Available online at www.sciencedirect.com

ScienceDirect

journal homepage: www.jfma-online.com

ORIGINAL ARTICLE

Atorvastatin alleviates cardiomyocyte apoptosis by suppressing *TRB3* induced by acute myocardial infarction and hypoxia

Wen-Pin Cheng [a], Huey-Ming Lo [b,c], Bao-Wei Wang [a],
Su-Kiat Chua [b,d,e], Ming-Jen Lu [f,g], Kou-Gi Shyu [b,*,g]

[a] Department of Medical Education and Research, Shin Kong Wu Ho-Su Memorial Hospital, Taipei, Taiwan
[b] Division of Cardiology, Shin Kong Wu Ho-Su Memorial Hospital, Taipei, Taiwan
[c] School of Medicine, Fu-Jen Catholic University, New Taipei City, Taiwan
[d] Graduate Institute of Clinical Medicine, College of Medicine, Taipei Medical University, Taipei, Taiwan
[e] Department of General Medicine, Shin Kong Wu Ho-Su Memorial Hospital, Taipei, Taiwan
[f] Division of Cardiovascular Surgery, Department of Surgery, Shin Kong Wu Ho-Su Memorial Hospital, Taipei, Taiwan

Background/Purpose: TRB3 (tribbles 3), an apoptosis-regulated gene, increases during endoplasmic reticulum stress. Hypoxia can induce inflammatory mediators and apoptosis in cardiomyocytes. However, the expression of *TRB3* in cardiomyocyte apoptosis under hypoxia is not thoroughly known. We investigated the regulation mechanism of *TRB3* expression and apoptosis induced by hypoxia in cardiomyocytes.
Methods: An *in vivo* model of acute myocardial infarction (AMI) was applied in adult Wistar rats to induce myocardial hypoxia. Rat neonatal cardiomyocytes were subjected to 2.5% O_2 to induce hypoxia.
Results: The expression of TRB3 was evaluated in cultured rat neonatal cardiomyocytes subjected to hypoxia. Hypoxia significantly enhanced TRB3 protein and mRNA expression. Adding c-jun N-terminal kinase (JNK) inhibitor SP600125, JNK small interfering RNA (siRNA), tumor necrosis factor-α (TNF-α) antibody, and atorvastatin 30 minutes before hypoxia reversed the induction of TRB3 protein. A gel-shift assay showed the DNA-binding activity of growth arrest and DNA damage-inducible gene 153 (GADD153), which increased after hypoxia. Hypoxia increased, whereas the *TRB3*-mut plasmid, SP600125, and TNF-α antibody abolished the hypoxia-induced *TRB3* promoter activity. Hypoxia increased the secretion of TNF-α from

cardiomyocytes. Exogenous administration of TNF-α recombinant protein to the cardiomyocytes without hypoxia increased TRB3 protein expression, similar to that observed after hypoxia. Hypoxia-induced cardiomyocyte apoptosis is inhibited by *TRB3* siRNA, the TNF-α antibody, and atorvastatin. Atorvastatin reduced the TRB3 expression and cardiomyocyte apoptosis induced by AMI. Hypoxia induces TRB3 through TNF-α, JNK, and the GADD153 pathway.

Conclusion: Treatment of atorvastatin inhibits the expression of TRB3 and cardiomyocyte apoptosis induced by AMI and hypoxia.

Introduction

Heart failure is a leading cause of mortality in Western countries.[1] The etiology of heart failure involves multiple conditions, but cardiomyocyte apoptosis is one of the most crucial pathogenic components.[2] In recent years, there has been a dramatic proliferation of studies on cardiomyocyte apoptosis.[3] At present, the role of cardiomyocyte apoptosis in the progression of heart failure is not thoroughly understood.

Endoplasmic reticulum (ER) stress results in the unfolded protein response (UPR), an adaptive response that promotes organelle recovery.[4] However, if ER stress is prolonged or UPR cannot restore homeostasis, UPR could induce transcription factor growth arrest and DNA damage-inducible gene 153 (GADD153) and activating transcription factor 4 (ATF4), which regulate the expression of genes involved in apoptosis.[5] *Tribble 3* (*TRB3*) (NM_175093), also named neuronal cell death–inducible putative protein kinase, is an apoptosis-related gene during ER stress.[6] TRB3 is expressed in the liver, thymus, prostate, and heart.[7] TRB3 expression was up-regulated under an *in vitro* model of ischemia–reperfusion injury in cardiomyocytes.[8] Experimental acute myocardial infarction (AMI) significantly increased TRB3 expression in murine heart tissue.[9] Ohoka et al noted that TRB3 is a novel target of GADD153 in the TRB3 promoter.[10] In addition, a previous study indicated that GADD153 could activate TRB3.[11] However, the role of TRB3 in cardiomyocytes is still debated.

The ER-related apoptotic pathway was demonstrated to be one of the mechanisms underlying AMI in cardiomyocytes.[12] Hypoxia could induce inflammatory mediators and apoptosis in cardiomyocytes.[13] Tumor necrosis factor-α (TNF-α) is known to trigger the inflammatory reaction caused by ischemia and could induce cardiomyocytes apoptosis.[14] A previous study has indicated that treatment with atorvastatin results in a significant reduction in the levels of TNF-α.[15] Atorvastatin, a 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitor, is a member of the statins used for treating hypercholesterolemia. Accumulated evidence demonstrates that atorvastatin not only reduces the lipid level but also provides antiinflammatory and antiapoptotic benefits.[16] However, the role of atorvastatin in inflammation and oxidative stress caused by hypoxia is not thoroughly understood. Therefore, the central purpose of the present study was to investigate the molecular mechanism of *TRB3* under hypoxia in cardiomyocytes. Moreover, we sought to determine the possibility of using atorvastatin to inhibit the TRB3 expression and cardiomyocyte apoptosis under hypoxia and AMI.

Materials and methods

In vivo rat model of AMI

A rat model of the left anterior descending coronary artery (LAD) occlusion was used in Wistar rats weighing 250–300 g as described previously.[17] Animals study protocols were followed according to the Guide for the Care and Use of Laboratory Animals (NIH publication No. 86–23, revised 2011) and were approved by the Committee of Animal Care and Use at Shin Kong Wu Ho-Su Memorial Hospital (permit number: 1021025015).

Cardiomyocytes primary culture

Cardiomyocytes were obtained from 2- to 3-day-old Wistar rats by trypsinization as previously described.[18] The obtained cultured cardiomyocytes were >95% pure, as revealed by observing contractile characteristics stained with an anti-desmin antibody under a light microscope (Dako Cytomation, Glostup, Denmark). Cardiomyocytes were seeded on a cultured dish in Ham's F-10 containing 20% fetal bovine serum. After 3 days in culture, cells were transferred to a serum-free Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich China Inc., Shanghai, China) and subjected to hypoxia.

Hypoxia apparatus

Hypoxic conditions (2.5% O_2) were applied as described previously.[17] To investigate the roles of ERK (extracellular signal–regulated kinase), p38, and JNK (c-Jun N-terminal kinase) mitogen-activated protein kinases (MAPK) in the protein expression of *TRB3* induced by hypoxia, cardiomyocytes were treated with PD98059 (50μM; Calbiochem, San Diego, CA, USA), SB203580 (3μM; Calbiochem), or SP600125 (20μM) (Calbiochem) 30 minutes before hypoxia.

Reverse transcription polymerase chain reaction

Reverse transcription polymerase chain reaction was performed as previously described.[18]

Real-time quantitative polymerase chain reaction

Real-time polymerase chain reaction was performed as previously described.[18] The primers used were as follows: TRB3, 5′-d(TCGCTGACCGTGAGAGGAA)-3′ (forward) and 5′-d(GCA AGATGAGCCCGTGCTT)-3′ (reverse); and glyderaldehyde-3-phosphate dehydrogenase (GAPDH), 5′-d(CATCACCATCT TCCAGGAGC) (forward) and 5′-d(GGATGATGTTCTGGGCT GCC)-3′ (reverse).

Western blot

Western blot was performed as previously described.[18] The antibodies used for Western blots were anti-TRB3 (1:200; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA); anti-hypoxia inducible factor-1α (HIF-1α) (1:200; Santa Cruz Biotechnology Inc.); and anti-TNF-α (1:1000, Bioss Inc., Woburn, MA, USA).

Electrophoretic mobility shift assay

Electrophoretic mobility shift assay (EMSA) was performed as previously described.[18] The GADD153 binding site used was TGGTGCAATCCCC.[18] The mutant oligonucleotides of GADD153 sequences were TGGTATAATCCCC.

Construction of small interfering RNA

Cardiomyocytes were transfected with 800 ng of TRB3 small interfering RNA (siRNA) (Dharmacon, Inc., Lafayette, CO, USA). TRB3 siRNA: sense: 5′-P.CAGCCUACCUCCCGCCUCA-3′ and antisense: 5′-P.UGAGGCGGGAGUAGGCUG-3′. Scramble siRNA of TRB3: sense: 5′-P.UCCACCCGUUCGACCCACC-3′ and antisense: 5′-P.GGUGGGUCGAAC GGUGGA-3′ (Dharmacon). JNK1 siRNA: sense: 5′-P.CGUGGAUUUAUGGUCUGUGdTdT-3′; antisense: 5′-P.CACAGACCAUAA AUCCACCdTdT-3′ (Dharmacon).

Promoter activity assay

We used a low-pressure accelerated gene gun (Bioware Technologies, Taipei, Taiwan) to transform the TRB3 promoter construct containing the GADD153 binding site into cardiomyocytes. The procedures were performed as previously described.[18]

Detection of TNF-α by enzyme-linked immunosorbent assay

A conditioned medium from cardiomyocytes was subjected to hypoxia and those from control (normoxia) cells were collected for detection of TNF-α. The lowest limit of detection of the TNF-α enzyme-linked immunosorbent assay kit is 45 pg/mL.

Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling assay

A Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay was performed as previously described.[18] DNA fragmentation was determined using the TUNEL assay with an ApopTag peroxidase in situ apoptosis detection kit (Chemicon International, Temecula, CA, USA). We then used a confocal microscope (Nikon D-ECLIPSE; Nikon, Tokyo, Japan) and its associated image processing/analysis software to obtain fluorescence signals.

Flow cytometric analysis for apoptosis

Cardiomyocytes stained with annexin V were determined to be apoptotic cells. The procedures were performed as previously described.[18]

Statistical analysis

All results are expressed as means $\pm$ S.E.M. Statistical significance was evaluated using analysis of variance (ANOVA) (GraphPad Software, La Jolla, CA, USA). Dunnett's test was used to compare multiple groups with a single control group. The Tukey—Kramer comparison was used for pairwise comparisons between multiple groups after the ANOVA; $P < 0.05$ was considered to denote statistical significance.

Results

### AMI increases the TRB3 protein and mRNA expression in rat myocardia

The myocardial TRB3 protein expression significantly increased in rats after AMI for 10 days (Figures 1A and 1B). As shown in Figure 1C, real-time polymerase chain reaction (PCR) demonstrated that TRB3 mRNA was up-regulated after AMI. These results indicated that myocardial TRB3 is induced by AMI in rat myocardia.

Atorvastatin reduces myocardial TRB3 protein expression and apoptosis induced by AMI

As shown in Figures 2A and 2B, treatment with atorvastatin significantly reversed the increase of TRB3 protein induced by AMI. However, treatment with atorvastatin in the sham group did not affect the myocardial TRB3 protein expression. As shown in Figure 2C and Figure S1A, atorvastatin markedly inhibited the TUNEL positive cardiomyocytes induced by AMI. These results demonstrated that atorvastatin inhibits myocardial TRB3 protein expression and apoptosis induced by AMI. Furthermore, treatment with atorvastatin reduced the infarct size induced by AMI (Figure S1B).

Hypoxia increases the protein and mRNA expression of TRB3 in cultured cardiomyocytes

To test the effect of hypoxia on TRB3 expression, we used different degrees of hypoxia (10%, 5%, and 2.5% O$_2$) (Figures S2A and S2B). TRB3 expression was observed to have the most evident effect under 2.5% O$_2$ hypoxia for 16 hours. Thus, we applied 2.5% O$_2$ as hypoxia in subsequent experiments. As shown in Figures 3A and 3B, hypoxia markedly
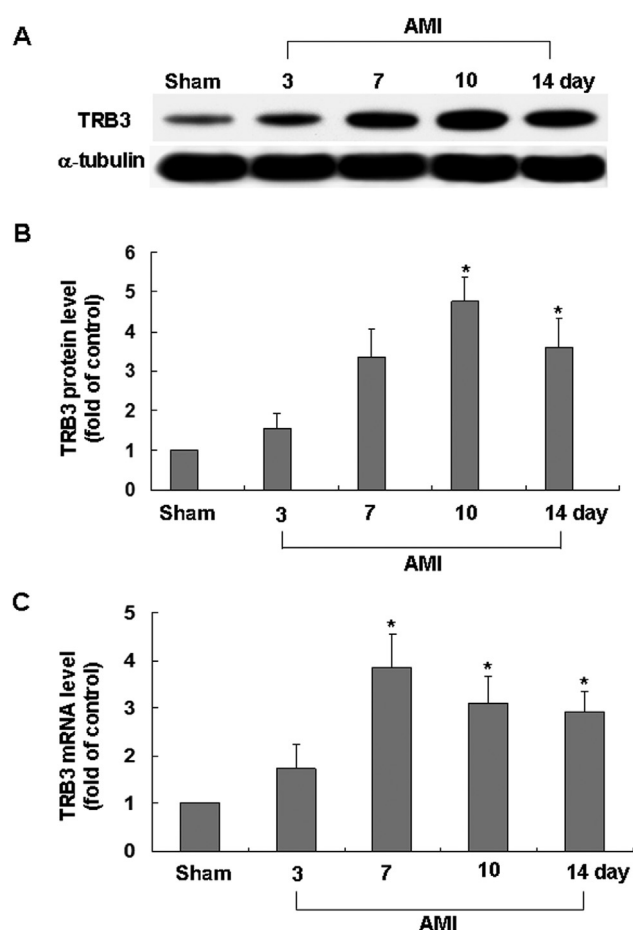
Figure 1 Effect of AMI on myocardial TRB3 protein levels and mRNA. (A) Representative Western blots for TRB3 in rat myocardia after AMI. (B) Quantitative analysis of TRB3 protein levels. Values for myocardia after AMI were normalized to match the α-tubulin measurement and then expressed as a ratio of normalized values to protein in the control group ($n = 3$ per group). *$P < 0.05$ vs. control group. (C) Fold increases in *TRB3* mRNA as a result of induction of AMI. The values for the experimental groups were normalized to match the GADPH measurement and then expressed as a ratio of normalized values to mRNA in the sham group. *$P < 0.05$ vs. sham group ($n = 3$ per group).

increased TRB3 protein expression in cardiomyocytes. The levels of *TRB3* mRNA also significantly increased from 8 to 16 hours after hypoxia treatment (Figure S2C). *TRB3* siRNA significantly attenuated the TRB3 protein expression induced by hypoxia (Figures S3A and S3B).

TRB3 protein expression induced by hypoxia in cardiomyocytes is mediated by JNK

To investigate the possible signaling pathway mediating the TRB3 induced by hypoxia in cardiomyocytes, the cardiomyocytes were subjected to 2.5% O_2 for 16 hours in the presence or absence of inhibitors, siRNA and a vehicle (DMSO 0.1%). As shown in Figure 3C and 3D, the hypoxia-induced increases of TRB3 protein were significantly

blocked after the addition of SP600125 (20 μM) 30 min before hypoxia. TRB3 protein induced by hypoxia were not affected by the addition of SB203580 (3 μM) or PD98059 (50 μM). In addition, treatment of the JNK siRNA before hypoxia significantly inhibited the TRB3 level induced by hypoxia. The scramble siRNA and DMSO alone as a vehicle control did not affect the hypoxia-induced TRB3 expression. Moreover, adding of atorvastatin (10 μM) significantly reversed the TRB3 protein expression induced by hypoxia (Figures S3C and S3D). These results indicated that the JNK pathway, but not ERK or p38 MAP kinases, mediates the induction of TRB3 protein by hypoxia in cardiomyocytes.

### Hypoxia increases GADD153 binding activity in cardiomyocytes

As shown in Figure 4A, cardiomyocytes subjected to hypoxia for 6 hours significantly increased the DNA—protein-binding activity of GADD153. An excess of unlabeled GADD153 oligonucleotide competed with the probe for binding GADD153 protein, whereas an oligonucleotide containing a 2-bp substitution in the GADD153 binding site did not compete for binding. Adding SP600125, JNK siRNA, TNF-α Ab (5 mg/mL; R&D Systems, Minneapolis, MN, USA), and atorvastatin before hypoxia significantly inhibited the DNA—protein-binding activity induced by hypoxia. Exogenous administration of TNF-α to the cardiomyocytes without hypoxia also increased the GADD153—DNA-binding activity. However, the addition of the TNF-α antibody abolished the increased binding activity induced by exogenous administration of TNF-α. These findings demonstrate that hypoxia enhanced the GADD153 binding activity in cardiomyocytes.

TRB3 promoter activity is induced by hypoxia through GADD153

To determine whether the *TRB3* expression induced by hypoxia is regulated at the transcriptional level, we used a luciferase reporter assay to identify the genetic transcription activity of *TRB3* in cardiomyocytes under hypoxia. The TRB3 promoter construct contains an amino-acid response element (AAREs), ATF4, and GADD153 binding sites (Figure 4B). As shown in Figure 4C, hypoxia increased the transcriptional activity of TRB3, but the *TRB3* mutant failed to exert the same effect. Moreover, adding the TNF-α antibody, SP600125. and atorvastatin inhibited the transcriptional activity of *TRB3*. These findings suggest that the GADD153 binding site in the *TRB3* promoter is essential for transcriptional regulation in cardiomyocytes under hypoxia.

Hypoxia induces TRB3 protein expression in cardiomyocytes through TNF-α

TNF-α secretion from cardiomyocytes under hypoxia significantly increased at 2 hours and remained elevated for 16 hours (Figure 5A). Adding the TNF-α antibody or atorvastatin 30 minutes before hypoxia significantly inhibited the expression of TNF-α induced by hypoxia. This result demonstrated that hypoxia increases secretion of TNF-α from cardiomyocytes.
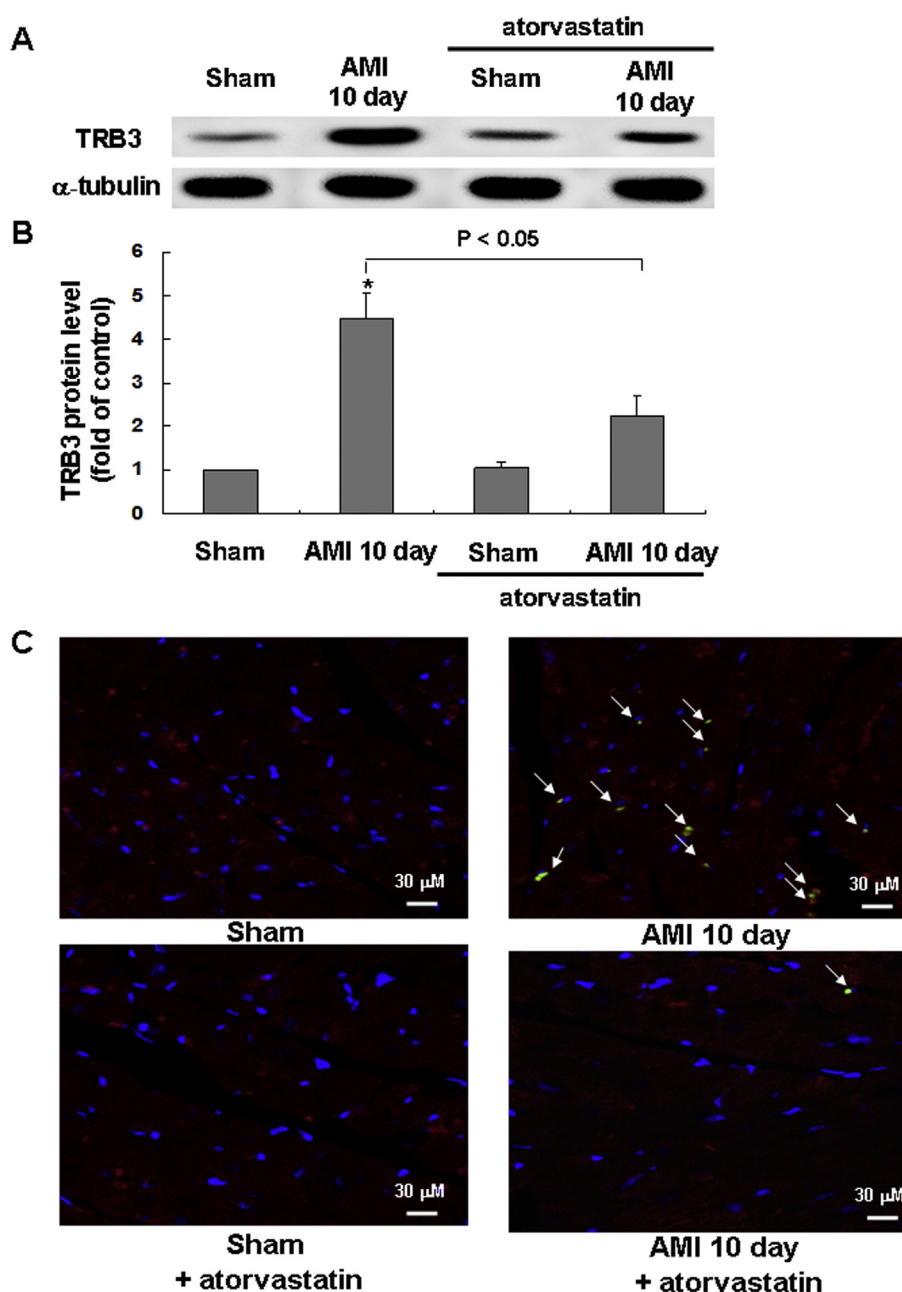
Figure 2   Effect of AMI and treatment with atorvastatin (30 mg/kg daily) on myocardial TRB3 protein levels and apoptosis. (A) Representative Western blots for TRB3 after induction of AMI with or without treatment with atorvastatin. (B) Quantitative analysis of TRB3 protein levels. Values for myocardia after induction of AMI with or without treatment with atorvastatin. $*P < 0.05$ vs. sham group ($n = 3$ per group). (C) Representative microscopic images for rat myocardia after AMI or treatment of atorvastatin before AMI then stained using a TUNEL kit. Blue indicates nuclei stained with DAPI. Red indicates desmin. Green indicates TUNEL positive nuclei. Similar results were observed in other two independent experiments.

To investigate the direct effect of TNF-α on TRB3 expression in cardiomyocytes, TNF-α at different concentrations was added to the cultured medium for 16 h. As shown in Figures 5B and 5C, the effect of TNF-α on TRB3 protein expression was dose-dependent. Adding the TNF-α antibody 30 minutes before hypoxia markedly inhibited the expression of hypoxia-induced TRB3. Furthermore, treatment of atorvastatin abolished the expression of TRB3 protein induced by exogenous administration of TNF-α for 16 hours. These findings suggested that TNF-α mediates TRB3 expression under hypoxia in cardiomyocytes.

Hypoxia-induced cardiomyocyte apoptosis is mediated by *TRB3* and TNF-α

The TUNEL assay was performed to investigate the cardiomyocyte apoptosis under hypoxia. A significant increase
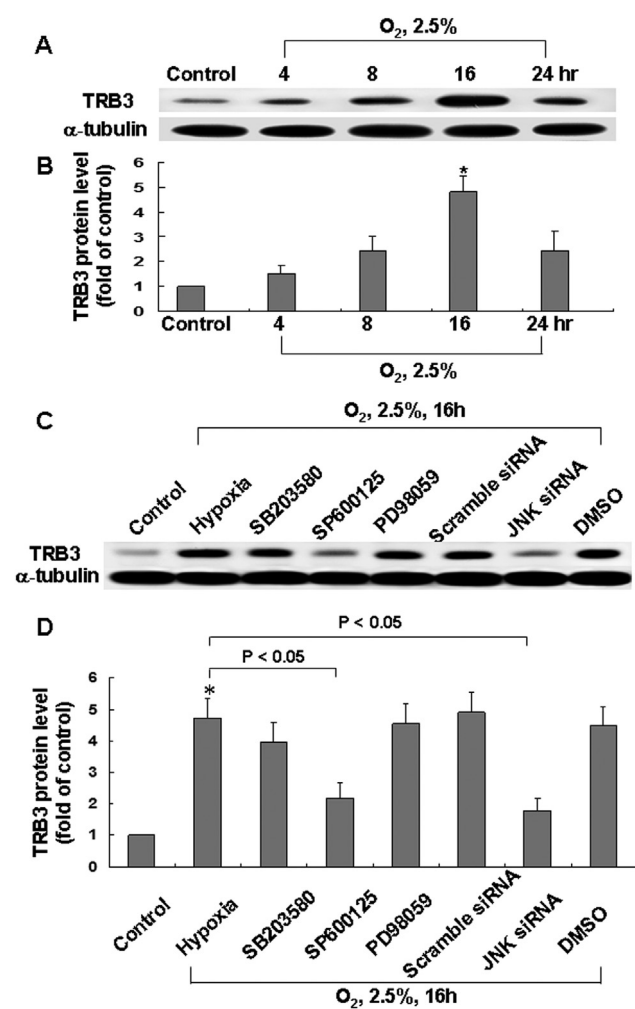
Figure 3 Effects of *in vitro* hypoxia and treatment with MAPK inhibitors on TRB3 protein expression in cardiomyocytes. (A) Representative Western blots for TRB3 in cardiomyocytes after 2.5% O_2 (hypoxia) treatment for various periods. (B) Quantitative analysis of TRB3 protein levels. Values for cardiomyocytes under hypoxia were normalized to match the α-tubulin measurement and then expressed as a ratio of normalized values to protein in the control group ($n = 3$ per group). *$P < 0.05$ vs. control. (C) Representative Western blots for TRB3 protein levels in cardiomyocytes subjected to hypoxia in the absence or presence of MAPK inhibitors, siRNA, and a vehicle (DMSO 0.1%). (D) Quantitative analysis of TRB3 protein levels. Values for cardiomyocytes under hypoxia were normalized to match the α-tubulin measurement and then expressed as a ratio of normalized values to protein in the control group ($n = 3$ per group). *$P < 0.05$ vs. control.

in TUNEL-positive nuclei was present after hypoxia for 16 hours (Figure 6A and Figure S4). The hypoxia-induced TUNEL-positive nuclei of cardiomyocytes were significantly inhibited by *TRB3* siRNA, TNF-α Ab, and atorvastatin. As shown in Figure 6B, apoptosis was determined through propidium iodide—annexin V double staining and FACS analysis. The percentage of cells stained with annexin V was increased after hypoxia for 16 hours. Adding the *TRB3* siRNA, TNF-α Ab, and atorvastatin before hypoxia significantly reversed the percentage of annexin V-positive cells
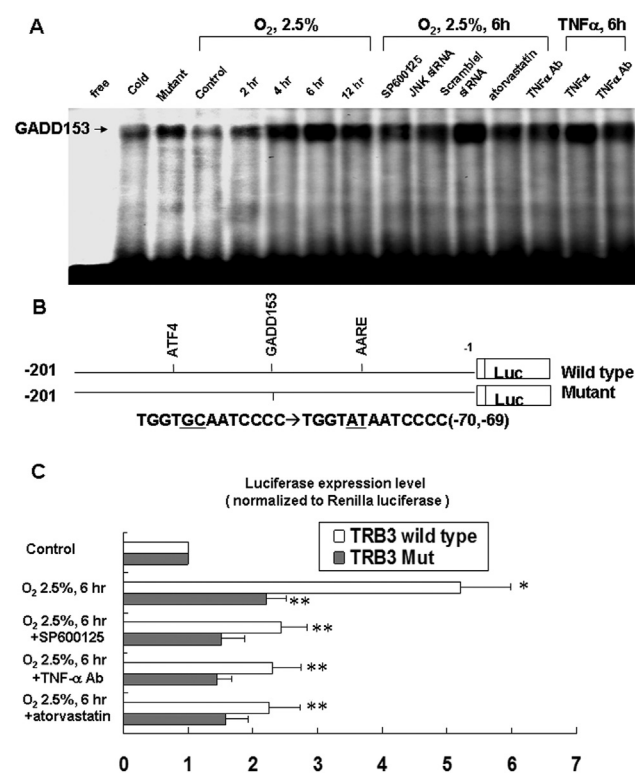

Figure 4 Effects of hypoxia on the GADD153-binding activity and *TRB3* promoter activity in cardiomyocytes. (A) Representative EMSA showing protein binding to a GADD153 oligonucleotide in nuclear extracts of cardiomyocytes under hypoxia during various periods and in the absence or presence of a JNK inhibitor, siRNA, TNF-α antibody, and atorvastatin (10 μM). Arrows indicate the mobility of the complex. Similar results were found in another independent experiment. Cold oligo means an unlabeled GADD153 oligonucleotide. (B) Constructs of the *TRB3* promoter gene. (C) Quantitative analysis of *TRB3* promoter activity. Cardiomyocytes were transiently transfected with p*TRB3*-luc by using a gene gun. The luciferase activity in cell lysates was measured and normalized with *Renilla* activity ($n = 3$ per group). *$P < 0.05$ vs. *TRB3* wild-type control; **$P < 0.05$ vs. *TRB3* wild-type for hypoxia 6 hours.

induced by hypoxia. These data demonstrated that cardiomyocytes apoptosis induced by hypoxia is mediated by *TRB3* and TNF-α.

Discussion

This paper presents the following significant results: (1) *in vitro* hypoxia and *in vivo* AMI up-regulates TRB3 expression in cardiomyocytes; (2) JNK MAP kinase and the GADD153 transcription factor are involved in the signaling pathway of TRB3 induction; (3) TRB3 expression in cardiomyocytes is induced by hypoxia through TNF-α; (4) hypoxia enhancing cardiomyocytes apoptosis is TRB3 dependent; and (5) atorvastatin inhibits TRB3 expression and apoptosis in cardiomyocytes induced by hypoxia and AMI.

In the present study, we demonstrate that TRB3 mRNA and protein expression in cardiomyocytes were up-regulated under hypoxia. TRB3 expression induced by
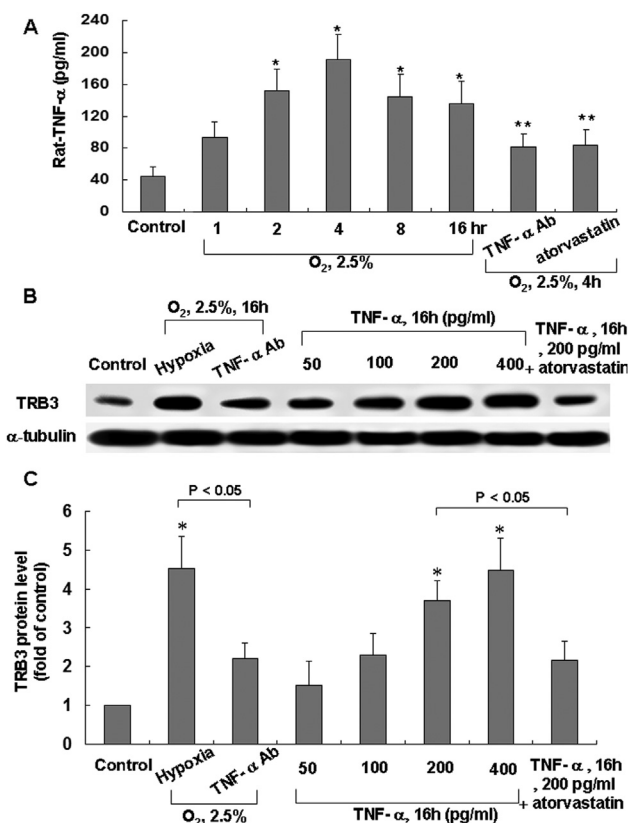
Figure 5 Effects of rat TNF-α on TRB3 in cardiomyocytes. (A) Release of rat TNF-α from cardiomyocytes subjected to hypoxia for various periods of time ($n = 3$ per group). *$P < 0.05$ vs. control; **$P < 0.05$ vs. hypoxia for 4 hours. (B) Representative Western blots for TRB3 in cardiomyocytes after exogenous administration of TNF-α. (C) Quantitative analysis of TRB3 protein levels. Values for cardiomyocytes were normalized to match the α-tubulin measurement and then expressed as a ratio of normalized values to control cells ($n = 3$ per group). *$P < 0.05$ vs. control.

hypoxia is time-dependent. We demonstrated previously that TRB3 expression in cardiomyocytes was induced by cyclic stretch and *in vivo* volume overload.[18] In addition, Avery et al reported that TRB3 was induced in response to an *in vitro* model of ischemia—reperfusion injury for 4 hours in HL-1 cardiomyocytes.[8] However, it was demonstrated that CoCl$_2$, a chemical inducer of hypoxia, did not affect the TRB3 expression in PC-3 cells.[19] A partial explanation for this may be that the cell was different. Another possible explanation for this is the difference between physical and chemical hypoxic stresses. Moreover, previous studies have demonstrated that AMI can induce TRB3 expression in cardiomyocytes.[8,9] These observations are consistent with our results regarding AMI-induced myocardial TRB3 expression.

Our data reveal that TRB3 protein expression induced by hypoxia is mediated by JNK because the JNK inhibitor and siRNA abolished the TRB3 expression. SP600125, a specific inhibitor of JNK, inhibited the TRB3 expression induced by hypoxia. However, inhibitors of ERK and p38 MAPK had no inhibitory effect. Previously, we determined that TRB3 expression induced by cyclic stretch is mediated by JNK.[17]

Furthermore, Humphrey et al have demonstrated that TRB3 is required for optimal MLK3-JNK activation in pancreatic beta cells.[20] Adding SB203580 was demonstrated as enhancing the expression of TRB3 in response to 4-hydroxy-trans-2-nonenal (4-HNE) in PC12 cells.[21] Furthermore, Zhang et al indicated that TRB3 may be involved in diabetic nephropathy by regulating the collagen type IV through the ERK1—2 MAPK pathway.[22] These findings suggest that TRB3 is closely linked with the MAPK signaling pathway. In the present study, we demonstrated that hypoxia-induced TRB3 expression acts via the GADD153 pathway. The TRB3 gene contains an AARE similar to genomic elements that are responsive to activation by GADD153.[10] Bromati et al noted that GADD153 cooperates with ATF4 to activate the *TRB3* gene.[23] In addition, a study indicated that hypoxia-induced TRIB3 is regulated by the GADD153 pathway of the UPR.[24] These findings are consistent with our results.

In this study, we provide the first evidence that TRB3 protein expression in cardiomyocytes is induced by hypoxia through TNF-α. Ord et al demonstrated that TRB3 is regulated by IL-3 in mouse bone marrow—derived mast cells.[25] In addition, it has been revealed that induction of TRB3 in hepatocytes is induced by IFN-gamma.[26] These studies complement each other because each has emphasized a different aspect of experimental model or cells. Notably, Steverson et al indicated that TRB3 overexpression suppressed inflammation by reduced expression and secretion of TNF-α.[27] In another study, knockdown of *TRB3* by siRNA significantly induced the production of TNF-α in RBL-CCR1 cells.[28] These results suggest a feedback relationship between TRB3 and TNF-α.

Hypoxia-induced cardiomyocyte apoptosis is regulated by *TRB3*. Wang et al have suggested that TRB3 mediates renal tubular-cell apoptosis induced by protein overload.[29] A previous study demonstrated that TRB3 was involved in lipoapoptosis of INS-1 β cells.[30] Moreover, TRB3 was reported to be a crucial mediator of palmitate-induced apoptosis in human liver cells.[31] The findings of these studies are consistent with our data that TRB3 was involved in apoptosis. By contrast, a study indicated that TRB3 contributes to the survival and activity of primary cultured-mast cells.[25] Furthermore, Zhou et al demonstrated that knocking down *TRB3* significantly induced lung-cancer cell apoptosis.[32] These findings implicate that *TRB3* plays a crucial role in not only cell death but also survival. In general, the characteristic of *TRB3* is injurious rather than protective.

In this study, we demonstrated that atorvastatin inhibits TRB3 expression and apoptosis induced by hypoxia and AMI in cardiomyocytes. A previous study demonstrated that atorvastatin protects cardiomyocyte H9C2 from oxidative stress through the inhibition of lectin-like oxidized low-density lipoprotein receptor-1 and apoptosis.[33] Abdel-Hamid et al also suggested that atorvastatin could preserve myocardial structure in diabetic cardiomyopathy by suppressing cardiomyocyte apoptosis and oxidative stress.[34] These results indicate that atorvastatin has heart-protective effects from oxidative stress. The findings of our study indicate that TNF-α induced by hypoxia is inhibited by atorvastatin. Moreover, it has been implicated that treatment with atorvastatin can reduce TNF-α in
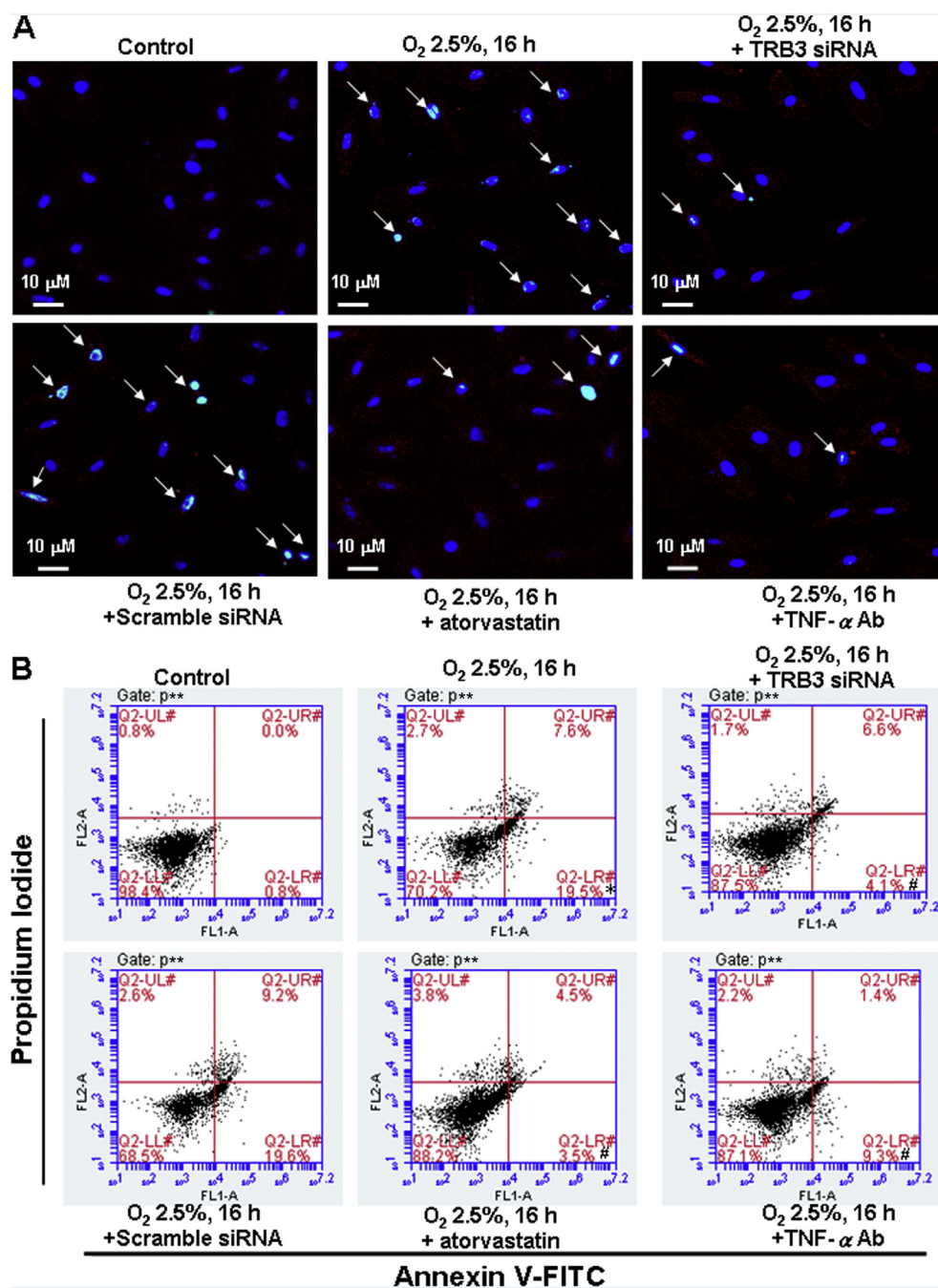
Figure 6 Effects of TRB3 on hypoxia-induced apoptosis in cardiomyocytes. (A) A TUNEL assay was used to detect the cardiomyocyte apoptosis after hypoxia. Blue indicates nuclei stained with DAPI. Red indicates the cytoskeleton stained with rhodamine. Green indicates TUNEL-positive nuclei. Representative microscopic images of cardiomyocytes after hypoxia for 16 hours, addition of TNF-α Ab, *TRB3* siRNA, or atorvastatin before hypoxia, then stained using the TUNEL kit. Similar results were observed in other two independent experiments. (B) Cardiomyocytes were subjected to hypoxia for 16 hours, TNF-α Ab, *TRB3* siRNA, or atorvastatin (10 μM) was added before hypoxia. Quantification of the apoptotic fractions was performed using FACScan. Cells that stained negative for both Annexin V and PI were alive. Cells that stained positive for Annexin V and negative for PI were undergoing apoptosis. Cells that stained positive for both Annexin V and PI were at the end stage of apoptosis called second apoptosis ($n = 3$). $*P < 0.05$ vs. control; $**P < 0.05$ vs. hypoxia for 16 hours.

ischemic heart-failure patients.[15] Moreover, Dai et al demonstrated that atorvastatin inhibits the production of TNF-α after hypoxia or AMI.[35] The findings of these studies are consistent with our results. We found that atorvastatin can abrogate the GADD153 binding activity induced by hypoxia. By contrast, Sun et al indicated that atorvastatin can enhance levels of nuclear factor erythroid 2-related factor 2 to alleviate oxidative stress induced by

ischemia—reperfusion.[16] These findings indicate that ator-vastatin can be considered a nonlipid-lowering benefit of statins to improve oxidative stress disease.

We found protein expression of HIF-1α is induced by hypoxia (Figures S5A and S5B). A previous study demon-strated that hypoxia can increase the expression of HIF-1α in cardiomyocyte H9$_C$2.[36] Treatment of atorvastatin before hypoxia significantly inhibited the protein expression of HIF-1α induced by hypoxia (Figures S5C and S5D). In addi-tion, adding the HIF-1α siRNA before hypoxia did not have a significant effect on the TRB3 and TNF-α protein expression induced by hypoxia (Figure S6). Wennemers et al indicated that knockdown of HIF-1α did not alter the anoxia induction of TRB3 expression in breast cell lines.[24] These results suggest TRB3 expression induced by hypoxia is HIF-1α independent.

In summary, our study reports that hypoxia induces TRB3 expression in rat cardiomyocytes. Hypoxia induces TRB3 through TNF-α, JNK MAPK, and the GADD153 pathway. Treatment of atorvastatin inhibits the TRB3 expression and cardiomyocyte apoptosis induced by hypoxia and AMI. This study concludes that atorvastatin can alleviate car-diomyocyte apoptosis by inhibiting TRB3 induced by hyp-oxia and AMI. The findings of this study may contribute toward a clearer understanding of the role of *TRB3* under ischemia in cardiomyocytes.

Acknowledgment

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jfma.2016.07.010.

References

1. Sun A, Zou Y, Wang P, Xu D, Gong H, Wang S, et al. Mitochon-drial aldehyde dehydrogenase 2 plays protective roles in heart failure after myocardial infarction via suppression of the cytosolic JNK/p53 pathway in mice. *J Am Heart Assoc* 2014;**3**: e000779.
2. Hashem SI, Perry CN, Bauer M, Han S, Clegg SD, Ouyang K, et al. Brief report: oxidative stress mediates cardiomyocyte apoptosis in a human model of Danon Disease and heart failure. *Stem Cells* 2015;**33**:2343—50.
3. Liu M, Xue M, Wang XR, Tao TQ, Xu FF, Liu XH, et al. *Panax quinquefolium* saponin attenuates cardiomyocyte apoptosis induced by thapsigargin through inhibition of endoplasmic re-ticulum stress. *J Geriatr Cardiol* 2015;**12**:540—6.
4. Doroudgar S, Völkers M, Thuerauf DJ, Khan M, Mohsin S, Respress JL, et al. Hrd1 and ER-associated protein degrada-tion, ERAD, are critical elements of the adaptive ER stress response in cardiac myocytes. *Circ Res* 2015;**117**:536—46.
5. Tao TQ, Wang XR, Liu M, Xu FF, Liu XH. Myofibrillogenesis regulator-1 attenuated hypoxia/reoxygenation-induced apoptosis by inhibiting the PERK/Nrf2 pathway in neonatal rat cardiomyocytes. *Apoptosis* 2015;**20**:285—97.
6. Pierre N, Fernández-Verdejo R, Regnier P, Vanmechelen S, Demeulder B, Francaux M. IRE1α and TRB3 do not contribute to

7. Zhang W, Zhong M, Tang MX, Ma X, Miao Y, Sun H, et al. Effect of valsartan on Tribble 3 gene expression in rats with experi-mental diabetic cardiomyopathy. *Zhonghua Xin Xue Guan Bing Za Zhi* 2006;**34**:212—6.
8. Avery J, Etzion S, DeBosch BJ, Jin X, Lupu TS, Beitinjaneh B, et al. TRB3 function in cardiac endoplasmic reticulum stress. *Circ Res* 2010;**106**:1516—23.
9. Muslin AJ. Akt2: a critical regulator of cardiomyocyte survival and metabolism. *Pediatr Cardiol* 2011;**32**:317—22.
10. Ohoka N, Yoshii S, Hattori T, Onozaki K, Hayashi H. TRB3, a novel ER stress-inducible gene, is induced via ATF4-CHOP pathway and is involved in cell death. *EMBO J* 2005;**24**: 1243—55.
11. Su N, Kilberg MS. C/EBP homology protein (CHOP) interacts with activating transcription factor 4 (ATF4) and negatively regulates the stress-dependent induction of the asparagine synthetase gene. *J Biol Chem* 2008;**283**:35106—17.
12. Li RJ, He KL, Li X, Wang LL, Liu CL, He YY. Salubrinal protects cardiomyocytes against apoptosis in a rat myocardial infarction model via suppressing the dephosphorylation of eukaryotic translation initiation factor 2α. *Mol Med Rep* 2015;**12**:1043—9.
13. Yu J, Lu Y, Li Y, Xiao L, Xing Y, Li Y, et al. Role of S100A1 in hypoxia-induced inflammatory response in cardiomyocytes via TLR4/ROS/NF-κB pathway. *J Pharm Pharmacol* 2015;**67**:1240—50.
14. Chen ZW, Qian JY, Ma JY, Chang SF, Yun H, Jin H, et al. TNF-α-induced cardiomyocyte apoptosis contributes to cardiac dysfunction after coronary microembolization in mini-pigs. *J Cell Mol Med* 2014;**18**:1953—63.
15. Oikonomou E, Siasos G, Zaromitidou M, Hatzis G, Mourouzis K, Chrysohoou C, et al. Atorvastatin treatment improves endo-thelial function through endothelial progenitor cells mobiliza-tion in ischemic heart failure patients. *Atherosclerosis* 2015; **238**:159—64.
16. Sun G, Li Y, Ji Z. Atorvastatin attenuates inflammation and oxidative stress induced by ischemia/reperfusion in rat heart via the Nrf2 transcription factor. *Int J Clin Exp Med* 2015;**8**: 14837—45.
17. Shyu KG, Cheng WP, Wang BW, Chang H. Hypoxia activates muscle-restricted coiled-coil protein (MURC) expression via transforming growth factor-β in cardiac myocytes. *Clin Sci (Lond)* 2014;**126**:367—75.
18. Cheng WP, Wang BW, Lo HM, Shyu KG. Mechanical stretch in-duces apoptosis regulator TRB3 in cultured cardiomyocytes and volume-overloaded heart. *PLoS One* 2015;**10**:e0123235.
19. Schwarzer R, Dames S, Tondera D, Klippel A, Kaufmann J. TRB3 is a PI 3-kinase dependent indicator for nutrient starvation. *Cell Signal* 2006;**18**:899—909.
20. Humphrey RK, Ray A, Gonguntla S, Hao E, Jhala US. Loss of TRB3 alters dynamics of MLK3-JNK signaling and inhibits cytokine-activated pancreatic beta cell death. *J Biol Chem* 2014;**289**:29994—30004.
21. Lin MH, Yen JH, Weng CY, Wang L, Ha CL, Wu MJ. Lipid per-oxidation end product 4-hydroxy-trans-2-nonenal triggers unfolded protein response and heme oxygenase-1 expression in PC12 cells: roles of ROS and MAPK pathways. *Toxicology* 2014; **315**:24—37.
22. Zhang L, Zhang J, Liu X, Liu S, Tian J. Tribbles 3 regulates the fibrosis cytokine TGF-β1 through ERK1/2-MAPK signaling pathway in diabetic nephropathy. *J Immunol Res* 2014;**2014**:240396.
23. Bromati CR, Lellis-Santos C, Yamanaka TS, Nogueira TC, Leonelli M, Caperuto LC, et al. UPR induces transient burst of apoptosis in islets of early lactating rats through reduced AKT phosphorylation via ATF4/CHOP stimulation of TRB3 expression. *Am J Physiol Regul Integr Comp Physiol* 2011;**300**:R92—100.
24. Wennemers M, Bussink J, Scheijen B, Nagtegaal ID, van Laarhoven HW, Raleigh JA, et al. Tribbles homolog 3 denotes a

poor prognosis in breast cancer and is involved in hypoxia response. *Breast Cancer Res* 2011;**13**:R82.

25. Ord T, Ord D, Kuuse S, Plaas M, Ord T. Trib3 is regulated by IL-3 and affects bone marrow-derived mast cell survival and function. *Cell Immunol* 2012;**280**:68—75.

26. Kanki K, Kawamura T, Watanabe Y. Control of ER stress by a chemical chaperone counteracts apoptotic signals in IFN-gamma-treated murine hepatocytes. *Apoptosis* 2009;**14**:309—19.

27. Steverson Jr D, Tian L, Fu Y, Zhang W, Ma E, Garvey WT. Tribbles homolog 3 promotes foam cell formation associated with decreased proinflammatory cytokine production in macrophages: evidence for reciprocal regulation of cholesterol uptake and inflammation. *Metab Syndr Relat Disord* 2016;**14**:7—15.

28. Kuo CH, Morohoshi K, Aye CC, Garoon RB, Collins A, Ono SJ. The role of TRB3 in mast cells sensitized with monomeric IgE. *Exp Mol Pathol* 2012;**93**:408—15.

29. Wang W, Cheng J, Sun A, Lv S, Liu H, Liu X, et al. TRB3 mediates renal tubular cell apoptosis associated with proteinuria. *Clin Exp Med* 2015;**15**:167—77.

30. Qin J, Fang N, Lou J, Zhang W, Xu S, Liu H, et al. TRB3 is involved in free fatty acid-induced INS-1-derived cell apoptosis via the protein kinase C δ pathway. *PLoS One* 2014;**9**:e96089.

31. Yan W, Wang Y, Xiao Y, Wen J, Wu J, Du L, et al. Palmitate induces TRB3 expression and promotes apoptosis in human liver cells. *Cell Physiol Biochem* 2014;**33**:823—34.

32. Zhou H, Luo Y, Chen JH, Hu J, Luo YZ, Wang W, et al. Knockdown of TRB3 induces apoptosis in human lung adenocarcinoma cells through regulation of Notch 1 expression. *Mol Med Rep* 2013;**8**:47—52.

33. Zhang L, Cheng L, Wang Q, Zhou D, Wu Z, Shen L, et al. Atorvastatin protects cardiomyocytes from oxidative stress by inhibiting LOX-1 expression and cardiomyocyte apoptosis. *Acta Biochim Biophys Sin (Shanghai)* 2015;**47**: 174—82.

34. Abdel-Hamid AA. Firgany Ael-D. Atorvastatin alleviates experimental diabetic cardiomyopathy by suppressing apoptosis and oxidative stress. *J Mol Histol* 2015;**46**:337—45.

35. Dai G, Xu Q, Luo R, Gao J, Chen H, Deng Y, et al. Atorvastatin treatment improves effects of implanted mesenchymal stem cells: meta-analysis of animal models with acute myocardial infarction. *BMC Cardiovasc Disord* 2015;**15**:170.

36. Jin HJ, Xie XL, Ye JM, Li CG. TanshinoneIIA and cryptotanshinone protect against hypoxia-induced mitochondrial apoptosis in H9c2 cells. *PLoS One* 2013;**8**:e51720.