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ORIGINAL ARTICLE

Regulation of GADD153 induced by mechanical stress in cardiomyocytes

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ABSTRACT

Background Growth arrest and DNA damage-inducible gene 153 (GADD153), an apoptosis regulated gene, increased during endoplasmic reticulum stress. However, the expression of GADD153 in cardiomyocytes under mechanical stress is little known. We aimed to investigate the regulation mechanism of GADD153 expression and apoptosis induced by mechanical stress in cardiomyocytes.

Materials and methods Aorta-caval shunt was performed in adult Sprague–Dawley rats to induce volume overload. Rat neonatal cardiomyocytes grown on a flexible membrane base were stretched by vacuum to 20% of maximum elongation, at 60 cycles min^{-1}.

Results The increased ventricular dimension measured using echocardiography in the shunt group (n = 8) was reversed to normal by treatment with chaperon 4-phenylbutyric acid (PBA) (n = 8) at 500 mg kg^{-1} day^{-1} orally for 3 days. GADD153 protein and mRNA were up-regulated in the shunt group when compared with sham group (n = 8). Treatment with PBA reversed the protein of GADD153 to the baseline values. The TUNEL assay showed that PBA reduced the apoptosis induced by volume overload. Cyclic stretch significantly increased GADD153 protein and mRNA expression after 14 h of stretch. Addition of c-jun N-terminal kinase (JNK) inhibitor SP600125, JNK small interfering RNA and tumour necrosis factor-α (TNF-α) antibody 30 min before stretch, reduced the induction of GADD153 protein. Stretch increased, while GADD153-Mut plasmid, SP600125 and TNF-α antibody abolished the GADD153 promoter activity induced by stretch. GADD153 mediated apoptosis induced by stretch was reversed by GADD153 siRNA, GADD153-Mut plasmid and PBA.

Conclusions Mechanical stress enhanced apoptosis and GADD153 expression in cardiomyocytes. Treatment with PBA reversed both GADD153 expression and apoptosis induced by mechanical stress in cardiomyocytes.

Keywords Cardiomyocytes, GADD153, mechanical stress, pressure overload, volume overload.

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Introduction

Cardiac hypertrophy, which occurs in response to increased mechanical load on the heart in the form of volume overload, is characterized by increased cell size, enhanced protein synthesis, and re-expression of foetal genes, including atrial natriuretic factor. Initially, hypertrophy is considered compensatory; however, it may activate signalling events leading to heart failure. Hypertrophic heart may lead to dilated cardiomyopathy, and finally leads to congestive heart failure after sustained overload [1]. Cardiac apoptosis may be an important factor during the transition from compensatory hypertrophy to heart failure [2].

Cardiomyocytes apoptosis has been reported in a variety of cardiovascular disease, including ischaemia/reperfusion, end-stage heart failure, myocardial infarction, right ventricular dysplasia and cardiomyopathy [3,4]. However, the role of cardiomyocytes apoptosis in the progression of cardiac disease remains controversial [5]. Therefore, the possibility of reducing cardiomyocytes loss by inhibiting apoptosis has potentially important implications in the treatment of heart failure [6].

Endoplasmic reticulum (ER) is considered as an organelle that participates in the folding of membrane and secretory proteins [7]. It has been demonstrated that the ER develops markedly in hypertrophic and failing hearts [8]. The synthesis of secretary proteins, such as brain natriuretic peptide (BNP), is up-regulated in hypertrophic and failing hearts [9]. When the acceleration of protein synthesis is prolonged, cells adapt to the increased protein synthesis load by the development of the ER,

which involves coordinated expression lots of genes encoding ER proteins [10]. Thus, the development of the ER in hypertrophic and failing hearts may implicate the compensatory response to the up-regulated protein synthesis. Another emerging function of the ER is to modulate apoptosis [11]. Protein folding in the ER is impaired under various physical and pathological conditions, called ER stress [12]. To overcome the ER stress, ER has a specific signal pathway termed the unfolding protein response (UPR). ER stress can not be reversed by UPR as the stress is excess and/or prolonged. The expression of C/EBP homologous protein (CHOP), also known as growth arrest and DNA damage inducible protein 153 (GADD153) is elevated and the initiation of the apoptotic processes begins [13]. The CCAAT/enhancer-binding protein (C/EBP) family of transcription factors play an important role in regulating the expression of various genes. CHOP is a small nuclear protein that heterodimerizes avidly with members of the C/EBP family of transcription factors.

The expression of GADD153 in cardiomyocytes under mechanical stress is little known. Sodium 4-phenlybutyrate (PBA) is a low molecular weight fatty acid that acts as a chemical chaperone reducing the load of mutant or unfolded proteins retained in the ER during cellular stress [14]. It is not known whether PBA could reduce GADD153 in cardiomyocytes after mechanical stress. Therefore, the aim of this study was to test whether GADD153 and apoptosis was induced by the aorta-caval (AV) shunt model in rat myocardium. Besides, we determined the molecular regulation of GADD153 in cardiomyocytes apoptosis induced by mechanical stretch. We also used chaperon PBA to inhibit the GADD153 expression and apoptosis under mechanical stretch.

Materials and methods

Rat model of aorta-caval shunt

On the day of surgery, the Sprague–Dawley rats weighing 280–330 g were anaesthetized with pentobarbital sodium (80 mg kg^{-1}) and the vena cava and aorta were exposed via abdominal midline incision. For volume overload study, rats were randomly divided into four groups (i) sham-operated and treatment with vehicle, (ii) sham-operated and treatment with PBA, (iii) volume-overloaded (AV shunt) and treatment with vehicle and (iv) volume-overloaded and treatment with PBA. AV shunt was performed as previously described [15]. In the treatment group, the rats were given 500 mg kg^{-1} body weight per day of PBA in dinking water after surgery for 3 days. For the sham group, drinking water was used as vehicle. All animal procedures were performed in accordance with institutional guideline and conformed to *Guide for the Care and Use of Laboratory Animals* as published by the US National Institutes of Health.

Haemodynamic monitor

Haemodynamic monitor of rats was performed with polyethylene catheters to measure through a Grass model tachograph preamplifier as described previously [15].

Assessment of cardiac hypertrophy and function

Cardiac function of rats was evaluated non-invasively by echocardiography performed with an Acuson Sequoia 512 machine (Siemens Medical Solutions, Mountain View, CA, USA) using a 15-MHz probe at the day of killing, 3 days (AV shunt) after the surgery as described previously [15]. The sonographer was blinded to the randomization of rats.

Cardiomyocytes culture

Cardiomyocytes were obtained from Sprague–Dawley rats aged 2–3 days old by trypsinization, as previously described [16]. Cultured cardiomyocytes thus obtained were > 95% pure as revealed by observation of contractile characteristics with a light microscope and stained with anti-desmin antibody (Dako Cytomation, Glostrup, Denmark). Cardiomyocytes were seeded on flexible membranes base of six culture wells at a cell density of 5×10^5 cells per well in Ham's F-10 containing 20% foetal calf serum. After 3 days in culture, cells were transferred to serum-free medium (Ham's F-10) and subjected to cyclic stretch.

In vitro cyclic stretch on cultured cardiomyocytes

The strain unit Flexcell FX-4000 (Flexcell International Co., Hillsborough, NC, USA) consists of a vacuum unit linked to a valve controlled by a computer program. Cardiomyocytes cultured on the flexible membrane base were subjected to cyclic stretch produced by this computer-controlled application of sinusoidal negative pressure as characterized and described in detail previously [16]. A frequency of 1 Hz (60 cycles per min) was used for cyclic stretch. To determine the roles of c-Jun N-terminal kinase (JNK), p38 mitogen-activated protein (MAP) kinase or p44 MAP kinase in the expression of stretch-induced GADD153 expression, cardiomyocytes were pretreated with SP600125 (20 μM; Calbiochem, San-Diego, CA, USA), SB203580 (3 μM, Calbiochem), or PD98059 (50 μM, Calbiochem) for 30 min, respectively, followed by cyclic stretch. SP600125 is a potent, cell-permeable, selective and reversible inhibitor of JNK. SB203580 is a highly specific, cell permeable inhibitor of p38 kinase. PD98059 is a specific and potent inhibitor of ERK kinase. The pretreatment of PBA and tauroursodeoxycholic acid (TUDCA), a hydrophilic bile acid derivative, 24 h before stretch were 1 mM (Sigma, St Louis, MO, USA) and 0·1 mM (Sigma) respectively.

Western blot analysis

Western blot was performed as previously described [16]. Mouse monoclonal anti-GADD153 antibody (1 : 200; Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used. Equal protein loading of the samples was verified by staining monoclonal antibody β-actin. All Western blots were quantified using densitometry.

Real-time PCR

The real-time PCR was performed as described previously [17]. The primers used were as follows: TNF-α, 5′-d(CCCACGTCGTAGCAAAC)-3′ (forward) and 5′-d(CGGACTCCGTGATGTC)-3′ (reverse); GAPDH, 5′-d(CATCACCATCTTCCA GGAGC) (forward) and 5′-d(GGATGATGTTCTGGGCTGCC)-3′ (reverse).

Electrophoretic mobility shift assay

Nuclear protein concentrations from cultured cardiomyocytes were determined using Bio-Rad protein assay (Bio-Rad Lab Inc., Hercules, CA, USA). Consensus and control oligonucleotides (Santa Cruz Biotechnology Inc.) were labelled by polynucleotides kinase incorporation of [γ^{32}-p] ATP. Oligonucleotides sequences included the activating factor 1 (AP-1) consensus 5′-CGCTTGATGACTCAGCCGGAA-3′. The AP-1 mutant oligonucleotides sequences were 5′-CGCTTGATGACTTGGCCGGAA-3′. Electrophoretic mobility shift assay (EMSA) was performed as described previously [18]. Controls were performed in each case with mutant oligonucleotides or cold oligonucleotides to compete with labelled sequence.

RNA interference

Cardiomyocytes were transfected with 800 ng GADD153 annealed siRNA oligonucleotide or siRNA of JNK1 (Dharmacon Inc., Lafayette, CO, USA). GADD153 siRNA is a target-specific 21 nt siRNA according to a computer program provided by Dharmacon. The GADD153 targeted base sequences were sense: 5′-GGUAUGA GGAUCUGCAGGAUU; antisense: 5′-P.UCCUGCAGAUCCUCAUACCUU and JNK1 siRNA were sense: 5′-CGUGGAUUUAUGGUCUGUGdTdT-3′; antisense: 5′-CACAGACCAUAAAUCCACCdTdT-3′. For negative control, a dsRNAi was purchased from Dharmacon. After overnight incubation, cells were stretched and subjected to analysis using Western blot, EMSA, immunohistochemistry and detection of apoptosis.

Promoter activity assay

A −845 to +85 bp rat GADD153 promoter construct was generated as follows. Rat genomic DNA was amplified with forward primer, CTCGAGGAAGGGCA TAAGAGCATCA and reverse primer, CCGCTTCTCCTCAGGTTCCGGCTGT. The GADD153 promoter contains AP-1 conserved sites (TGACTCA) at −246 to −240 bp. For the mutant, the AP-1 binding sites were mutated using the mutagenesis kit (Stratagene, La Jolla, CA, USA). Site specific mutations were confirmed by DNA sequencing. Plasmids were transfected into cardiomyocytes using a low pressure-accelerated gene gun (Bioware Technologies, Taipei, Taiwan) essentially following the protocol from the manufacturer. Test plasmid 2 μg and control plasmid (pGL4-Renilla luciferase) 0·02 μg were cotransfected with gene gun in each well at a helium pressure of 15 psi, and then replaced by normal cultured medium. The transfection efficiency using this method is 25%. Following 14 h of cyclic stretch, cell extracts were prepared using Dual-Luciferase Reporter Assay System (Promega Corp., Madison, WI, USA) and measured for dual luciferase activity using luminometer (Turner Designs Inc., Sunnyvale, CA, USA).

Cytotoxicity study

Cardiomyocytes were adjusted to 3×10^4 cells mL^{-1} in medium M199. Aliquots (20 mL) of cell suspension were plated in 40-mm Petri dishes. Cytotoxicity study was performed as previously described [18]. For detection of cell injury induced by stretch, cell viability after application of cyclic stretch was monitored using trypan blue staining.

Flow cytometric analysis for apoptotic quantitation

Apoptotic cells were quantified as the percentage of cells with hypodiploid DNA (sub-G1). Cardiomyocytes were fixed with 70% ethanol and treated with RNase. Then nuclei were stained with propidium iodide. The DNA content was measured using a FACSCalibur flow cytometer and Cell Quest software (Becton Dickinson, Franklin Lakes, NJ, USA). For all assays, 10 000 cells were counted.

Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling assay

DNA fragmentation was determined by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL) using the ApopTag peroxidase *in situ* apoptosis detection kit (Chemicon International, Temecula, CA, USA). At the end of cyclic stretch, cardiomyocytes were fixed in 4% paraformaldehyde for 10 min followed by a staining procedure according to manufacturer's protocol.

Statistical analysis

All results were expressed as mean ± SEM. Statistical significance was evaluated with analysis of variance (GraphPad Software Inc., San Diego, CA, USA). The Dunnett's test was used to compare multiple groups to a single control group. Tukey–Kramer comparison was used for pairwise comparisons between multiple groups after the ANOVA. A value of $P < 0{\cdot}05$ was considered to denote statistical significance.

Results

Haemodynamic and echocardiographic change after AV shunt and treatment with PBA

A total of 30 rats were studied. The surgical AV shunt significantly increased the heart rate, heart weight and ratio of heart weight and body weight at 3 days as shown in Table 1. Mean arterial pressure decreased from 81 ± 6 mmHg in the sham group to 62 ± 5 mmHg in the shunt group ($P < 0{\cdot}05$). Echocardiography showed that the AV shunt increased left ventricular end-diastolic and end-systolic dimensions, but maintained the left ventricular fraction shortening ($P < 0{\cdot}05$). These findings indicate the presence of volume-overload in the AV-shunt group.

Treatment with PBA significantly reduced the heart weight and the ratio of heart weight and body weight. Mean arterial pressure was reversed to baseline levels after treatment with PBA. The increased left ventricular dimension in the shunt group was reversed to normal by PBA.

Table 1 Haemodynamic and echocardiographic parameters at the end of study

			PBA	
	Sham	**Shunt**	**Sham**	**Shunt**
Number	8	8	8	6
Body weight (BW), g	337 ± 5	321 ± 9	334 ± 10	324 ± 7
Heart weight (HW), mg	1064 ± 82	1349 ± 118*	1099 ± 78	1120 ± 108†
HW/BW, mg g^{-1}	3·2 ± 0·2	4·2 ± 0·4*	3·3 ± 0·3	3·5 ± 0·4†
Heart rate	347 ± 19	407 ± 18*	349 ± 12	334 ± 7†
MAP, mmHg	81 ± 6	62 ± 5*	82 ± 13	79 ± 8†
IVSTd, mm	1·7 ± 0·1	1·5 ± 0·4	1·8 ± 0·2	1·8 ± 0·3
LVPWT, mm	1·6 ± 0·2	1·4 ± 0·4*	1·4 ± 0·6	1·5 ± 0·3
LVEDD, mm	6·6 ± 0·7	8·7 ± 0·4*	6·4 ± 0·4	6·9 ± 0·5†
LVESD, mm	3·1 ± 0·6	4·3 ± 0·4*	3·5 ± 0·8	3·2 ± 0·2†
FS, %	53 ± 3	50 ± 3	45 ± 9	54 ± 5
LV mass, mg	662 ± 52	1250 ± 98*	675 ± 66	850 ± 105†

Data are means ± SD; PBA: 500 mg kg^{-1} day^{-1}.
MAP, mean arterial pressure; IVSTd, interventricular Septum thickness; LVPWT, left ventricular posterior wall thickness; LVEDD, left ventricular end-diastolic dimension; LVESD, left ventricular end-systolic dimension; FS, fraction shortening.
*$P < 0{\cdot}05$ vs. sham group; †$P < 0{\cdot}05$ vs. shunt group.

AV shunt enhances myocardial GADD153 protein and mRNA expression

As shown in Fig. 1, the GADD153 protein expression in rat myocardium significantly increased in rats with AV shunt for 3 days. Real-time PCR also showed that GADD153 mRNA was up-regulated after AV shunt (data not shown). These findings

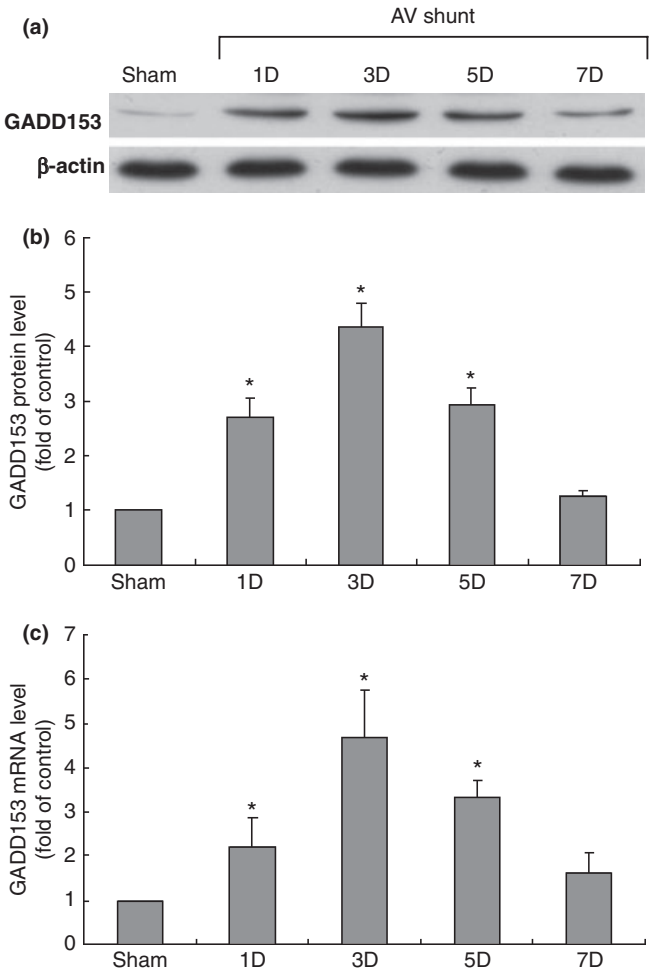

Figure 1 Effect of *in vivo* model of aorta-caval shunt (AV shunt) on myocardial GADD153 protein and mRNA levels. (a) Representative Western blots for GADD153 in rat myocardium after induction of AV shunt. (b) Quantitative analysis of GADD153 protein levels. The values have been normalized to β-actin measurement and then expressed as a ratio of normalized values to GADD153 protein in sham. ($n = 5$ per group). *$P < 0{\cdot}01$ vs. sham group. (c) Fold increases in GADD153 mRNA as a result of induction of AV shunt. The values from experiment groups have been normalized to match GADPH measurement and then expressed as a ratio of normalized values to mRNA in sham group. *$P < 0{\cdot}05$ vs. sham group. ($n = 5$ per group).

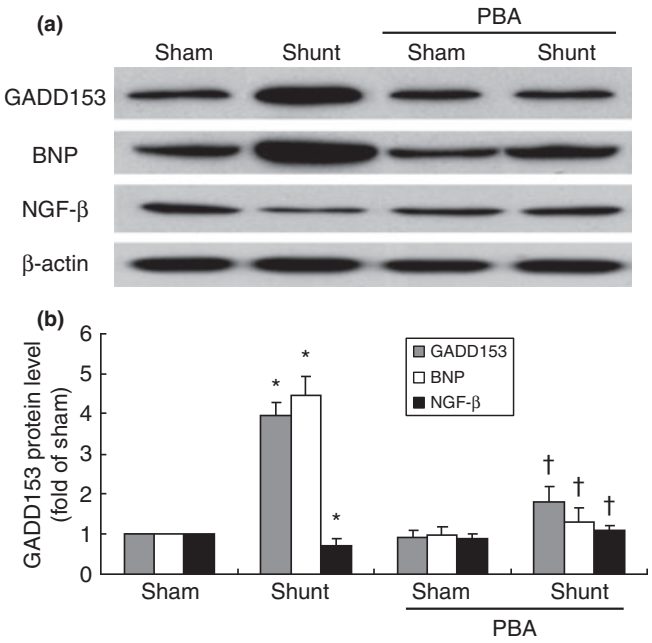

Figure 2 Effect of AV shunt and treatment with PBA on protein expression. (a) Representative Western blot analysis for GADD153, BNP and NGF-β after induction of AV shunt with or without treatment with PBA. (b) Quantitative analysis of GADD153, BNP and NGF-β protein levels. The values from experimental groups have been normalized to values in sham group. (n = 5 per group). *P < 0·01 vs. sham group. †P < 0·05 vs. shunt group.

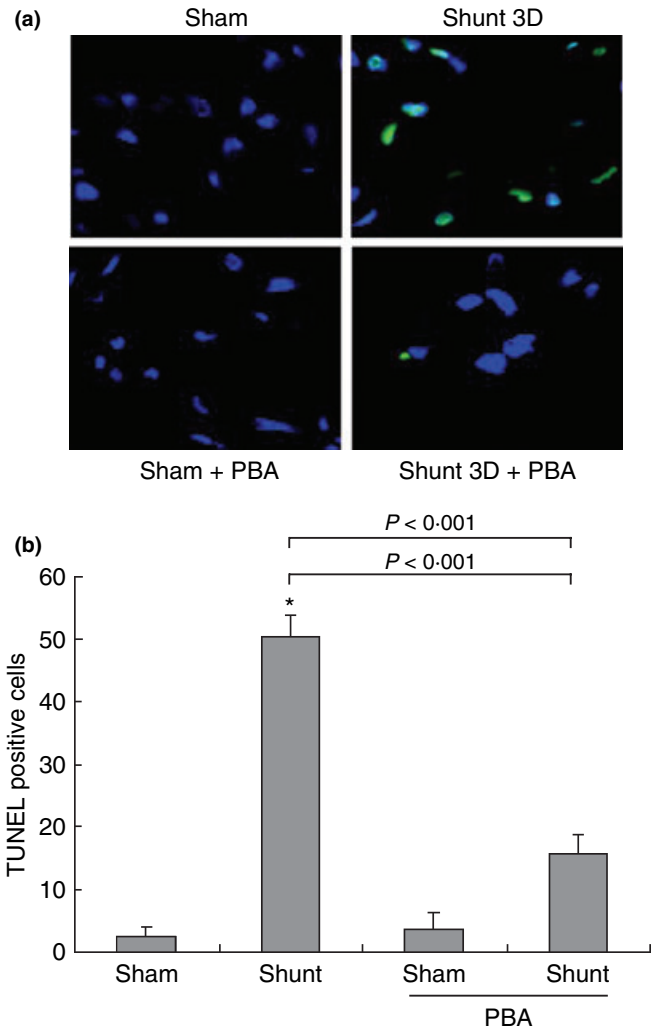

Figure 3 Effect of AV-shunt and treatment with PBA on cardiac myocytes apoptosis. (a) TUNEL staining of cardiac myocytes after induction of AV shunt with or without treatment with PBA. (b) Quantitative analysis of TUNEL positive cells. *P < 0·001 vs. sham group. (n = 5 per group).

indicated GADD153 was induced by volume overload in rat myocardium.

Western blot analysis after AV shunt and treatment with PBA

As shown in Fig. 2, the GADD153 protein increased 4·1-fold at 3 days of AV shunt when compared with the sham group (P < 0·05). Treatment with PBA significantly blocked the increase of GADD153 protein induced by AV shunt. AV shunt significantly increased BNP protein expression and decreased NGF-β protein expression. Treatment with PBA in the AV shunt reversed the BNP and NGF-β to the baseline levels. However, treatment with PBA in the sham group did not affect the protein expression of GADD153, BNP and NGF-β.

PBA inhibits apoptosis induced by AV shunt

As shown in Fig. 3, AV shunt significantly increased apoptotic nuclei. Treatment with PBA significantly reduced TUNEL-positive nuclei. This result indicates that PBA inhibits myocardium apoptosis induced by AV shunt.

Cyclic stretch enhances GADD153 protein and mRNA expression in cardiomyocytes

The level of GADD153 protein began to increase as early as 2 h after stretch to 20% elongation was applied, reached a maximum of 4-fold over the control by 14 h and remained elevated up to 18 h. When cardiomyocytes were stretched at 10% elongation, the level of GADD153 protein was similar to that of control without stretch (Fig. 4a). The real-time PCR showed that GADD153 mRNA increased significantly after 6–14 h of stretch at 20% elongation (Fig. 4c).

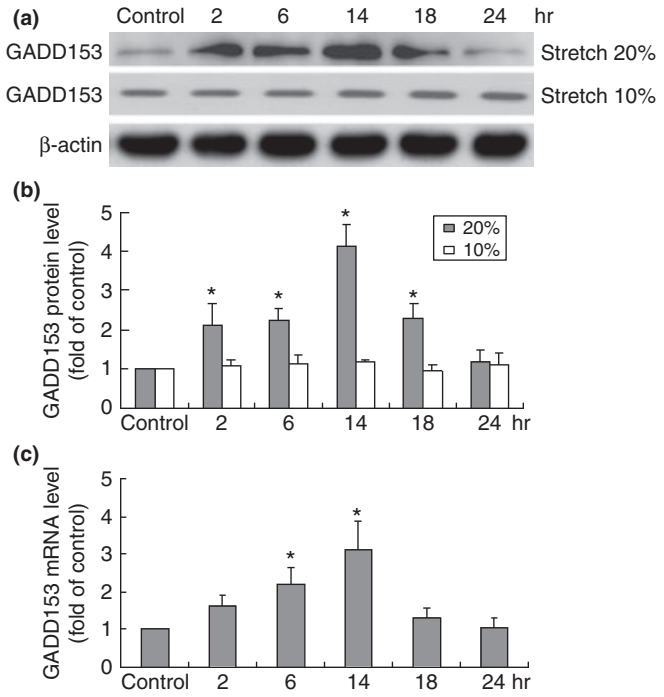

Figure 4 Effects of cyclic stretch on GADD153 mRNA and protein expression in cardiomyocytes. (a) Representative Western Blots for GADD153 in cardiomyocytes subjected to cyclic stretch by 20% or 10% for various periods of time. (b) Quantitative analysis of GADD153 protein levels. The values from stretched cardiomyocytes have been normalized to values in control cells (n = 5 per group). (c) Fold increases in GAD153 mRNA. The values from stretched cardiomyocytes have been normalized to GAPDH levels and then expressed as a ratio of normalized values to mRNA in control cells (n = 4 per group). *P < 0·01 vs. control.

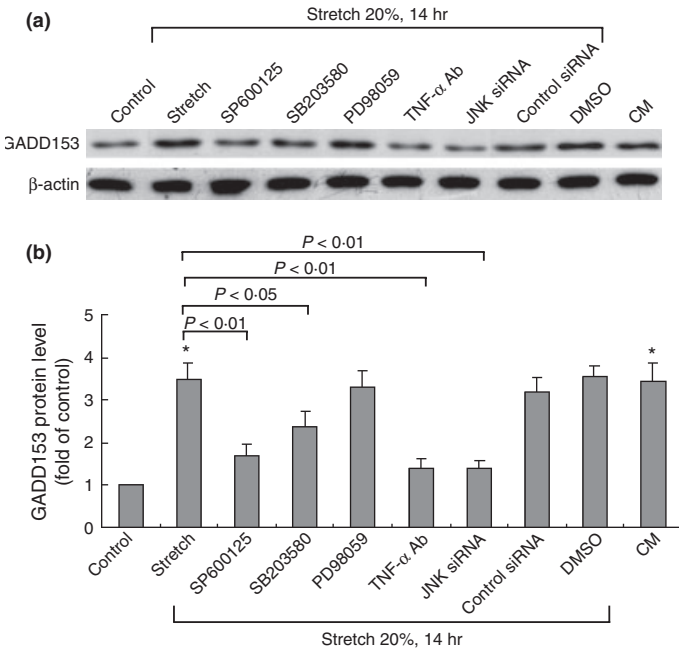

Figure 5 Effects of MAPK inhibitors on GADD153 protein expression induced by cyclic stretch in cardiomyocytes. (a) Representative Western Blots for GADD153 protein levels in cardiomyocytes subjected to cyclic stretch in the absence or presence of inhibitors, siRNA, TNF-α monoclonal antibody and vehicle (DMSO 0·1%). CM = conditioned medium. (b) Quantitative analysis of GADD153 protein levels. The values from stretched cardiomyocytes have been normalized to values in the control cells (n = 4 per group). *P < 0·01 vs. control.

Stretch-induced GADD153 protein expression in cardiomyocytes is mediated by JNK and TNF-α

As shown in Fig. 5, the stretch induced increases of GADD153 proteins were significantly blocked after the addition of SP600125 30 min before stretch. The GADD153 proteins induced by stretch were not affected by the addition of PD98059, but partially blocked by the addition of SB203580. Moreover, JNK siRNA also completely blocked the GADD153 expression induced by cyclic stretch. DMSO alone as a vehicle control and control siRNA did not affect the GADD153 expression induced by cyclic stretch. Addition of TNF-α monoclonal antibody 30 min before stretch also significantly blocked the expression of GADD153 induced by cyclic stretch. Conditioned medium from stretched cardiomyocytes significantly increased GADD153 protein expression when compared with control group.

As shown in Fig. 5c, cyclic stretch significantly enhanced the expression of TNF-α mRNA. Besides, cyclic stretch also significantly began to increase the TNF-α secretion from cardiomyocytes at 1 h after stretch and reached a maximum at 2 h and remained elevated for 24 h (Fig. S1).

Cyclic stretch increases AP-1 binding activity

Cyclic stretch significantly began to increase the DNA-protein binding activity of AP-1 in cardiomyocytes at 1 h after stretch and reached a maximum at 2 h and remained elevated for 6 h (Fig. 6a). An excess of unlabeled AP-1 oligonucleotide competed with the probe for binding AP-1 protein, whereas an oligonucleotide containing a 2-bp substitution in the AP-1 binding site did not compete for binding. Addition of SP600125 and TNF-α antibody 30 min before stretch abolished the DNA-protein binding activity induced by cyclic stretch. JNK siRNA, similar to SP600125, also abolished the DNA-protein binding activity induced by stretch (Fig. 6b). Moreover, exogenous

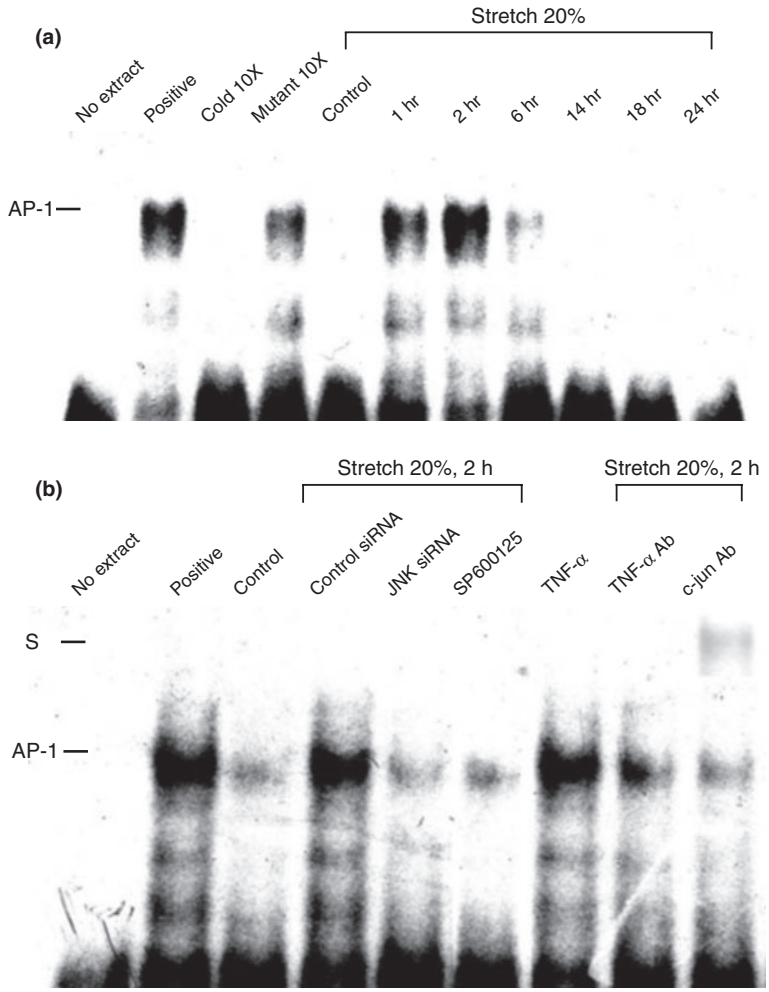

Figure 6 Effects of cyclic stretch on GADD153 binding activity in cardiomyocytes. (a) Representative EMSA showing protein binding to AP-1 oligonucleotide in nuclear extracts of cardiomyocytes after cyclic stretch for various times. (b) Representative EMSA showing protein binding to AP-1 oligonucleotide in nuclear extracts of cardiomyocytes after cyclic stretch in the absence or presence of JNK inhibitors or siRNA. Arrows indicate the mobility of the complex. Similar results were found in another two independent experiments. Cold oligo means unlabelled AP-1 oligonucleotide. A significant supershifted complex after incubation with c-jun antibody was observed.

TNF-α also induced AP-1 binding activity. These results demonstrated that stretch enhanced AP-1 binging activity was mediated by TNF-α and JNK in cardiomyocytes.

Cyclic stretch increases GADD153 promoter activity through AP-1 in cardiomyocytes

The GADD153 promoter construct contains SP-1, NF-IL6, NF1 and AP-1 binding sites. As shown in Fig. 7, cyclic stretch for 2 h significantly activated GADD153 promoter. This result indicates that GADD153 expression is induced at transcriptional level during cyclic stretch in cardiomyocytes. Besides, transient transfection of GADD153-Mut plasmid and addition of SP600125 and TNF-α abolished the promoter activity induced by stretch.

PBA reduces GADD153 expression and apoptosis induced by cyclic stretch in cardiomyocytes

As shown in Fig. 8a, PBA and TUDCA significantly reduced the GADD153 protein expression induced by stretch. As GADD153 is an apoptosis related gene and enhanced by volume overload, we speculated that GADD153 is involved in apoptosis during cyclic stretch. As shown in Fig. S2, cyclic

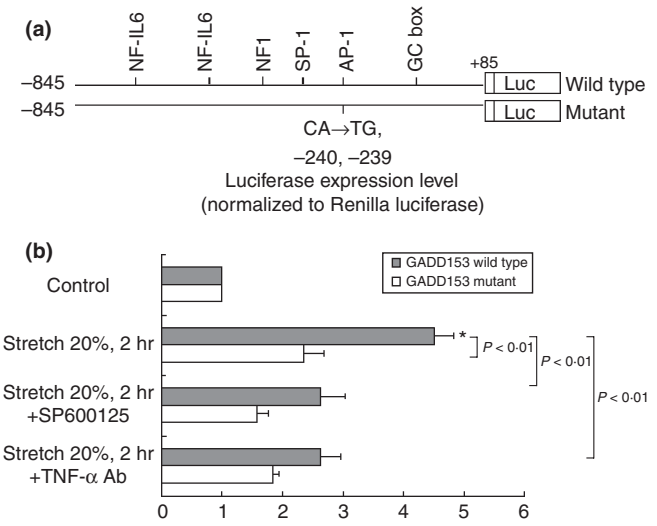

Figure 7 Effect of cyclic stretch on GADD153 promoter activity in cardiomyocytes. (a) Constructs of GADD153 promoter gene. Positive +1 demonstrates the initiation site for the GADD153 transcription. (b) Quantitative analysis of GADD153 promoter activity. The luciferase activity in cell lysates was measured and was normalized with renilla activity (n = 4 per group). *P < 0·01 vs. control.

stretch decreased the viability of cardiomyocytes measured using a cell counter and MTT assay. These results suggest that cyclic stretch induces cell death of cardiomyocytes. Addition of PBA or TUDCA reversed the cell death induced by cyclic stretch.

Apoptosis was assessed using propidium iodide stain and TUNEL assay (Fig. 8c,d). The apoptotic cells increased after stretch for 14 h and addition of TNF-α. These increases of apoptosis in cardiomyocytes induced by stretch were significantly reversed by PBA, TUDCA, GADD153 siRNA and transient transfection of GADD153-Mut plasmid. Then we used colormetric activity assay kit to determine caspase activity of cardiomyocytes under cyclic stretch. The result showed that addition of GADD153 siRNA or transient transfection of GADD153-Mut reduced caspase 3 and 9 activity induced by stretch. Moreover, PBA and TUDCA inhibited not only caspase 3 and 9 activity but also caspase 8 activities (Fig. 8e). These findings demonstrated that GADD153 mediated stretch-induced apoptosis of cardiomyocytes. Besides, PBA and TUDCA reversed the apoptosis induced by cyclic stretch in cardiomyocytes.

Discussion

In this study, we demonstrated that myocardium GADD153 protein and mRNA expression were up-regulated in a model of volume overload. GADD153 was upregulated in both a time- and load-dependent manner by cyclic stretch. Cyclic stretch of cardiomyocytes increased both GADD153 protein and mRNA expression. Okada *et al.* have also demonstrated that GADD153 was activated in failing heart by transverse aortic constriction [19]. Besides, pressure overload did not alter but volume overload enhanced the expression of CCAAT/enhancer binding protein (C/EBP)δ [20]. It indicated that C/EBP family protein may involve in the hypertrophy as result of mechanical stress.

In this study, we found that apoptosis was up-regulated in a model of volume overload. PBA reduced GADD153 expression and apoptosis in myocardium. Hang *et al.* demonstrated that uncoupling protein-2 played a critical role in cardiomyocytes apoptosis in pressure overload-induced left ventricular hypertrophy [21]. Besides, volume overload produced in a mouse model for 8 weeks induced endothelial apoptosis because of the activation of matrix metalloproteinase 9. During the early phase of reperfusion, mechanical stress is a critical determinant form of cardiomyocytes death [22]. Previously, we found that AV shunt could induce apoptosis in aorta [16]. According to these studies, we may suggest that volume overload enhance apoptosis of cardiovascular cells.

We have demonstrated that cyclic stretch induces apoptosis in cardiomyocytes. Liao *et al.* demonstrated that endogenous NO signalling also plays an important role in stretch-induced cardiomyocytes apoptosis. [23]. Although we and Liao *et al.* used 20% cyclic stretch, Liao's results indicated that 4 h was enough to induce cardiomyocytes apoptosis but our results indicated that 14 h was needed. Besides, it has been demonstrated that 20% stretch for 24 h activated the mitochondrial dependent apoptosis pathway in cardiomyocytes including release of cytochrome *c* [24]. Moreover, cyclic stretch for 24 h induced apoptosis in myocytes of young rats but necrosis in old rats [25].

Our study has revealed that TNF-α acts as an autocrine mediator in response to cyclic stretch in cardiomyocytes. We found that cyclic stretch enhanced TNF-α expression in cardiomyocytes. Our results suggested that TNF-α is responsible for AP-1-DNA binding in cardiomyocytes. AP-1, a well-characterized downstream target of JNK, has been demonstrated to be needed for GADD153 promoter activity in rat. Cyclic stretch-enhanced AP-1-DNA binding activity in cardiomyocytes required at least phosphorylation of JNK, because JNK inhibitor and JNK siRNA abolished the AP-1 binding activity induced by stretch. We further demonstrated that the JNK1 siRNA significantly inhibited GADD153 expression induced by stretch. These results indicated that the JNK MAP kinase pathway is the major pathway involved in the induction of GADD153 by stretch and mediates the increased binding activity of AP-1 in cardio

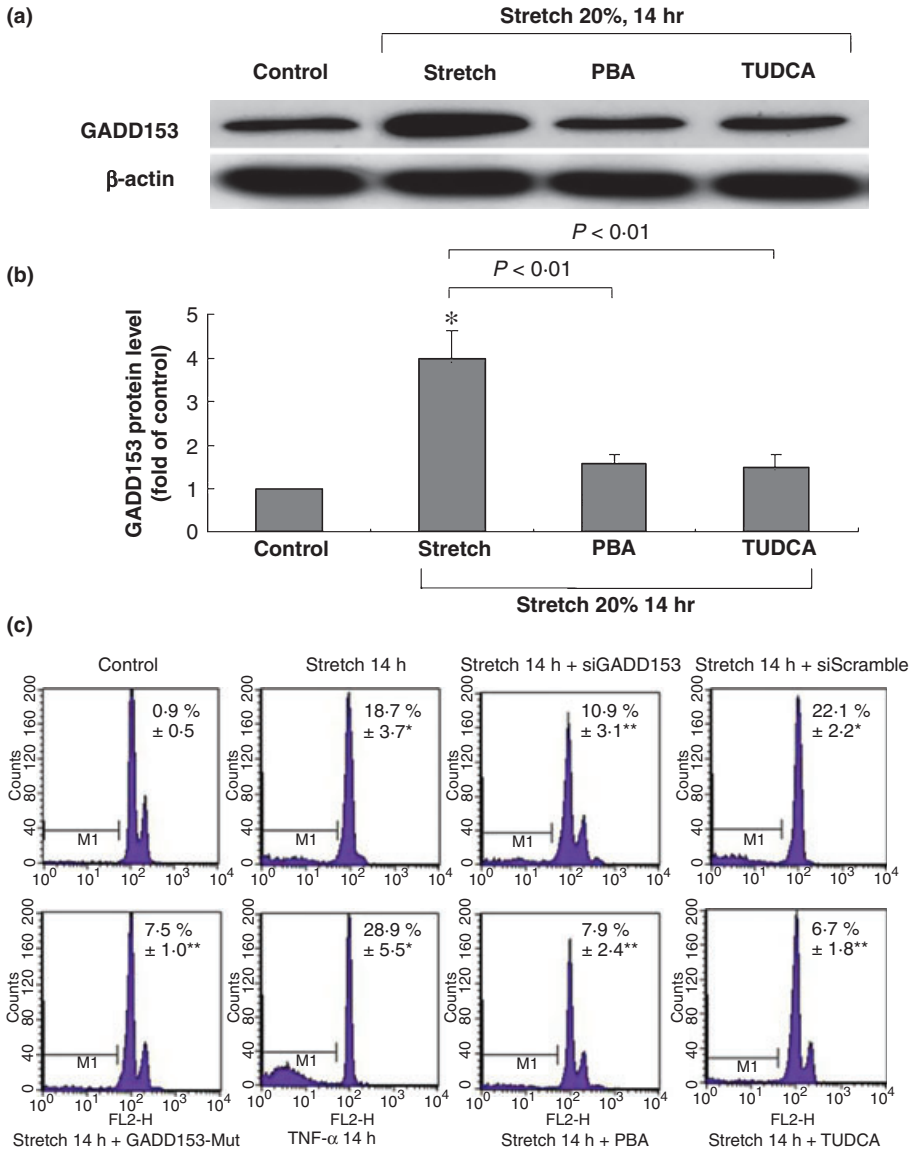

Figure 8 Effect of GADD153 on stretch-induced apoptosis in cardiomyocytes. (a) Representative Western blots for GADD153 protein levels in cardiomyocytes subjected to cyclic stretch in the absence or presence of PBA and TUDCA. (b) Quantitative analysis of GADD153 protein levels ($n = 6$). *$P < 0{\cdot}01$ vs. control. (c) Quantification of the apoptotic fractions of cardiomyocytes using FACScan ($n = 4$). *$P < 0{\cdot}01$ vs. control; **$P < 0{\cdot}05$ vs. stretch 14 h. (d) Representative microscopy images of TUNEL assay in cardiomyocytes. Similar results were observed in another two independent experiments. (e) Quantification of caspase activity induced by cyclic stretch ($n = 4$). *$P < 0{\cdot}01$ vs. caspase 3 control; **$P < 0{\cdot}01$ vs. caspase 3 stretch 14 h; $^{\#}P < 0{\cdot}01$ vs. caspase 8 control; $^{\#\#}P < 0{\cdot}05$ vs. caspase 8 stretch 14 hr; $^{\dagger}P < 0{\cdot}01$ vs. caspase 9 control; $^{\ddagger}P < 0{\cdot}01$ vs. caspase 9 stretch 14 h.

myocytes. Besides, our reporter gene assay found that increased transcriptional activity of GADD153 promoter by cyclic stretch was AP-1 dependent.

In this study, our results revealed that cardiomyocytes apoptosis induced by stretch is mediated by ER stress-related gene GADD153. Mao *et al.* showed that ER stress induced cardiomyocytes apoptosis in autoimmune cardiomyopathy [26]. Application of chemical chaperones which are low molecular mass, protein stabilizing agents that can help to correct protein misfolding [27,28]. PBA and TUDCA are considered as chemical chaperones. This kind of chaperon was known to stabilize protein conformation, enhance ER folding capacity,

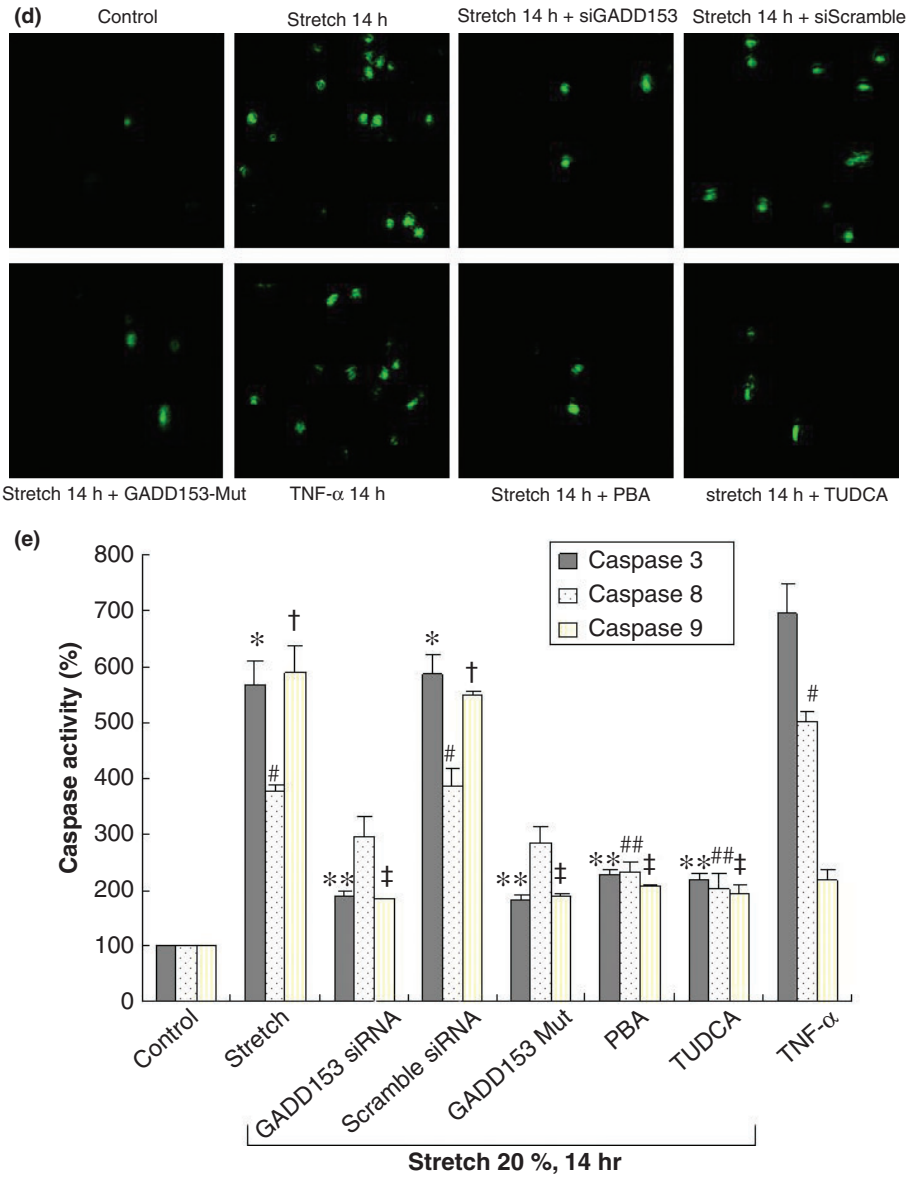

Figure 8 *Continued*

and facilitate the trafficking of mutant proteins [29]. *In vitro* study, TUDCA, a hydrophilic bile acid and formed in the conjugation pathway of ursodeoxycholic acid, inhibits apoptosis in a dose-dependent manner. Chen *et al.* demonstrated the most effective concentration of TUDCA to reduce apoptosis is 100 μM [30]. In this study, we have found that PBA and TUDCA reduce apoptosis induced by stretch. Our results revealed that TNF-α level was down-regulated by PBA and TUDCA (data not shown). TUDCA can also regulate ER function through regulation of Bcl-2 family and survival signalling process [31]. TUDCA could inhibit apoptosis by preventing the binding of Bax to mitochondria [32]. Recently, TUDCA has been shown to reduce apoptosis following myocardial infarction in rats [33].

In summary, our result reveals that PBA inhibits myocardium GADD153 expression and apoptosis induced by AV shunt and aortic banding. Cyclic stretch enhances GADD153 expression in cultured cardiomyocytes. The stretch-induced GADD153 is mediated by TNF-α, JNK MAP kinase and AP-1 pathway. Besides, we find that PBA and TUDCA reduce the

GADD153-mediated apoptosis induced by stretch. Our data indicate that ER stress may play an important role in mechanical stress and volume overload. Targeting ER function for therapeutic application in cardiac hypertrophy and failure may warrant further studies and clinical investigation.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Cyclic stretch increases TNF-α mRNA in cardiac myocytes. TNF-α mRNA was measured using real-time PCR. *$P < 0{\cdot}01$ vs. control. ($n = 5$ per group).

Figure S2 Effect of cyclic stretch on viability of cardiac myocytes. Cyclic stretch decreased the viability of cardiomyocytes measured by a cell counter (a) and MTT assay (b). *$P < 0{\cdot}01$ vs. control. ($n = 4$ per group).

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