Chemical Endoplasmic Reticulum Chaperone Alleviates Doxorubicin-Induced Cardiac Dysfunction

Hai Ying Fu, Shoji Sanada, Takashi Matsuzaki, Yulin Liao, Keiji Okuda, Masaki Yamato, Shota Tsuchida, Ryo Araki, Yoshihiro Asano, Hiroshi Asanuma, Masanori Asakura, Brent A. French, Yasushi Sakakibara, Masafumi Kitakaze, Tetsuo Minamino

Rationale: Doxorubicin is an effective chemotherapeutic agent for cancer, but its use is often limited by cardiotoxicity. Doxorubicin causes endoplasmic reticulum (ER) dilation in cardiomyocytes, and we have demonstrated that ER stress plays important roles in the pathophysiology of heart failure.

Objective: We evaluated the role of ER stress in doxorubicin-induced cardiotoxicity and examined whether the chemical ER chaperone could prevent doxorubicin-induced cardiac dysfunction.

Methods and Results: We confirmed that doxorubicin caused ER dilation in mouse hearts, indicating that doxorubicin may affect ER function. Doxorubicin activated an ER transmembrane stress sensor, activating transcription factor 6, in cultured cardiomyocytes and mouse hearts. However, doxorubicin suppressed the expression of genes downstream of activating transcription factor 6, including X-box binding protein 1. The decreased levels of X-box binding protein 1 resulted in a failure to induce the expression of the ER chaperone glucose-regulated protein 78 which plays a major role in adaptive responses to ER stress. In addition, doxorubicin activated caspase-12, an ER membrane–resident apoptotic molecule, which can lead to cardiomyocyte apoptosis and cardiac dysfunction. Cardiac-specific overexpression of glucose-regulated protein 78 by adeno-associated virus 9 or the administration of the chemical ER chaperone alleviated cardiac apoptosis and dysfunction induced by doxorubicin.

Conclusions: Doxorubicin activated the ER stress–initiated apoptotic response without inducing the ER chaperone glucose-regulated protein 78, further augmenting ER stress in mouse hearts. Cardiac-specific overexpression of glucose-regulated protein 78 or the administration of the chemical ER chaperone alleviated the cardiac dysfunction induced by doxorubicin and may facilitate the safe use of doxorubicin for cancer treatment.

Key Words: apoptosis ■ cardiac dysfunction ■ doxorubicin ■ endoplasmic reticulum chaperone ■ endoplasmic reticulum stress

Doxorubicin, an anthracycline antibiotic, is commonly used to treat a wide range of cancers. However, the clinical use of doxorubicin is often limited by its cardiotoxicity. Although inhibition of DNA/RNA synthesis, excessive oxidative stress, and the disturbance of Ca2+ homeostasis are known to be involved in doxorubicin-induced cardiotoxicity, the precise mechanisms have not yet been identified.

Histological analysis has demonstrated that doxorubicin causes marked endoplasmic reticulum (ER) dilation in human hearts. The ER is a principal site for protein folding and Ca2+ homeostasis. Perturbation of ER function results in the accumulation of unfolded proteins and Ca2+ disturbance, which is collectively referred to as ER stress. When ER stress is transient and mild, adaptive responses are induced to maintain ER function. Three ER transmembrane sensors, activating transcription factor 6 (ATF6), inositol-requiring enzyme 1 (IRE1), and protein kinase RNA-like ER kinase, are involved in mediating adaptive responses. On ER stress, ATF6 migrates to the Golgi, where it is cleaved to release an active transcription factor into the nucleus. The cleaved ATF6 then induces the expression of various genes, including X-box binding protein 1 (XBPI). IRE1 functions as an endoribonuclease and mediates the unconventional splicing of XBPI mRNA to generate spliced XBPI (sXBPI). sXBPI mRNA encodes a potent...
transcription factor which induces glucose-regulated protein 78 (GRP78) that is the most abundant ER chaperone and plays a major role in adaptive responses to ER stress. However, when ER stress is prolonged or severe, 3 ER stress–initiated apoptotic signaling pathways are activated, including caspase-12, c-JUN NH2-terminal kinase (JNK), and C/EBP homologous protein (CHOP). We and others have demonstrated that ER stress is involved in the pathophysiology of heart failure induced by pressure overload and ischemic injury.

Because doxorubicin can generate reactive oxygen species and disturb Ca2+ homeostasis, both of which potentially disturb ER function and doxorubicin can cause ER dilation in the heart, we investigated whether this drug can activate adaptive and apoptotic responses to ER stress. We also investigated whether cardiac-specific overexpression of the ER chaperone GRP78 or the administration of the chemical ER chaperone 4-phenylbutyrate on doxorubicin-induced cardiac dysfunction, 10-week–old male ICR mice received a single intraperitoneal injection of saline or doxorubicin (15 mg/kg). One day before receiving doxorubicin, male ICR mice received a single intraperitoneal injection of saline, 4-phenylbutyrate (100 mg/kg per day), or sodium butyrate (BS, 100 mg/kg per day) was administered once a day for 1 week by intraperitoneal injection (Online Figure IB). Thereafter, the mice were subjected to echocardiography, and heart samples were obtained for Western blot and histological analyses.

All protocols and procedures were approved by the Osaka University School of Medicine Standing Committee for Animal Use.

Bioluminescence Imaging
Luciferase activity in the mouse hearts was noninvasively assessed using a previously reported in vivo bioluminescence imaging system. All bioluminescence imaging was performed with the IVIS 100 system (Caliper Life Sciences, Hopkinton, MA).

Statistical Analysis
Data are expressed as mean±SEM. On the basis of the sample size and distribution, parametric tests (unpaired 2-tailed t test and 1-way ANOVA followed by the Tukey–Kramer post-test) and nonparametric tests (the Mann–Whitney U test and the Kruskal–Wallis test followed by the Dunn post-test) were applied as appropriate. For all analyses, P<0.05 was considered statistically significant.

Results

Doxorubicin Caused ER Dilation and Induced ER Stress in Mouse Hearts
Consistent with previous reports, we confirmed that doxorubicin resulted in marked ER dilation in mouse hearts (Figure 1A). In addition, mitochondrial alterations as well as autophagosome and lysosome formation were also observed in mouse hearts (Online Figure II). Among the adaptive responses to ER stress, doxorubicin induced ATF6 cleavage and IRE1 phosphorylation but not protein kinase RNA-like ER kinase phosphorylation in mouse hearts (Figure 1B). Although doxorubicin induced ATF6 cleavage, it suppressed the expression of genes downstream of ATF6 at mRNA levels, such as the regulator of calcineurin 1.4, Derlin-3, and XBP1, in mouse hearts (Figure 1C). Because of the suppression of the XBP1 mRNA levels, the sXBP1 mRNA levels were also decreased in mouse hearts after doxorubicin administration (Figure 1D). Because GRP78 that can reduce ER stress as an ER chaperone is regulated by sXBP1, the lack of sXBP1 failed to increase the protein level of GRP78 (Figure 1E). Moreover, doxorubicin also failed to induce GRP94 or protein disulfide isomerase, either of which is regulated by sXBP1 in the same manner as GRP78 (Online Figure IIIA).

Next, we investigated the effects of doxorubicin on the 3 signaling pathways involved in ER stress: activated caspase-12 cleavage and JNK phosphorylation but not CHOP induction were detected in mouse hearts after doxorubicin administration (Figure 1F).

Doxorubicin Induced ER Stress in Cultured Cardiomyocytes
Doxorubicin induced ATF6 cleavage and IRE1 phosphorylation but not protein kinase RNA-like ER kinase phosphorylation in cultured cardiomyocytes in a dose-dependent manner (Figure 2A). Although doxorubicin increased ATF6 cleavage, it suppressed the expression of genes downstream of ATF6, including XBP1 (Figure 2B). Accordingly, doxorubicin decreased mRNA levels of sXBP1 in a dose-dependent manner (Figure 2C). Importantly, doxorubicin failed to induce the protein levels of GRP78 as well as GRP94 and protein disulfide isomerase (Figure 2D; Online Figure IIIIB). Moreover, doxorubicin induced caspase-12 cleavage in a dose-dependent manner.
manner in cultured cardiomyocytes, which was consistent with the increase in caspase-12 activity induced by doxorubicin (Figure 2E; Online Figure IVA). Furthermore, doxorubicin increased JNK phosphorylation but not CHOP induction in cultured cardiomyocytes (Figure 2E).

**Overexpression of sXBP1 Increased the Protein Levels of GRP78 and Attenuated Doxorubicin-Induced Caspase-12 Cleavage and Cell Death in Cultured Cardiomyocytes**

Doxorubicin decreased the mRNA levels of sXBP1 and failed to induce GRP78 protein level; therefore, we overexpressed sXBP1 with adenovirus to evaluate the roles of sXBP1 and GRP78 in doxorubicin-induced cardiomyocyte death. As shown in Figure 3A, overexpressed sXBP1 was distributed mainly in the nucleus of the cardiomyocytes. The overexpression of sXBP1 increased the protein levels of the ER chaperone GRP78 and attenuated caspase-12 cleavage but not JNK phosphorylation or CHOP induction (Figure 3B). Moreover, cardiomyocyte death induced by doxorubicin was reduced after sXBP1 overexpression (Figure 3C). Importantly, GRP78 knockdown blunted the protective effects of sXBP1, which included the attenuation of caspase-12 cleavage and the reduction of cardiomyocyte death (Online Figure V). These results suggested that failure of GRP78 induction because of the suppression of sXBP1 augments the doxorubicin-induced cardiomyocyte death.

**Cardiac-Specific Overexpression of GRP78 or 4-Phenylbutyrate Treatment Attenuated Doxorubicin-Induced Caspase-12 Cleavage and Reduced Cell Death in Cultured Cardiomyocytes**

Doxorubicin activated ER stress-initiated apoptotic signaling pathways, such as the caspase-12 cleavage and c-JUN NH₂-terminal kinase (JNK) phosphorylation but not the C/EBP homologous protein (CHOP) induction, in mouse hearts. Tunicamycin (Tu), the positive control sample obtained from cardiomyocytes treated with Tu (1.0 mg/L), a pharmacological ER stress inducer, for 24 hours. The graphs summarize data from 3 to 5 mice per group; *P<0.05 vs Control group.
Acetylation, the concentration (0.5 mmol/L) of 4-phenylbutyrate or BS is known to induce histone acetylation in cultured cardiomyocytes (Online Figure VIIIA), suggesting that histone acetylation is not involved in the cardiac protective effect of 4-phenylbutyrate. Similar to 4-phenylbutyrate, tauroursodeoxycholic acid, another chemical ER chaperone,22 also attenuated caspase-12 cleavage and reduced cardiomyocyte death induced by doxorubicin (Online Figure IX), which indicated that the administration of the chemical ER chaperone tauroursodeoxycholic acid as well as 4-phenylbutyrate could reduce ER stress and protect cardiomyocyte from doxorubicin. Neither the cardiac-specific overexpression of GRP78 nor 4-phenylbutyrate treatment affected the release of cytochrome C from the mitochondria to the cytosol induced by doxorubicin.19,20 Cardiac-specific overexpression of GRP78 suppressed caspase-12 cleavage and reduced cell death by inhibiting the activation of caspase-12. ER stress is known to induce JNK phosphorylation in an IRE1-dependent pathway.18 Knockdown of IRE1 did not affect JNK phosphorylation in cultured cardiomyocytes treated with doxorubicin (Online Figure VI), suggesting that it may have induced JNK phosphorylation in an ER-independent manner. Heat shock proteins (HSP) 70 and 90, 2 chaperone proteins belonging to the HSP superfamily, showed protective effects against doxorubicin.21,22 Cardiac-specific overexpression of GRP78 did not change the protein level of HSP70 or 90 (Online Figure VIIIA), suggesting that HSP70 or 90 was not involved in the protective effects of GRP78 in doxorubicin-induced cardiomyopathy.

The chemical ER chaperone 4-phenylbutyrate attenuated caspase-12 cleavage and reduced cell death in cultured cardiomyocytes treated with doxorubicin (Figure 4B and 4C, right). However, BS, an analog of 4-phenylbutyrate that does not function as an ER chaperone,23 neither attenuated caspase-12 cleavage nor reduced cardiomyocyte death induced by doxorubicin (Figure 4B and 4C, right). Although a high dose of 4-phenylbutyrate or BS is known to induce histone acetylation,21 the concentration (0.5 mmol/L) of 4-phenylbutyrate or BS used in the present study did not affect histone 3 acetylation in cultured cardiomyocytes (Online Figure VIIIIA), suggesting that histone acetylation is not involved in the cardiac protective effect of 4-phenylbutyrate. Similar to 4-phenylbutyrate, tauroursodeoxycholic acid, another chemical ER chaperone, also attenuated caspase-12 cleavage and reduced cardiomyocyte death induced by doxorubicin (Online Figure IX), which indicated that the administration of the chemical ER chaperone tauroursodeoxycholic acid as well as 4-phenylbutyrate could reduce ER stress and protect cardiomyocyte from doxorubicin. Neither the cardiac-specific overexpression of GRP78 nor 4-phenylbutyrate treatment affected the release of cytochrome C from the mitochondria to the cytosol induced by doxorubicin (Online Figure X), suggesting that GRP78 or 4-phenylbutyrate protects cardiomyocytes by suppressing ER-dependent but not mitochondria-dependent pathways.

Cardiac-Specific Overexpression of GRP78 or 4-Phenylbutyrate Administration Alleviated Doxorubicin-Induced Cardiac Dysfunction

Consistent with previous reports,14 an in vivo bioluminescence imaging system showed that the systemic administration of AAV9-luciferase specifically increased luciferase activity in the heart (Figure 5A). The luciferase protein was also confirmed in mouse hearts at 9 weeks after systemic administration of AAV9-luciferase (Figure 5B). The immunofluorescence analysis showed that the ratio of GRP78 immunofluorescence intensity in troponin I–positive cells to that in troponin I–negative ones was increased after AAV9-GRP78 administration (Figure 5C), which suggested that GRP78

**Figure 2. Doxorubicin induced endoplasmic reticulum (ER) stress in cultured cardiomyocytes.** A. Doxorubicin induced activating transcription factor 6 (ATF6) cleavage and inositol-requiring enzyme 1 (IRE1) phosphorylation but not protein kinase RNA-like ER kinase (PERK) phosphorylation in cultured cardiomyocytes in a dose-dependent manner. B. Doxorubicin suppressed the expression of genes downstream of ATF6, such as regulator of calcineurin (RCAN1.4), Derlin-3, and X-box binding protein 1 (XBP1). C. Doxorubicin decreased the mRNA levels of spliced XBP1 (sXBP1) in cultured cardiomyocytes in a dose-dependent manner. D. Doxorubicin did not increase the protein levels of glucose-regulated protein 78 (GRP78) in cultured cardiomyocytes. E. Doxorubicin increased caspase-12 cleavage and c-JUN NH2-terminal kinase (JNK) phosphorylation but not C/EBP homologous protein (CHOP) induction in cultured cardiomyocytes in a dose-dependent manner. Cardiomyocytes were treated with doxorubicin at 1.0 μmol/L (B) or the indicated doses (A, C, D, and E) for 24 hours. The graphs summarize data from 3 independent experiments; * P<0.05 vs Control group.
was specifically overexpressed in cardiomyocytes by AAV9-GRP78. The protein level of GRP78 increased in mouse heart after systemic administration of AAV-GRP78 (Figure 5D). Moreover, the histological analysis revealed no pathophysiological changes, such as cardiac hypertrophy or fibrosis, in mouse hearts after AAV9-GRP78 administration (Figure 5E).

Cardiac-specific overexpression of GRP78 or 4-phenylbutyrate administration reduced caspase-12 cleavage in mouse hearts after doxorubicin administration, but the administration of AAV9-luciferase or BS did not (Figure 6A). JNK phosphorylation and CHOP induction were not changed by cardiac-specific overexpression of GRP78 or 4-phenylbutyrate administration (Figure 6A). The cardiodynamic-specific overexpression of GRP78 did not change the protein level of HSP70 or HSP90 in mouse hearts (Figure 6B). The graphs summarize data from 3 independent experiments; \( P < 0.05 \) vs Control group; \( P < 0.05 \) vs Doxorubicin group. DAPI indicates 4', 6-diamidino-2-phenylindole.

Cardiac protective effect of 4-phenylbutyrate. The percentage of terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)-positive cells in troponin I-positive ones increased from 0.3% to 2.1% in doxorubicin-administered mouse hearts, suggesting cardiomyocyte apoptosis was increased by doxorubicin in mouse hearts. Cardiac-specific overexpression of GRP78 or 4-phenylbutyrate administration reduced the number of TUNEL-positive cells in troponin I-positive ones (Figure 6B; Online Figure XI). Moreover, cardiac-specific overexpression of GRP78 or 4-phenylbutyrate administration attenuated ER dilation induced by doxorubicin (Figure 6C).

Echocardiographic examination showed that doxorubicin increased the left ventricular end-systolic dimension and decreased the left ventricular fractional shortening and left ventricular ejection fraction, which were attenuated by cardiac-specific overexpression of GRP78 or 4-phenylbutyrate administration (Table). Taken together, these results suggest that the cardiac-specific overexpression of GRP78 or 4-phenylbutyrate administration alleviated doxorubicin-induced cardiac dysfunction.
Discussion

In the present study, doxorubicin activated the ER transmembrane sensor ATF6, which failed to induce GRP78 that plays a major role in adaptive responses to ER stress. The failure of GRP78 induction augmented ER-initiated apoptosis induced by doxorubicin in mouse hearts. Cardiac-specific overexpression of GRP78 or the administration of the chemical ER chaperone 4-phenylbutyrate alleviated doxorubicin-induced cardiomyocyte apoptosis and cardiac dysfunction (Figure 7).

Doxorubicin Exerts Pleiotropic Effects on Cardiotoxicity

Doxorubicin induces cardiotoxicity through multiple mechanisms, including transcription inhibition, mitochondria damage, reactive oxygen species production, and Ca²⁺ disturbance. In addition, a recent study revealed that doxorubicin caused myofibrillar nitrilation, which inhibited myofibrillar force development and led to left ventricular dysfunction. Furthermore, Zhang et al. reported that the deletion of topoisomerase-IIβ protected mice from doxorubicin-induced cardiac dysfunction, suggesting that topoisomerase-IIβ...
mediates doxorubicin-induced cardiotoxicity. These studies indicate that doxorubicin has multiple toxic effects on cardiomyocytes. However, the therapeutic approach for doxorubicin-induced cardiomyopathy has not been established in the clinical practice.

**Doxorubicin Can Cause ER Dilation and ER Stress**

Doxorubicin has been shown to cause ER dilation in both human and mouse hearts,

indicating that ER dysfunction is involved in doxorubicin-induced cardiotoxicity. There are 2 possible mechanisms by which doxorubicin may induce ER stress. First, increased reactive oxygen species levels induced by doxorubicin can alter the oxidative environment in the ER, inducing ER stress.

Second, abnormal Ca²⁺ homeostasis induced by doxorubicin can cause ER stress.

Doxorubicin can disturb Ca²⁺ homeostasis by suppressing the expression of the Ca²⁺ ATPase pump and by directly activating ryanodine Ca²⁺ release channels, resulting in decreased Ca²⁺ levels in the ER and potentially leading to increased ER stress in cardiomyocytes.

To our knowledge, we are the first to show that doxorubicin activates ER transmembrane stress sensors in cardiomyocytes, that is, ATF6 and IRE1, but not protein kinase RNA-like ER stress sensors. Although these 3 ER transmembrane stress sensors are often activated at the same time on ER stress, recent studies have revealed that some of these sensors are specifically activated depending on the stimuli involved.

On ER stress, ATF6 is cleaved, and the cleaved ATF6 activates ER transmembrane stress sensors in cardiomyocytes, including ATF6 and IRE1 but not protein kinase RNA-like ER stress sensors. These studies indicate that doxorubicin has multiple toxic effects on cardiomyocytes.

In the present study, however, we found that doxorubicin suppressed the expression of genes downstream of ATF6, although it induced ATF6 cleavage in cardiomyocytes. First, doxorubicin may have bound to the DNA by intercalation and blocked the synthesis of DNA, including the transcription of genes downstream of ATF6. Second, doxorubicin may have induced transcription factors that bind to the same promoter region as the cleaved ATF6. For example, doxorubicin-induced p53 recognizes the same CCAAT (cytosine-cytosine-adenine-thymine) boxes that exist within the promoters of ATF6-induced genes and may have competitively suppressed ATF6-induced gene expression in this study. Further investigation is needed to elucidate the mechanism by which doxorubicin suppresses the expression of genes downstream of ATF6 in cardiomyocytes.

Doxorubicin decreased the mRNA level of XBP1 in the cardiomyocytes (Figures 1C and 2B), consistent with previous research in which the mRNA level of XBP1 was inhibited by doxorubicin in breast cancer cells. In addition, the sXBP1 mRNA levels decreased because of the suppression of XBP1 mRNA levels by doxorubicin (Figures 1D and 2C). sXBP1 overexpression increased the protein levels of GRP78 and attenuated doxorubicin-induced cardiomyocyte death (Figure 3). GRP78 knockdown blunted the protective effect of sXBP1 (Online Figure V), suggesting that the lack of GRP78 induction because of the decreased expression of sXBP1 may have increased doxorubicin-induced cardiotoxicity. The decreased sXBP1 failed to induce the expression of GRP94 and protein disulfide isomerase as well as GRP78 (Figures 1E and 2B).
Previous studies have shown that GRP78 and 4-phenylbutyrate (4-PBA) can assist with protein folding and reduce the extent of ER stress, thereby attenuating caspase-12 cleavage and cell death.21,30 In the development of heart failure,5,29 ER stress–initiated apoptotic signaling plays an important role in the development of heart failure. In the present study, we found that doxorubicin activated caspase-12 and JNK but not 4-PBA administration (100 mg/kg per day, right) attenuated doxorubicin-induced caspase-12 cleavage but not c-JUN NH2-terminal kinase (JNK) phosphorylation or C/EBP homologous protein (CHOP) induction in mouse hearts. Cardiac-specific overexpression of GRP78 using adeno-associated virus 9 (AAV9; left) or 4-PBA administration (100 mg/kg per day, left) attenuated doxorubicin-induced caspase-12 cleavage and reduced cardiomyocyte death in mouse hearts.

**Doxorubicin Induces ER Stress–Initiated Apoptotic Signaling**

ER stress–initiated apoptotic signaling plays an important role in the development of heart failure.32,52 In the present study, we found that doxorubicin activated caspase-12 and JNK but not CHOP (Figures 1F and 2E), suggesting that ER stress–initiated apoptosis signaling is involved in doxorubicin-induced cardiomyocyte apoptosis. Caspase-12 is an ER membrane–resident proapoptotic molecule.7 On ER stress, caspase-12 activates caspase cascades that primarily induce cell death.9 Previous studies have shown that GRP78 and 4-phenylbutyrate can assist with protein folding and reduce the extent of ER stress, thereby attenuating caspase-12 cleavage and cell death.31 Consistent with previous reports that the suppression of caspase-12 cleavage is associated with the improvement of cardiac function,32 we demonstrated that the cardiac-specific overexpression of GRP78 or 4-phenylbutyrate administration attenuated caspase-12 cleavage (Figure 6A), leading to reduced cardiomyocyte death (Figure 6B) and improved cardiac function (Table). Caspase-12 has been detected only in mouse tissues, and caspase-4 may be its functional counterpart in humans.33 The elucidation of mouse caspase-12 function may reveal the nature of caspase-4 in humans.

JNK is phosphorylated in both ER-dependent and ER-independent pathways.34 Although IRE1 is involved in JNK phosphorylation in an ER-dependent pathway, knockdown of IRE1 did not have significant effects on JNK phosphorylation induced by doxorubicin (Online Figure VI). In addition, neither the cardiac-specific overexpression of GRP78 nor 4-phenylbutyrate administration affected JNK activation.
Mild ER stress 

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LVEF, left ventricular ejection fraction; LVESD, left ventricular end-systolic dimension; LVFS, left ventricular fractional shortening; and 4-PBA, 4-phenylbutyrate.

| Parameters | Saline (n=6) | AAV9-GRP78 (n=6) | AAV9-Luciferase (n=5) | 4-PBA (n=7) | BS (n=7) | Doxorubicin+ AA V9-GRP78 (n=5) | Doxorubicin+ AAV9-Luciferase (n=6) | Doxorubicin+ 4-PBA (n=5) | Doxorubicin+ BS (n=5) |
|------------|-------------|-----------------|----------------------|------------|---------|---------------------------|-----------------------------|----------------|----------------|---|
| LVEDD, mm  | 3.43±0.12   | 3.55±0.10       | 3.50±0.13            | 3.34±0.14  | 3.20±0.24| 3.10±0.15                 | 3.27±0.11                   | 3.02±0.08      | 3.22±0.08      | 3.14±0.14 |
| LVESD, mm  | 1.48±0.05   | 1.55±0.03       | 1.55±0.04            | 1.46±0.10  | 1.41±0.09| 2.02±0.12                 | 1.75±0.1                   | 1.90±0.04      | 1.74±0.05      | 2.02±0.13 |
| LVFS, %    | 56.0±0.9    | 56.0±1.0        | 55.3±0.2             | 56.7±2.0   | 55.4±1.0 | 34.0±3.7                  | 45.7±1.5†                   | 36.6±2.0*      | 45.6±1.2†     | 35.6±1.4* |
| LVEF, %    | 90.7±0.6    | 90.7±0.8        | 90.3±0.2             | 90.6±0.8   | 90.6±0.8 | 69.2±5.8*                 | 82.8±1.4†                   | 73.0±2.5*      | 83.0±0.9†     | 72.0±2.0* |
| HR, bpm    | 594±25      | 558±51          | 595±14               | 617±28     | 601±53  | 554±20                    | 611±17                     | 605±22         | 533±21         | 566±18   |

AAV9 indicates adeno-associated virus 9; BS, sodium butyrate; GRP78, glucose-regulated protein 78; HR, heart rate; LVEDD, left ventricular end-diastolic dimension; LVESD, left ventricular end-systolic dimension; LVFS, left ventricular fractional shortening; and 4-PBA, 4-phenylbutyrate.

*P<0.05 vs doxorubicin group.
†P<0.05 vs doxorubicin+saline group.

(Figures 4B and 6A), suggesting that doxorubicin primarily induces JNK phosphorylation in an ER-independent manner. In addition to ER stress–initiated apoptotic signaling, we observed that doxorubicin increased the release of cytochrome C from the mitochondria to the cytosol (Online Figure X), suggesting that both the ER- and mitochondria-dependent apoptosis pathways are involved in cardiomyocyte apoptosis caused by doxorubicin.

In the present study, we found that the percentage of apoptotic cardiomyocytes increased from 0.3% to 2.1% in doxorubicin-administered mouse hearts (Figure 6B), which is consistent with previous reports using a similar stimulation with doxorubicin.35,36 Because cardiomyocytes are terminally differentiated cells without the capacity to divide, apoptosis, even at a low percentage, would lead to consistent loss of cardiomyocytes and result in cardiac dysfunction.37 Moreover, cardiomyocyte apoptosis seems to be quantitatively related to the clinical severity of deterioration in dilated cardiomyopathy.38 These results and our findings indicate cardiac apoptosis may play an important role in doxorubicin-induced cardiomyopathy.

Cardiac-Specific Overexpression of GRP78 or a Chemical ER Chaperone Reduces ER Stress and Alleviates Cardiac Apoptosis and Dysfunction Induced by Doxorubicin

Cardiac-specific overexpression of GRP78 or 4-phenylbutyrate administration partially but significantly decreased...
caspase-12 cleavage (Figure 6A), reduced cardiomyocyte death (Figure 6B), and alleviated doxorubicin-induced cardiac dysfunction (Table). Importantly, GRP78 overexpression or 4-phenylbutyrate administration did not affect cytochrome C release induced by doxorubicin (Online Figure X), suggesting that the GRP78 overexpression or 4-phenylbutyrate administration alleviated cardiac dysfunction by doxorubicin in an ER- but not a mitochondria-dependent manner. Because both GRP78 and 4-phenylbutyrate are known to reduce ER stress functioning as an ER chaperone, the potential mechanisms by which GRP78 overexpression or 4-phenylbutyrate ameliorated doxorubicin-induced cardiac apoptosis and dysfunction would be promoting protein folding. However, we need careful consideration about these results because of the multiple functions of GRP78 and 4-phenylbutyrate.

GRP78, the most abundant chaperone in the ER, plays a critical role in the adaptive responses to ER stress by promoting protein folding and maintaining ER transmembrane sensors. Accumulation of misfolded or unfolded proteins in the ER induces ER stress and causes the dissociation of GRP78 from ER transmembrane sensors including ATF6, leading to the sensor activation and the subsequent upregulation of GRP78 to resolve ER stress. Beside the function as an ER chaperone, GRP78 can preserve ER Ca$^{2+}$ homeostasis and it is recently assumed to have novel functions such as control proliferation, apoptosis, and immunity. Further investigation will be required to clarify whether effects of GRP78 other than an ER chaperone are involved in its cardioprotective effects against doxorubicin-induced cardiomyopathy.

In addition to the function as a chemical ER chaperone, 4-phenylbutyrate, an aromatic fatty acid, has other effects such as waste nitrogen excretion by forming phenylacetylglutamine and histone acetylation by inhibiting histone deacetylase. 4-Phenylbutyrate is used to treat patients with urea cycle disorders. 4-Phenylbutyrate is metabolized in kidney and liver to phenylacetate that can form phenylacetylglutamine by conjugating with glutamine, which makes it an alternative pathway to urea for waste nitrogen excretion. Because 4-phenylbutyrate effectively reduced doxorubicin-induced cardiotoxicity in cultured cardiomyocytes (Figure 4C right), 4-phenylbutyrate may exert cardioprotective effects against doxorubicin-induced cardiomyopathy independent of nitrogen excretion. Daosukho et al showed that a high dose of 4-phenylbutyrate (400 mg/kg per day) upregulated the antioxidant enzyme manganese superoxide dismutase with increased histone acetylation activity and protected against doxorubicin-induced cardiac injury. They concluded that 4-phenylbutyrate may exert the cardiac protection against doxorubicin as a histone deacetylase inhibitor. On the contrary, another study demonstrated that 4-phenylbutyrate (200 mg/kg per day) reversed defects in associative learning in a mouse model of Alzheimer disease by functioning as both an ER chaperone and a histone deacetylase inhibitor. Thus, it is likely that a high dose of 4-phenylbutyrate (400 mg/kg per day) can function as an ER chaperone as well as a histone deacetylase inhibitor. In the present study, we used 4-phenylbutyrate at 100 mg/kg per day, which has been reported to be beneficial for the treatment of cerebral ischemia and aortic stiffening as a chemical ER chaperone. Consistently, we found that 4-phenylbutyrate (100 mg/kg per day) protected against doxorubicin-induced cardiomyopathy without histone acetylation (Table; Online Figure VIIIIB). These findings suggest that a low dose of 4-phenylbutyrate used in the present study may ameliorate doxorubicin-induced cardiac dysfunction through an ER- but not a histone acetylation-dependent mechanism. Moreover, another chemical ER chaperone, tauroursodeoxycholic acid also protected cardiomyocyte from doxorubicin (Online Figure IX), suggesting that the chemical ER chaperone other than 4-phenylbutyrate or tauroursodeoxycholic acid can also ameliorate cardiac dysfunction by doxorubicin.

**Doxorubicin Affects Protein Quality Control in Cardiomyocytes**

Protein quality control plays an important role in the pathophysiology of cardiovascular diseases. The proper functioning of the protein quality control system relies on protein folding mediated by chaperones in the ER and the degradation of misfolded protein by autophagy or the ubiquitin proteasome system. In addition to ER stress, doxorubicin induces autophagy and lysosome formation (Online Figure IIC and IID) and ubiquitin proteasome system has been reported to be activated by doxorubicin in cardiomyocytes. Although it is not yet fully understood whether autophagy and ubiquitin proteasome system are adaptive or maladaptive responses for doxorubicin-induced cardiomyopathy, doxorubicin has a strong impact on protein quality control in cardiomyocytes, which is involved in the pathophysiology of cardiac dysfunction. Further investigation is needed to clarify how the ER, autophagy, and ubiquitin proteasome system interact with each other and to determine their potential role in doxorubicin-induced cardiomyopathy.

**Conclusions**

We demonstrated that ER stress plays an important role in doxorubicin-induced cardiotoxicity. The cardiotoxicity overexpression of GRP78 or 4-phenylbutyrate administration reduces cardiomyocyte apoptosis and alleviates cardiac dysfunction induced by doxorubicin. Considering that 4-phenylbutyrate is already used in the treatment of urea cycle disorders in clinical settings, 4-phenylbutyrate represents a safe and promising candidate for the treatment of doxorubicin-induced cardiomyopathy. Future “proof-of-concept” clinical trials are strongly warranted.

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**Disclosures**

None.
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What Is Known?

- Doxorubicin causes the cumulative dose-dependent cardiotoxicity.
- Endoplasmic reticulum (ER) stress contributes to cardiac pathology, including hypertrophy and ischemia/reperfusion injury.
- ER chaperones can prevent cardiomyocyte death induced by ER stress.

What New Information Does This Article Contribute?

- Doxorubicin activated the ER stress–initiated apoptotic response without inducing the ER chaperone protein, glucose-regulated protein 78, which plays a major role in adaptive response to ER stress.
- The cardiac-specific overexpression of glucose-regulated protein 78 attenuated doxorubicin-induced cardiomyocyte death and cardiac dysfunction.
- The chemical chaperone 4-phenylbutyrate also attenuated doxorubicin-induced cardiomyocyte death and cardiac dysfunction.

Novelty and Significance

Doxorubicin is widely used for cancer treatment, but its application is often limited by the cumulative dose-dependent cardiotoxicity. Although several mechanisms have been proposed to account for doxorubicin-induced cardiotoxicity, the role of ER stress has not been elucidated. We found that in mouse hearts, doxorubicin activates an ER transmembrane stress sensor, activating transcription factor 6, but it suppresses the expression of genes downstream of activating transcription factor 6, such as X-box binding protein 1, which leads to failure to induce ER chaperone glucose-regulated protein 78. In addition, doxorubicin activates caspase-12, an ER membrane–resident apoptotic molecule, which can lead to cardiomyocyte apoptosis and cardiac dysfunction. Cardiac-specific overexpression of glucose-regulated protein 78 or the administration of the chemical chaperone 4-phenylbutyrate reduced ER stress and attenuated cardiomyocyte death and cardiac dysfunction induced by doxorubicin. Thus, the therapy targeting ER stress might be a new cardioprotective approach for reducing cardiotoxicity from doxorubicin. The chemical chaperone, 4-phenylbutyrate, which is already in clinical use, represents a safe and promising candidate for the treatment of doxorubicin-induced cardiomyopathy.
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Hai Ying Fu, Shoji Sanada, Takashi Matsuzaki, Yulin Liao, Keiji Okuda, Masaki Yamato, Shotaro Tsuchida, Ryo Araki, Yoshihiro Asano, Hiroshi Asanuma, Masanori Asakura, Brent A. French, Yasushi Sakata, Masafumi Kitakaze and Tetsuo Minamino

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Supplemental Materials

Materials
Doxorubicin and sodium butyrate (BS) were purchased from Sigma (St Louis, MO, USA) and Wako Pure Chemical Industries (Osaka, Japan), respectively. 4-phenylbutyrate (4-PBA) and tauroursodeoxycholic acid (TUDCA) were from BIOMOL International (Plymouth Meeting, PA, USA). The antibodies for CHOP, ATF6, XBP1, ATP5α, HSP70, and HSP90 were obtained from Santa Cruz (Santa Cruz, CA, USA). The antibodies for luciferase, α-actinin, Troponin I, and acetyl-histone H3 were from Abcam (Tokyo, Japan). The antibodies for GRP78 and cytochrome C were from BD Biosciences (Tokyo, Japan). The antibodies for GRP94, PDI, P-JNK, JNK, P-PERK, PERK, IRE1, and histone H3 were obtained from Cell Signaling Technology (Danvers, MA, USA). The antibodies for caspase-12, P-IRE1, and GAPDH were obtained from Sigma, Novus Biologicals (Littleton, CO, USA), and Millipore (Billerica, MA, USA), respectively. Z-ATAD-FMK, an inhibitor of caspase-12, was purchased from Biovision (Milpitas, CA, USA).

Electron microscopy
Hearts were excised under anesthesia and perfused with 2.5% glutaraldehyde and 1N Phosphate buffered saline (PBS). Small tissue blocks (1 mm³) were cut out, rinsed in 0.1M PBS (pH 7.4), and postfixed in 2% osmium tetroxide. Then they were dehydrated in alcohol and infiltrated overnight with epoxy resin. The samples were cured for 48 hours at 60°C. Ultrathin sections were cut at 60 nm by ultramicrotome (Reichert UltracutN, Vienna, Austria) and stained with uranyl acetate and lead citrate. Then, the samples were examined under an electron microscopy (H-7650, Hitachi, Tokyo, Japan).

Western blot analysis
Neonatal rat cardiomyocytes or mouse hearts 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 (Nacalai Tesque, Kyoto). Electrophoresis, immunoblotting, and detection were performed as described previously. 1 Protein levels were quantified with ImageJ and normalized to the control.

Real-time quantitative-PCR
Real-time quantitative PCR was performed with an ABI PRISM 700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Primer pairs are listed
in the table below. Melting curve analysis was performed to ensure purity of the PCR products and relative quantification was determined using the comparative CT method with data normalized to GAPDH.

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Preparation of neonatal rat cardiomyocytes
Primary cardiomyocyte cultures were prepared from neonatal rat hearts as described previously.²

Adenovirus transduction
Recombinant adenovirus harboring human spliced XBP1 gene or LacZ gene was constructed as described previously.³ Adenovirus was transfected 24 hours after
cardiomyocyte isolation at a multiplicity of infection (MOI) of 60 for XBP1 and LacZ as optimized in our pilot studies (data not shown), and the experiments were performed after an additional 24 hours.

**Immunofluorescence**
Cultured cardiomyocytes were stained with XBP1 antibody (1:100) and DAPI. F-actin was labelled with Rhodamine Phalloidin (Life Technologies, Tokyo, Japan, 1:1000). Cells were observed with confocal laser microscopy (Olympus, Tokyo, Japan) Alexa488 (green) was captured by Argon laser (wavelength 488 nm laser line) with band path 500–550 IR filter (500–550 nm excitation). Rhodamine was scanned by helium/neon laser (wavelength 543 nm laser line) with long path 590 filter (560–700 nm excitation). DAPI (blue) for nuclei staining of all cells was obtained in range of 400–470 nm excitation.

**Cell viability**
Cardiomyocytes were seeded at 3 x10⁴/well in 96-well plates. After drug treatment at appropriate conditions for 24 hours, cell numbers were measured by microplate reader (Corona, Ibaraki, Japan) with a water-soluble tetrazolium reagent [WST-8; 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulphophenyl) 2H-tetrazolium, monosodium salt] (Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer’s instructions. The wavelengths used in this assay were 450 nm (sample) and 630 nm (reference). Data for cell viability were normalized to the control.

**Luciferase activity**
Luciferase activity was measured using a Firefly Luciferase Flash Assay Kit (Thermo Fisher Scientific, MA, USA). Briefly, cell lysate was harvested with lysis buffer, luciferase assay regent was added to cell lysate and luciferase activity was measured using luminescent microplate reader (Berthold Technologies, Germany) according to the manufacturer’s protocol. Data for luciferase activity were expressed as relative light units per µg of cell proteins (RLU/µg).

**Caspase-12 activity**
Caspase-12 activity was measured using a Fluorometric Assay Kit (BioVision). Briefly, cell lysate was harvested with lysis buffer. The substrate, ATAD-7-amino-4-trifluoromethyl coumarin, was added to cell lysate and incubated at 37°C in reaction buffer for 1 hour. Subsequently, the fluorescence was measured by fluorescence
microplate reader (Corona) with 400 nm excitation and 505 nm emission filter according to the manufacturer’s protocol. Data for caspase-12 activity were normalized to the control.

**siRNA Knockdown**

siRNA knockdown was performed as described previously. Cardiomyocytes were seeded in 6-well or 96-well plates at $2 \times 10^6$ or $3 \times 10^4$ cells per well, respectively. IRE1 or GRP78 siRNA was added 4 hours after cardiomyocyte isolation at 5 nmol/L with RNAi Max (Invitrogen, Carlsbad, CA). Nontargeting scrambled siRNAs were used as a negative control (Bioneer, Korea). Experiments were performed after an additional 24 hours.

**Immunohistological analysis**

Immunohistological analysis was performed as described previously. Briefly, harvested cardiac tissues were fixed with 4% paraformaldehyde. For GRP78 immunofluorescence analysis, the fixed cardiac tissue were perfused with 30% sucrose, and frozen on dry ice. Then, the frozen tissue were sectioned at 4-μm thickness and subsequently stained with GRP78 (1: 50) antibody, Troponin I (1:200) antibody, and DAPI. The intensity of GRP78 immunofluorescence was quantified using ImageJ. The average intensity of GRP78 immunofluorescence was obtained from 3 areas of Troponin I-negative and Troponin I-positive cells in the same section, respectively. The relative intensity of GRP78 immunofluorescence was obtained by dividing the average intensity of GRP78 immunofluorescence in Troponin I-positive cells by that in Troponin I-negative ones. For hematoxylin and eosin (HE) and masson’s trichrome (MTC) staining, the fixed cardiac tissue were embedded in paraffin, sectioned at 4-μm thickness, and stained with hematoxylin, eosin, and Weigert's iron hematoxylin, respectively.

**TUNEL assay**

The terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay was performed using an ApopTag Peroxidase In Situ Apoptosis Detection Kit (Millipore). The heart sections were treated according to the manufacturer’s instructions and co-stained with cardiomyocyte specific marker Troponin I (1:200) and DAPI. The number of TUNEL-positive cells was expressed as a percentage of troponin I-positive cells, as previously described.
Echocardiography
Transthoracic echocardiography was performed with a Sonos 4500 and a 15–6 L MHz transducer (Philips, Netherlands). Mice were anesthetized with inhaled isoflurane and placed in a supine position. And then, echocardiography was performed after being awake. Two-dimensional short-axis views of the left ventricle (LV) were obtained for guided M-mode measurement of LV end-diastolic diameter (LVEDD) and LV end-systolic diameter (LVESD). LV fractional shortening (LVFS) was calculated as follows: (LVEDD – LVESD)/LVEDD × 100. LV ejection fraction (LVEF) was calculated as follows: \[\frac{[(LVEDD)^{3} – (LVESD)^{3}]/(LVEDD)^{3}}{LVEDD} \times 100.\]
References


Online Figure Legends

Online Figure I. In vivo experimental protocol.
(A) One-week-old mice received a single intravenous injection of saline or AAV9 expressing GRP78 or luciferase. Nine weeks later, the mice received a single intraperitoneal injection of saline or doxorubicin (15 mg/kg). After 6 days, the mice were subjected to echocardiography, and heart samples were obtained for western blotting and histological analysis. (B) Ten-week-old mice received a single intraperitoneal injection of saline or doxorubicin (15 mg/kg). They also received daily intraperitoneal injections of saline, 4-phenylbutyrate (4-PBA) (100 mg/kg), or sodium butyrate (BS) (100 mg/kg) once a day for one week. The mice were then subjected to echocardiography, and heart samples were obtained for western blotting and histological analysis.

Online Figure II. Doxorubicin induced mitochondria alteration and the formation of autophagosome and lysosome in mouse hearts.
(A) Electron microscopy analysis of mouse hearts at 6 days after the administration of saline. (B-D) Electron microscopy analysis of mouse hearts at 6 days after the administration of doxorubicin (15 mg/kg). Doxorubicin caused degeneration of mitochondria with disorganized cristae (B, arrow) and induced autophagosome (C, arrow) and lysosome formation (D, arrow) in mouse hearts. Scale bar indicates 1.0 µm.

Online Figure III. Doxorubicin failed to induce the expressions of GRP94 and protein disulfide isomerase (PDI) in cultured cardiomyocytes.
(A) GRP94 and PDI expressions in mouse hearts at 6 days after the administration of saline or doxorubicin (15 mg/kg) (B) GRP94 and PDI expressions in cultured cardiomyocytes. Cardiomyocytes were treated with doxorubicin at the indicated doses for 24 hours. The graphs summarize data from 3 mice per group in A and 3 independent experiments in B, respectively.

Online Figure IV. Doxorubicin increased caspase-12 activity in a dose-dependent manner and Z-ATAD-FMK, an inhibitor of caspase-12, suppressed caspase-12 cleavage, caspase-12 activity, and cardiomyocyte death induced by doxorubicin.
(A) Doxorubicin dose-dependently increased caspase-12 activity in cultured cardiomyocytes. (B) Z-ATAD-FMK, an inhibitor of caspase-12, dose-dependently suppressed caspase-12 cleavage induced by doxorubicin. (C) Z-ATAD-FMK inhibited
caspase-12 activity induced by doxorubicin. (D) Z-ATAD-FMK increased cell viability in cultured cardiomyocytes treated with doxorubicin (n=6 wells per group). Cardiomyocytes were treated with doxorubicin at the indicated doses (A) or 1.0 µmol/L (B, C and D) for 24 hours. Z-ATAD-FMK was added 1 hour before doxorubicin treatment at the indicated doses (B) or 1.0 µmol/L (C and D). Tunicamycin (Tu): the positive control sample obtained from cardiomyocytes treated with Tu (1.0 mg/L), a pharmacological ER stress inducer, for 24 hours. The graphs summarize data from 3 independent experiments. *P < 0.05 vs. Control group, †P < 0.05 vs. Doxorubicin group.

**Online Figure V.** GRP78 knockdown suppressed the protective effect of sXBP1 on cardiomyocyte death induced by doxorubicin.

(A) The knockdown efficiency of three different GRP78 siRNA and control siRNA was checked by real-time PCR analysis. GRP78 siRNA-1 most efficiently knocked down GRP78 at mRNA levels. (B) GRP78 siRNA-1 efficiently knocked down GRP78 at protein level, when sXBP1 was overexpressed in cultured cardiomyocytes. sXBP1 overexpression attenuated caspase-12 cleavage in cultured cardiomyocytes treated with doxorubicin, which was blunted by GRP78 knockdown. (C) sXBP1 overexpression increased cell viability in cultured cardiomyocytes treated with doxorubicin, which was blunted by GRP78 knockdown (n=6 wells per group). Cardiomyocytes were treated with doxorubicin (1.0 µmol/L) for 24 hours. The graphs summarize data from 3 independent experiments. *P < 0.05 vs. Control group, †P < 0.05 vs. Doxorubicin group.

**Online Figure VI.** IRE1 knockdown did not prevent JNK phosphorylation in cultured cardiomyocytes.

(A) The knockdown efficiency of three different IRE1 siRNA and control siRNA was checked by real-time PCR analysis. IRE1 siRNA-1 most efficiently knocked down IRE1 at mRNA levels. (B) IRE1 siRNA-1 efficiently knocked down IRE1 at protein levels, but not changed the extent of JNK phosphorylation. Cardiomyocytes were treated with doxorubicin (1.0 µmol/L) for 24 hours. The graphs summarize data from 2 or 3 independent experiments. *P < 0.05 vs Control group.
Online Figure VII. Cardiac-specific overexpression of GRP78 did not change the protein level of HSP70 or HSP90 in either cultured cardiomyocytes or mouse hearts.

(A) HSP70 and HSP90 expressions in cultured cardiomyocytes. AAV9-GRP78 or AAV9-luciferase (3 ×10^{10} vg) was transfected 24 hours after cardiomyocyte isolation. The experiments were performed after an additional 24 hours. (B) HSP70 and HSP90 expressions in mouse hearts at 9 weeks after saline, AAV9-GRP78, or AAV9-luciferase (1 x 10^{11} vg per mouse) administration. The graphs summarize data from 3 independent experiments in A and 3 mice per group in B, respectively.

Online Figure VIII. Neither 4-PBA nor sodium butyrate (BS) induced histone acetylation at the indicated doses in cultured cardiomyocytes or mouse hearts.

(A) Histone acetylation in cultured cardiomyocytes. 4-PBA or BS was added at 0.5 mmol/L. Cardiomyocytes were treated with doxorubicin (1.0 µmol/L) for 24 hours. (B) Histone acetylation in mouse hearts at 6 days after the administration of saline or doxorubicin (15 mg/kg). 4-PBA or BS was administrated at 100 mg/kg/day for 1 week. The graphs summarize data from 3 independent experiments in A and 3 mice per group in B, respectively. PC: positive control sample obtained from cardiomyocytes treated with BS (5.0 mmol/L) for 24 hours. *P < 0.05 vs Control group.

Online Figure IX. Tauroursodeoxycholic acid (TUDCA) treatment attenuated doxorubicin-induced caspase-12 cleavage and reduced cell death in cultured cardiomyocytes.

(A) Treatment with TUDCA (0.5 mmol/L) attenuated caspase-12 cleavage induced by doxorubicin. (B) TUDCA treatment increased cell viability in cultured cardiomyocytes treated with doxorubicin (n=6 wells per group). Cardiomyocytes were treated with doxorubicin (1.0 µmol/L) for 24 hours. The graphs summarize data from 3 independent experiments. *P < 0.05 vs. Control group, †P < 0.05 vs. Doxorubicin group.

Online Figure X. Neither cardiac-specific overexpression of GRP78 nor 4-PBA treatment affected doxorubicin-activated mitochondria-dependent pathways.

(A) The release of cytochrome C (CytC) from mitochondria (mit) to cytosol (cyt) in cultured cardiomyocytes. AAV9-GRP78 or AAV9-luciferase was transfected at 3 ×10^{10} vg 24 hours after cardiomyocyte isolation. The experiments were performed after an additional 24 hours. (B) The release of CytC from mitochondria to cytosol after the treatment with 4-PBA (0.5 mmol/L) or sodium butyrate (BS) (0.5 mmol/L) in cultured
cardiomyocytes. GAPDH and ATP5α were used as loading control for cytosolic and mitochondrial protein, respectively. Cardiomyocytes were treated with doxorubicin (1.0 µmol/L) for 24 hours. The graphs summarize data from 3 independent experiments. *P < 0.05 vs Control group.

Online Figure XI. Cardiac-specific overexpression of GRP78 or 4-PBA administration reduced cardiomyocyte death in mouse hearts.

Immunohistological analysis showed TUNEL-positive cells in cardiomyocytes. TUNEL-positive nuclei, DAPI-stained nuclei, and cardiomyocytes labeled with antibody to Troponin I were stained green, blue, and red, respectively. Scale bar indicates 100 µm. Arrowheads indicate TUNEL-positive cardiomyocytes.
Online Figure I

A

Saline  Saline

AAV9-GRP78  Saline

AAV9-luciferase  Saline

Saline  Doxorubicin

AAV9-GRP78  Doxorubicin

AAV9-luciferase  Doxorubicin

0  1  10  11  (Weeks old)

• Echocardiography
• Western blot
• Histological analysis

B

Saline

Saline

Saline

4-PBA

BS

Doxorubicin

Saline

Doxorubicin

4-PBA

BS

0  10  11  (Weeks old)

• Echocardiography
• Western blot
• Histological analysis
Online Figure II

A

B

C

D
Online Figure III

A. Mouse hearts

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B. Cultured cardiomyocytes

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Control Doxorubicin
Online Figure IV

A

![Graph showing relative caspase-12 activity vs. doxorubicin concentration](image)

B

![Graph showing pro-caspase-12 levels](image)

C

![Graph showing relative caspase-12 activity vs. Z-ATAD-FMK and doxorubicin](image)

D

![Graph showing relative cell viability](image)
Online Figure V

A

![Bar chart showing relative expression of GRP78.](image)

B

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C

![Bar chart showing relative cell viability.](image)

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* indicates statistical significance compared to control
† indicates statistical significance compared to siGRP78
Online Figure VI

A

B

siIRE1  --  --  +  --  --  +
siCTL   --  +  --  --  +  --
Doxorubicin --  --  +  +  +  +

IRE1  
GAPDH
p-JNK
JNK

Relative expression
IRE1

Doxorubicin  siCTL  siIRE1  siRNA-1  siRNA-2  siRNA-3

0  0.2  0.4  0.6  0.8  1.0  1.2

0  0.5  1.0  1.5

0  1  2  3

siIRE1  --  --  +  --  --  +
siCTL   --  +  --  --  +  --
Doxorubicin --  --  +  +  +  +
Online Figure VII

A. Cultured cardiomyocytes

B. Mouse hearts
Online Figure VIII

A. Cultured Cardiomyocytes

B. Mouse Hearts

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Online Figure IX

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Doxorubicin + TUDCA

pro-caspase-12

GAPDH

Relative cell viability
Online Figure X

A

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<td>Doxorubicin</td>
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<td>+</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

B

<table>
<thead>
<tr>
<th>Treatment</th>
<th>CytC (cyt)</th>
<th>GAPDH</th>
<th>CytC (mit)</th>
<th>ATP5aα</th>
</tr>
</thead>
<tbody>
<tr>
<td>BS</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>4-PBA</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Doxorubicin          | -          | +     | -          | +      |
Online Figure XI

<table>
<thead>
<tr>
<th></th>
<th>TUNEL</th>
<th>Troponin I</th>
<th>DAPI</th>
<th>Merge</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Control</strong></td>
<td><img src="image1" alt="TUNEL" /></td>
<td><img src="image2" alt="Troponin I" /></td>
<td><img src="image3" alt="DAPI" /></td>
<td><img src="image4" alt="Merge" /></td>
</tr>
<tr>
<td><strong>Doxorubicin</strong></td>
<td><img src="image5" alt="TUNEL" /></td>
<td><img src="image6" alt="Troponin I" /></td>
<td><img src="image7" alt="DAPI" /></td>
<td><img src="image8" alt="Merge" /></td>
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<tr>
<td><strong>Doxorubicin + AAV9-GRP78</strong></td>
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<td><img src="image10" alt="Troponin I" /></td>
<td><img src="image11" alt="DAPI" /></td>
<td><img src="image12" alt="Merge" /></td>
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<tr>
<td><strong>Doxorubicin + AAV9-luciferase</strong></td>
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<td><img src="image14" alt="Troponin I" /></td>
<td><img src="image15" alt="DAPI" /></td>
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<td><strong>Doxorubicin + PBA</strong></td>
<td><img src="image17" alt="TUNEL" /></td>
<td><img src="image18" alt="Troponin I" /></td>
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<td><strong>Doxorubicin + BS</strong></td>
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<td><img src="image23" alt="DAPI" /></td>
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