Cardiovascular Research (2008) **79**, 600–610
doi:10.1093/cvr/cvn128

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

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Received 17 December 2007; revised 8 April 2008; accepted 13 May 2008; online publish-ahead-of-print 28 May 2008

Time for primary review: 28 days

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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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

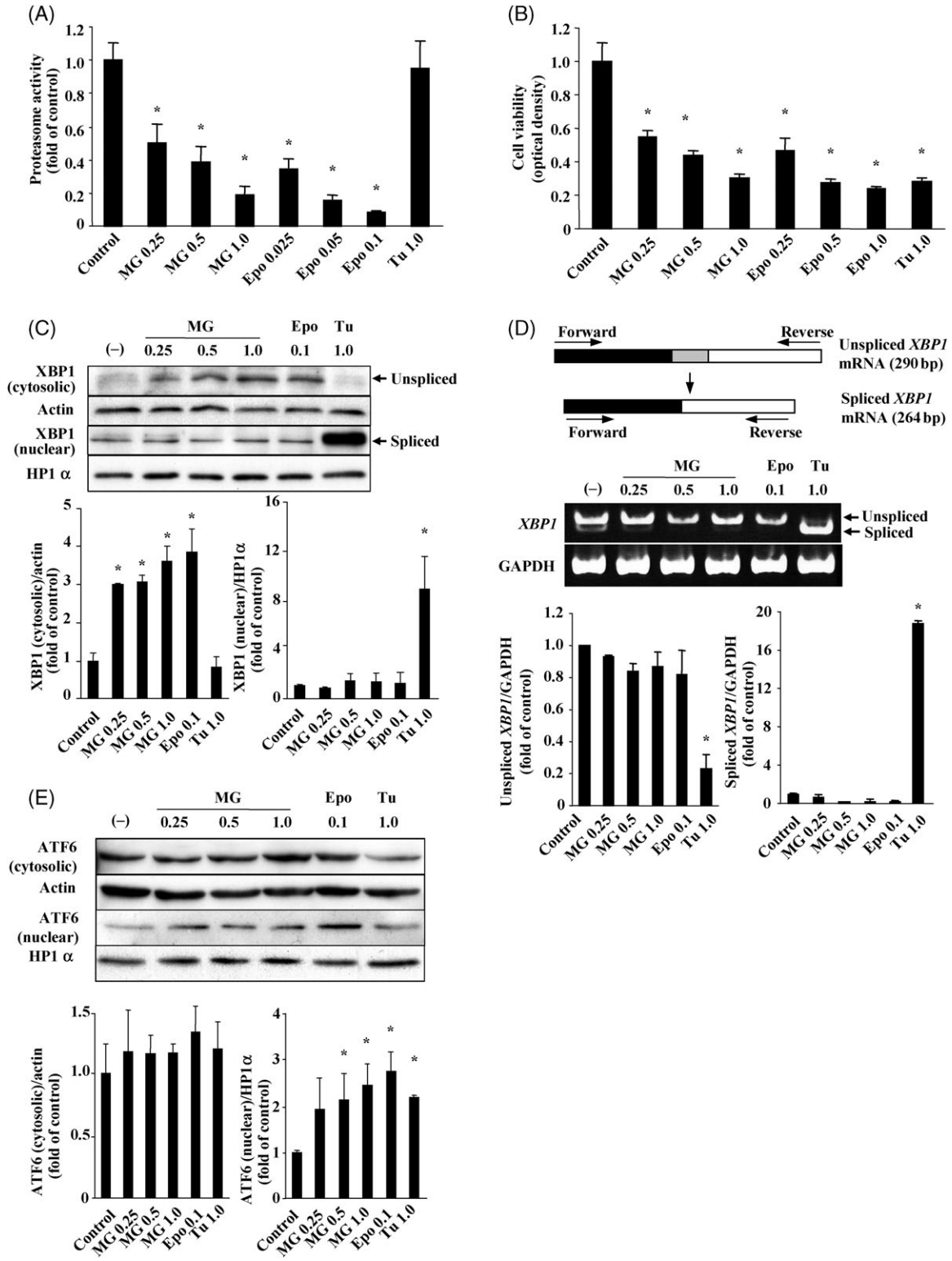

Figure 1 Effects of pharmacological proteasome inhibitors on the proteasome activity, cell death and endoplasmic reticulum stress-induced transcriptional factors in cultured cardiomyocytes. (*A*) Proteasome activity after the treatment with MG132 (MG) (0.25, 0.5, 1.0 μmol/L), epoxomicin (Epo) (0.025, 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.

(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 FACScan 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.

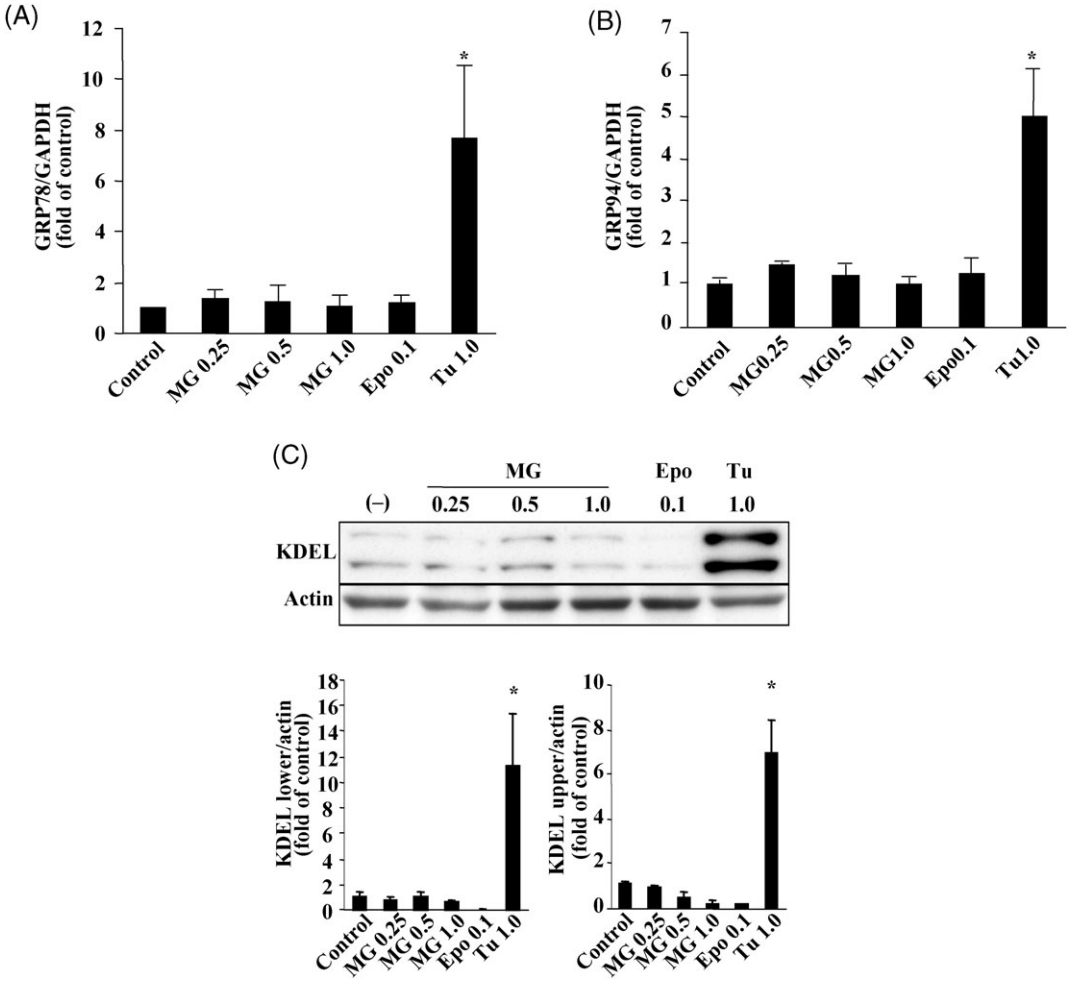

Figure 2 Endoplasmic reticulum chaperon expression by proteasome inhibition in cultured cardiomyocytes. Real-time polymerase chain reaction analysis of glucose-regulated protein (GRP) 78 (*A*) and GRP94 (*B*) ($n = 3$ in each experiment) and western blot analysis of Lys-Asp-Glu-Leu (KDEL) proteins (*C*) (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.

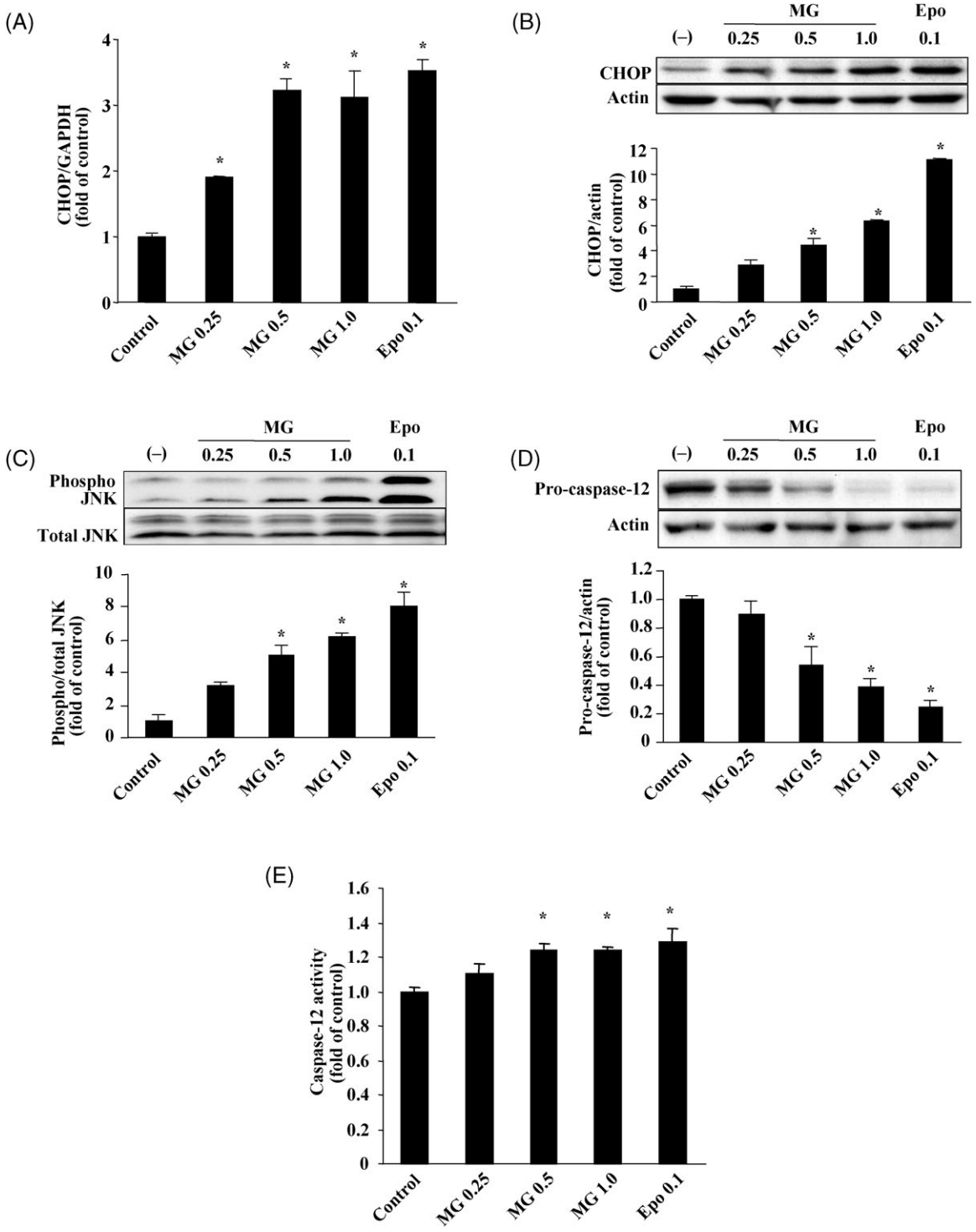

Figure 3 Activation of endoplasmic reticulum-initiated apoptosis signalling by proteasome inhibition in cultured cardiomyocytes. Real-time polymerase chain reaction (*A*) ($n = 3$ in each experiment) and western blot (*B*) analysis of CHOP [cytosine-cytosine-adenine-adenine-thymine (CCAAT) enhancer-binding 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.

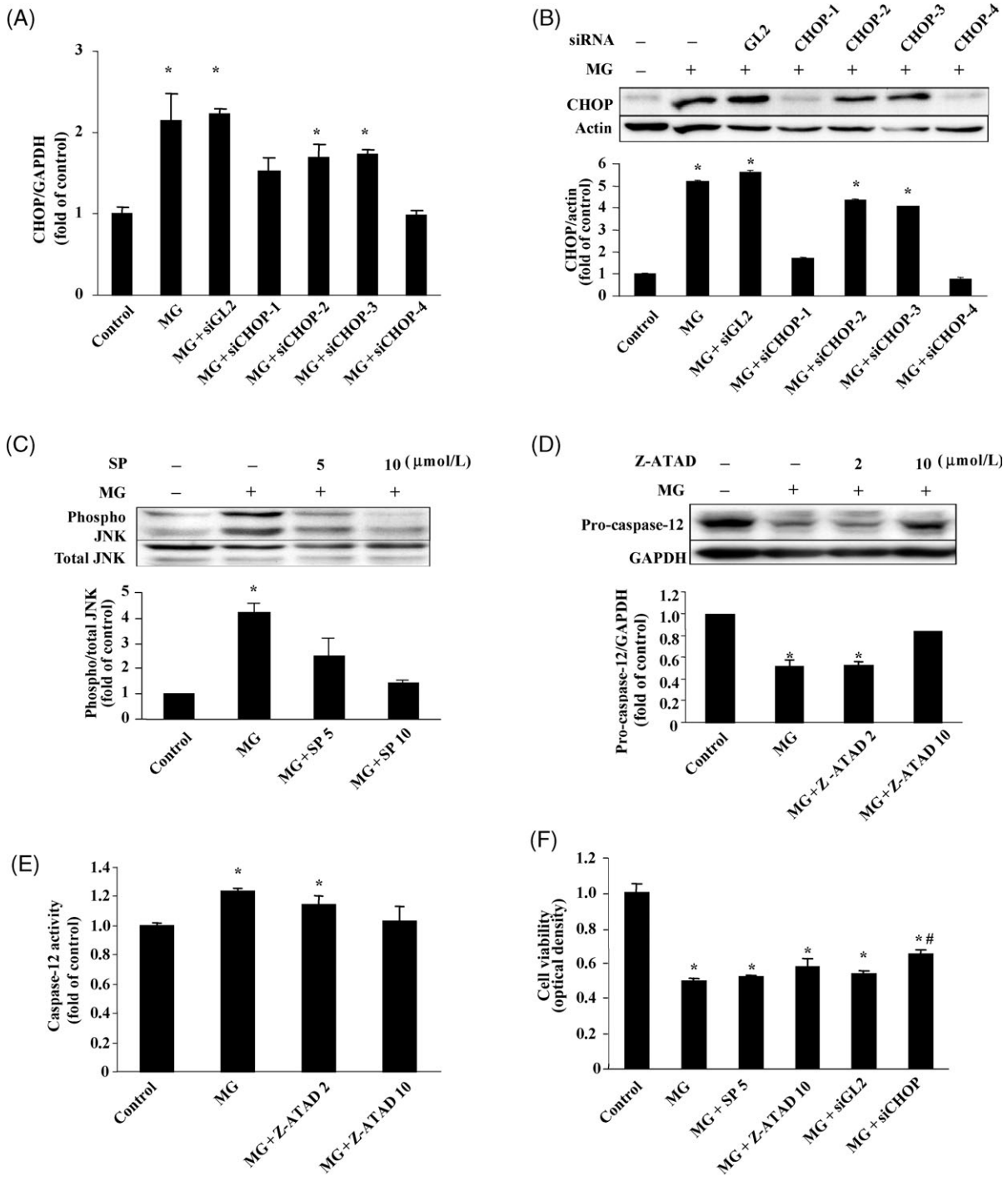

Figure 4 Effects of blockade of endoplasmic reticulum (ER)-initiated apoptosis signalling on apoptosis by proteasome inhibition in cultured cardiomyocytes. Effects of four different types of siRNA (short interfering ribonucleic acid) targeting CHOP [CCAAT enhancer-binding protein (C/EBP) homologous protein] 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).

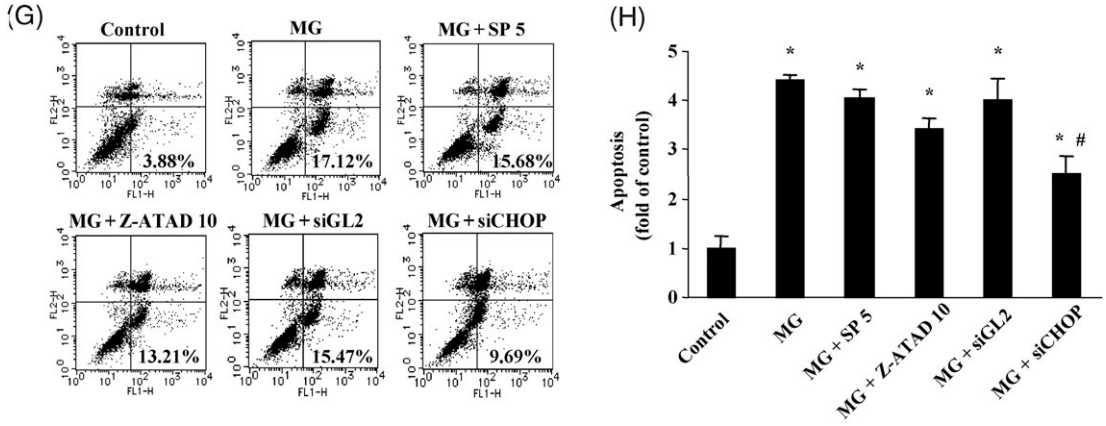

Figure 4 Continued.

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

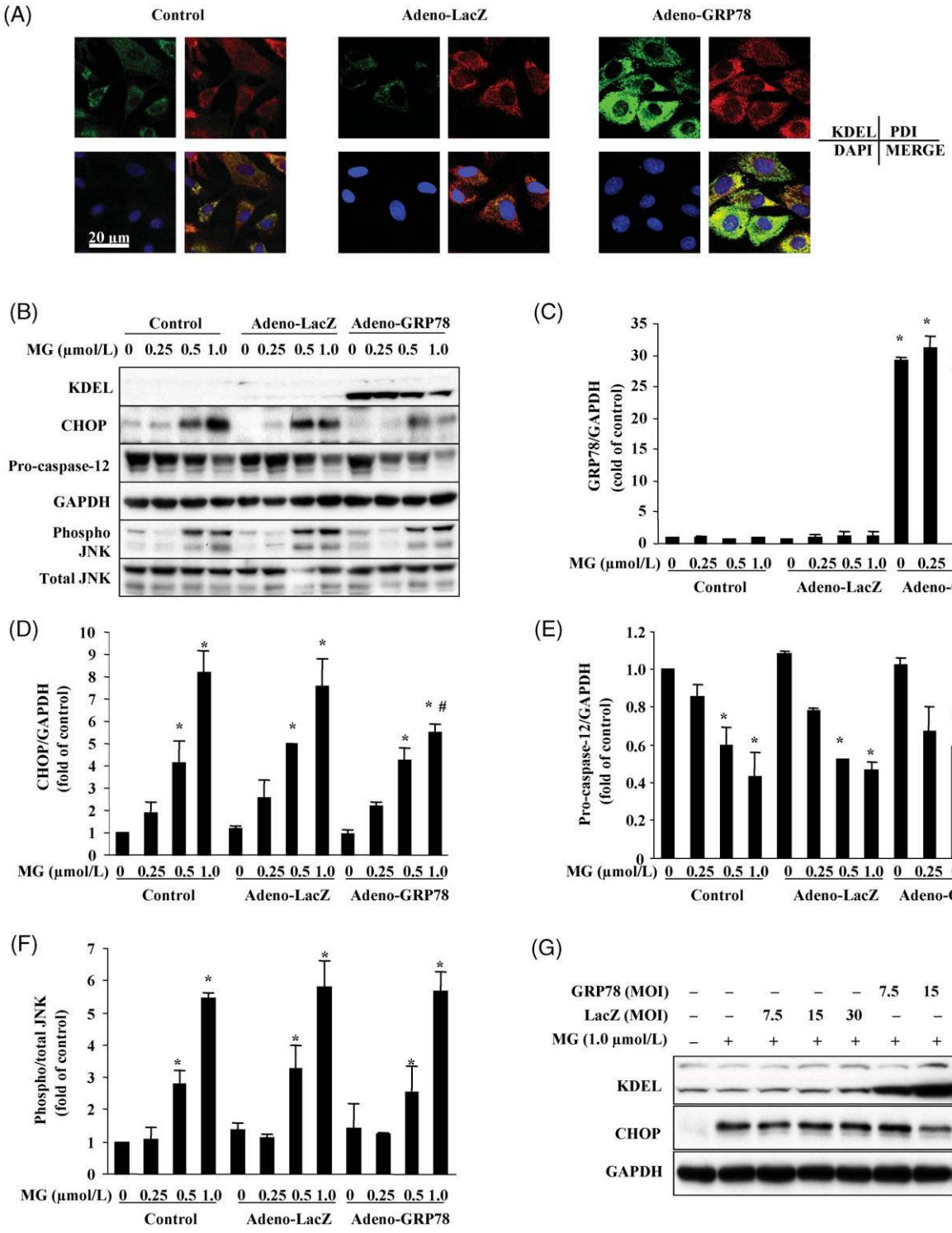

Figure 5 Overexpression of glucose-regulated protein (GRP) 78 reduced cardiomyocyte death by proteasome inhibition. (*A*) GRP78 was overexpressed by adenovirus at multiplicity of infection (MOI) 30 in cultured cardiomyocyte. Confocal fluorescence microscopy revealed that KDEL, PDI (protein disulphide 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).

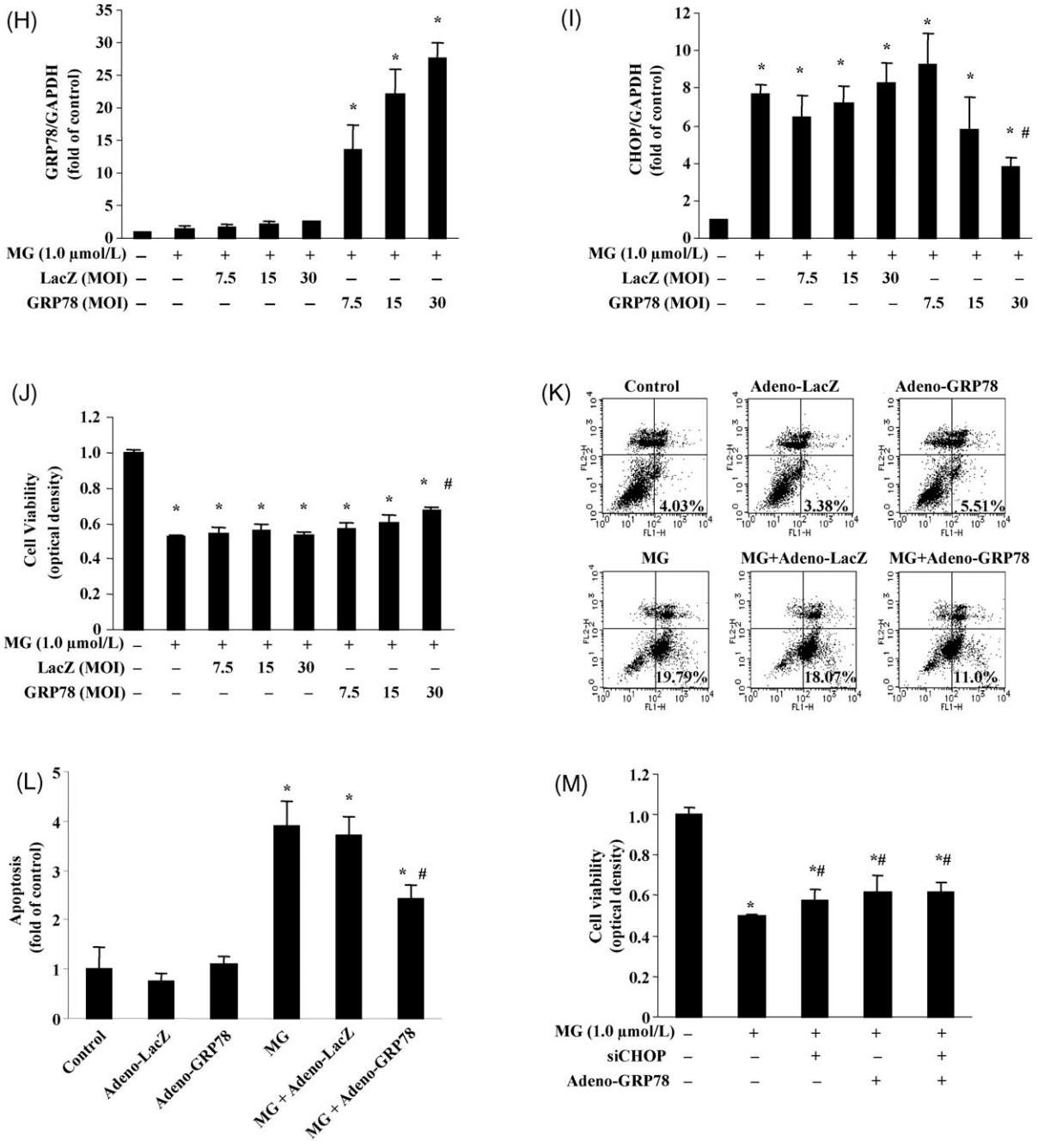

Figure 5 Continued.

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

may prevent the induction of ER chaperone. No compensatory increase in the ER chaperone may deteriorate the ER function to cope with ER stress when proteasome activity is inhibited (*Figure 6*).

In the present study, proteasome inhibition activated ER-initiated apoptotic signalling such as CHOP, caspase-12, and JNK. Using siRNA targeting CHOP and pharmacological inhibitors for caspase-12 and JNK, we found that CHOP knockdown partially, but significantly, inhibited cardiac apoptosis, while other pharmacological inhibitors did not. These findings suggest that CHOP, but not caspase-12 or JNK, mainly mediated cardiac apoptosis by proteasome inhibition. Recent research showed that the importance of three ER-initiated apoptotic signals is not equivalently involved in the pathophysiology in ER stress-related diseases.[25–27] Importantly, CHOP knockdown only partially prevented cardiomyocyte death by proteasome inhibition, suggesting that other mechanisms to induce cell death would be involved. Indeed, we have previously demonstrated that proteasome deactivation increased pro-apoptotic regulatory protein levels, such as p53 and Bax, and their knockdown also partially, but significantly, attenuated cardiac apoptosis.[15] These findings suggest that proteasome inhibition may cause cardiac apoptosis via the ER stress-dependent and -independent pathways.

We found overexpression of GRP78 could attenuate both CHOP expression and cell death by proteasome inhibition in cultured cardiomyocytes. In addition, the combination of GRP78 overexpression and CHOP knockdown did not show additional effects on preventing cardiomyocyte death, indicating that cell survival by GRP78 overexpression is predominantly through CHOP-dependent pathway. Further investigation is needed to elucidated why GRP78 specifically blocked CHOP induction among ER-initiated apoptotic signals. In the present study, although CHOP knockdown or GRP78 overexpression showed the small improvement of cell survival when cardiomyocytes were treated with proteasome inhibitors, these findings have some clinical relevance. Since patients will repeatedly receive the proteasome inhibitor for much longer time in the clinical settings, even a small size of improvement will exert the beneficial effects on the patients who need to receive the proteasome inhibitors.

We have previously demonstrated that both CHOP and GRP78 expression were induced in samples from human failing hearts and mouse failing hearts due to the pressure overload.[28] These findings suggest that ER stress may be involved in the pathogenesis in developing heart failure. Although we did not have the opportunity to check the ER-stress related signalling in the animal or human model when proteasome is inhibited, our *in vitro* data strongly suggest that proteasome inhibition may play an important role in the cardiomyocyte death via the ER stress-dependent pathways. The difference in the activation of ER stress-related signalling may be dependent on the pathophysiology of heart failure, and it is necessary to clarify how ER stress is involved in pathogenesis of cardiac diseases.

The ubiquitin-proteasome system is impaired in pathological cardiovascular conditions, such as ischaemia/reperfusion and failing hearts resulting from pressure overload.[15,29] Here, we found that proteasome inhibition induced ER-initiated apoptosis in cultured cardiomyocytes, supporting the idea that the impairment of the ubiquitin-proteasome system may play a crucial role in the development of heart disease. Bortezomib (PS-341) is clinically used as a novel class of anticancer agents against haematological malignancy and solid

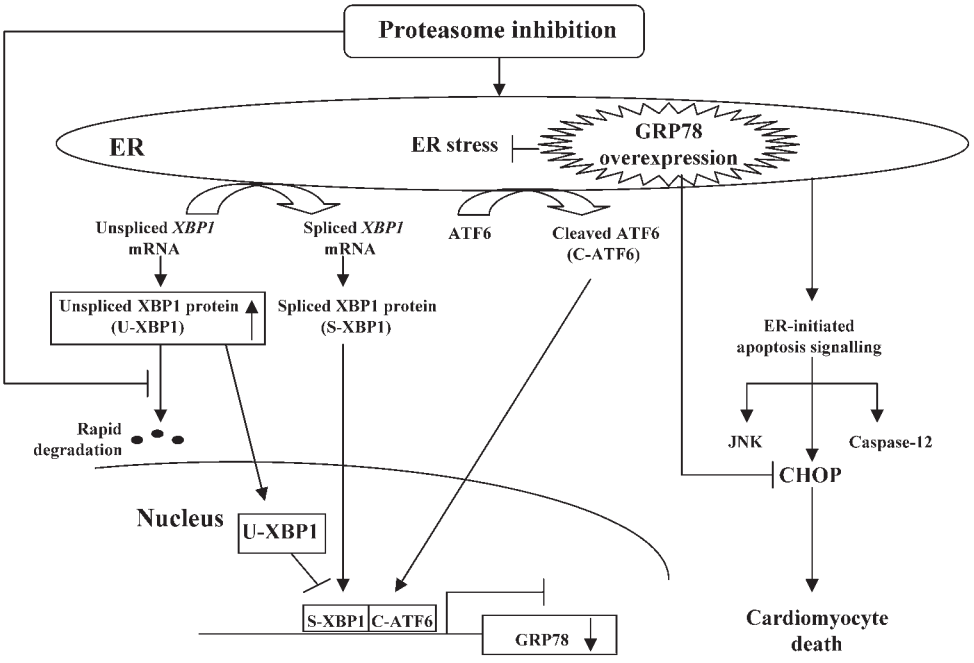

Figure 6 Schematic diagram of endoplasmic reticulum (ER)-chaperone glucose-regulated protein (GRP) 78 attenuating cardiomyocyte death by proteasome inhibition. Proteasome inhibition induces ER stress with the activation of activating transcription factor 6 (ATF6), but not X-box binding protein 1 (XBP1), in cardiomyocytes. Furthermore, proteasome inhibition activates ER-initiated apoptosis signalling such as CCAAT enhancer-binding protein (C/EBP) homologous protein (CHOP), JNK (c-Jun-N-terminal kinase) and caspase-12. Importantly, the expression of GRP78 was not enhanced probably due to the increased protein level of unspliced XBP1, which may further deteriorate ER stress. Overexpression of GRP78 attenuated cardiomyocyte death by proteasome inhibition via CHOP-dependent pathway. U-XBP1, S-XBP1, and C-ATF6 indicate unspliced XBP1, spliced XBP1, and cleaved ATF6, respectively.

tumour. Although bortezomib is not available currently in our hands, MG132 or epoxomicin used in the present study has similar characteristics as bortezomib to cause cell death via ER stress-related signalling.[30,31] Recently, some studies reported that the treatment with bortezomib was associated with cardiac dysfunction.[13,14] In addition, imatinib mesylate, a tyrosine kinase inhibitor used as an anticancer drug, was also reported to cause ER stress and heart failure.[32] Therefore, based on these findings, we need to monitor cardiac function carefully while using anticancer drugs that potentially disrupt protein quality control.

Conflict of interest: none declared.

Funding

This work was supported by a grant for Scientific Research from the Japanese Ministry of Education, Culture, Sports, Science and Technology (No. 17590731) and a grant from Japan Cardiovascular Research Foundation (No. 19390220).

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