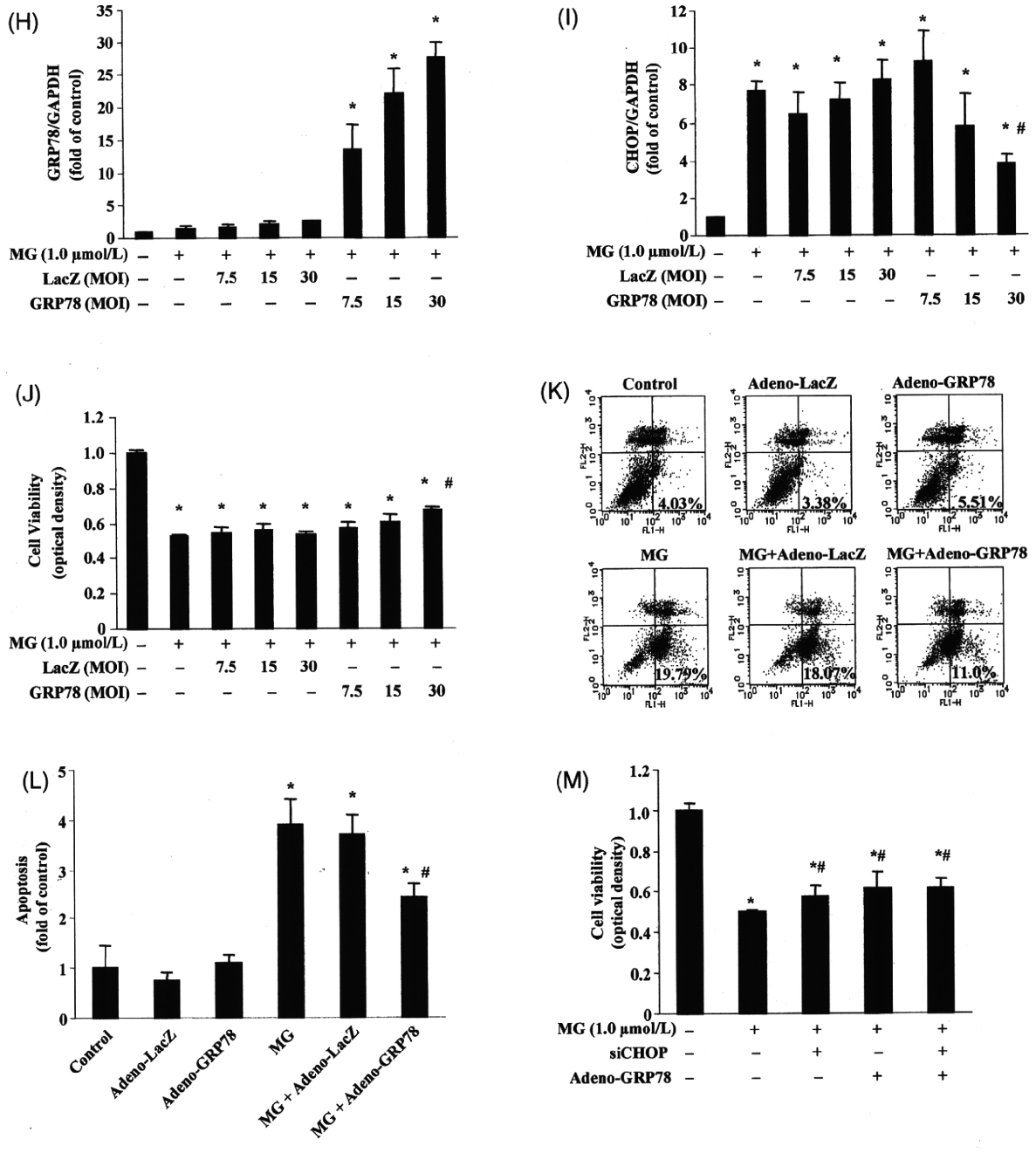

Figure 5 Continued.

caspase-12-, dependent pathway. Adenovirus-mediated GRP78 overexpression attenuated CHOP expression and rescued cardiomyocyte death by proteasome inhibition. These results suggest that proteasome inhibition caused ER stress without a compensatory increase in ER chaperones and induced cardiac apoptosis via the CHOP-dependent pathway. Supplement and/or pharmacological induction of GRP78 may be a potential therapeutic tool to attenuate cardiac damage by proteasome inhibition.

After proteasome inhibition, cleaved ATF6 protein in the nuclear fraction was increased, which might be due to the decrease in ATF6 degradation by proteasome inhibition and/or increase in the ATF6 cleavage.[18] However, consistent with the previous report,[19] we could not detect the increase of spliced XBP1 at either mRNA or protein level, suggesting that XBP1 was not activated by proteasome inhibition. Since overexpression of cleaved ATF6 could up-regulate ER chaperone expression,[20,21] ER chaperone should be induced due to the increase in cleaved ATF6 by proteasome inhibition. In our study, however, ER chaperons were not up-regulated after proteasome inhibition, suggesting there are some mechanisms that may prevent up-regulation of ER chaperone by cleaved ATF6. Since unspliced XBP1 protein acts as a dominant negative inhibitor of the spliced form and deactivates ATF6 by heterodimerization,[19,22-24] one possible mechanism is that increased protein levels of unspliced XBP1 probably due to the decelerated degradation by proteasome inhibition

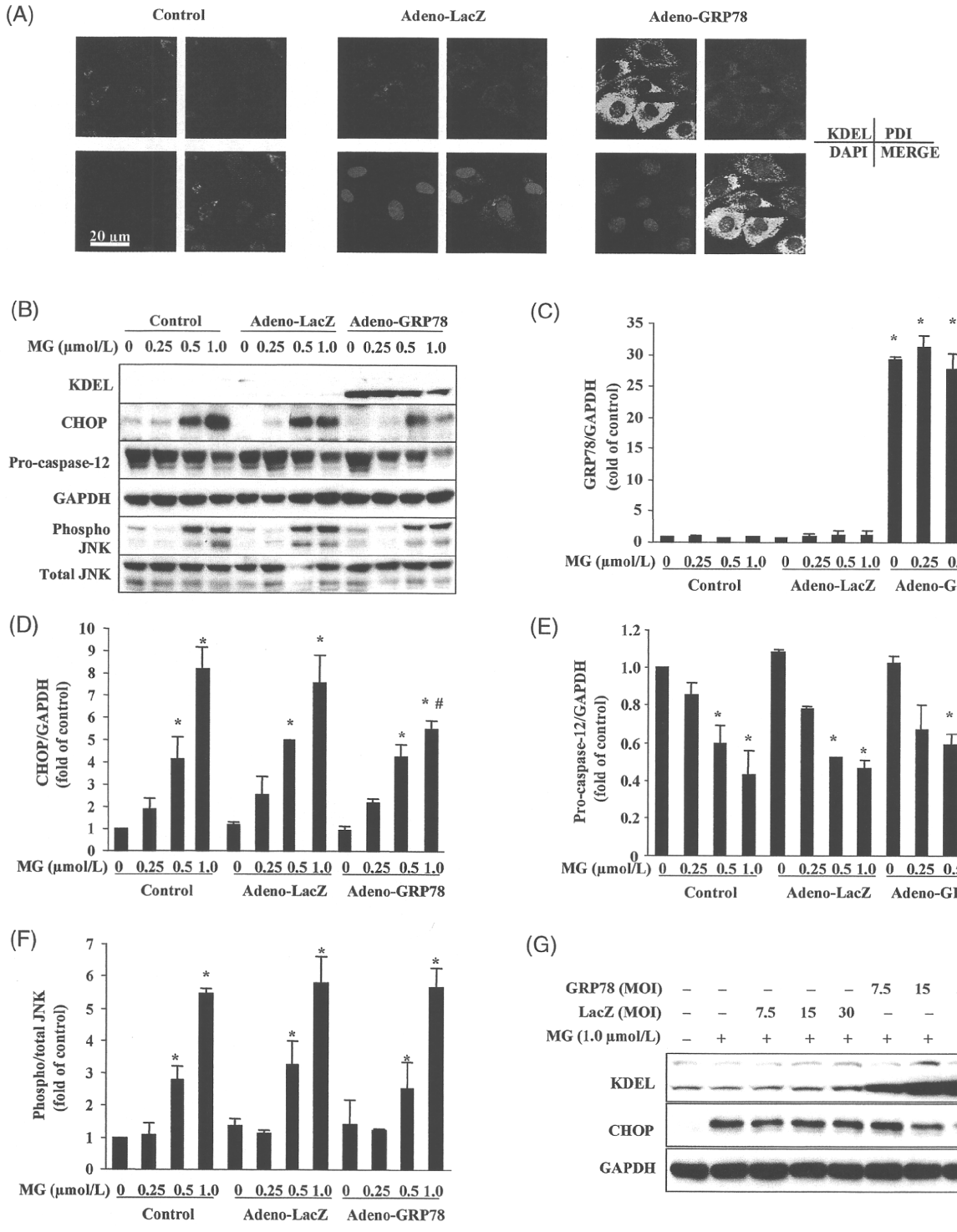

Figure 5 Overexpression of glucose-regulated protein (GRP) 78 reduced cardiomyocyte death by proteasome inhibition. (*A*) GRP78 was overexpressed by adenovirus at multiplicity of infection (MOI) 30 in cultured cardiomyocyte. Confocal fluorescence microscopy revealed that KDEL, PDI (protein disulphide isomerase) and DAPI were stained green, red and blue, respectively. (*B*) GRP78 expression, CCAAT-enhancer-binding protein (C/EBP) homologous protein (CHOP) expression and activation of capase-12 were investigated after the treatment with MG132 (MG) (1.0 μmol/L) for 6 h at appropriate concentrations, while phospho-c-Jun-N-terminal kinase (JNK) was detected 1 h after MG administration. (*C–F*) Quantitative data of GRP78 expression (*C*), CHOP expression (*D*), casapase-12 activation (*E*) and JNK phosphorylation (*F*). (*G–I*) Representative (*G*) and quantitative (*H, I*) data for the expressions of endoplasmic reticulum chaperone (KDEL) and CHOP protein after GRP78 was overexpressed in a dose-dependent manner. MG (1.0 μmol/L) was administrated for 6 h. (*J–L*) Effects of overexpression of GRP78 on cardiomyocyte viability by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenol tetrazolium bromide (MTT) analysis (*J*) ($n = 6$ in each experiment) and cardiomyocytes apoptosis by flow cytometry (*K, L*) ($n = 3$ in each experiment) after MG (1.0 μmol/L) administration. (*M*) Effects of GRP78 overexpression combined with CHOP knockdown on cardiomyocyte viability by MTT analysis after proteasome inhibition ($n = 5$ in each group). Results of western blot and flow cytometry analysis represented three independent experiments, while the result of cell viability was from four independent experiments, respectively. (Asterisk) $P < 0.05$ vs. control; (Hash) $P < 0.05$ vs. MG (1.0 μmol/L).