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Original Research

Involvement of TRPC1 in Nampt-induced cardiomyocyte hypertrophy through the activation of ER stress

J. Li, W. Wu, M. Zhao, X. Liu*

Laboratory of Cardiovascular Diseases, Regenerative Medicine Research Center, West China Hospital, Sichuan University, Chengdu 610041,China

Correspondence to: liuxq@scu.edu.cn

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Abstract: Nicotinamide phosphoribosyltransferase (Nampt) is involved in the development of cardiac hypertrophy. Transient receptor potential canonical channel 1 (TRPC1) and endoplasmic reticulum stress (ER stress) are regarded as critical pathways in cardiac hypertrophy. Therefore, we hypothesizedthat TRPC1 might be associated with ER stress in Nampt-induced cardiac hypertrophy. CulturedH9c2cardiomyocyteswereexposed to Namptfor different timesand the expression of markers of cardiomyocyte hypertrophy and ER stress, as well as TRPC1 were detected. Moreover, specific TRPC1-shRNA (short hairpin RNA) expressing plasmid was transfected to knockdown TRPC1 expression before Nampt stimulation. Thapsigargin was used as an agonist and pravastatin was employed as an inhibitor of ER stress. The results demonstrated that exposure of H9c2 cells to 100 ng/mL Nampt for 24h, 48h or 72h significantly increased the expression of atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), markers of ER stress and TRPC1. The Nampt-induced expression of TRPC1 was attenuated by pre-treatment with pravastatin, whereas promoted by pre-treatment with thapsigargin. Furthermore, transfection of TRPC1-shRNA for 48h partially inhibited Nampt-induced expression of ER stress markers and BNP in H9c2 cells. Our data suggest that TRPC1 might play an important role in cardiomyocyte hypertrophy induced by Namptinan ER stress-dependent way.

Key words: Nampt; Cardiomyocyte hypertrophy; TRPC1; ER stress.

Introduction

Nicotinamide phosphoribosyltransferase (Nampt) is a pleiotropic molecule that functions as an enzyme, cytokine, growth factor and hormone (1). It is also reported as a kind of adipocytokins secreted from visceral fat (visfatin) (2), and is a cytokine (pre-cell colony enhancing factor, PBEF) acting on early B lineage precursor cells subsequently (3). Nampt participates in various pathological conditions, such as inflammation, obesity, and type 2 diabetes (4). Elevated circulating levels of Nampt are reported in some cardiovascular diseases, including heart failure and cardiac hypertrophy (5). Importantly, a recent study has shown that cardiomyocytes are capable of secreting Nampt during stresses, and exogenous Nampt is a regulator of cardiac hypertrophy and adverse ventricular remodeling(1). But so far, the mechanism of Nampt in cardiac hypertrophy remains largely unknown.

Cardiomyocyte hypertrophy is initially as a compensatory response to enhanced cardiac workload.However, sustained increase in cardiac workload and activation of neuro humoral factors lead to pathological cardiomyocyte hypertrophy (6). ER stress is increasingly merged as a pathologic factor responsible for triggering the cardiac hypertrophy. It is reported that ER stressoccurs in cardiomyocytes by inducing the expression of ER chaperones, C/EBP-homologous protein/growth arrest and DNA damage-inducible gene 153 (CHOP/Gadd153) (7). Moreover, amelioration of ER stress by4-phenyl butyric acid reduces chronic hypoxia-induced cardiac damage and improves hypoxic tolerance through up regulation of hypoxia-inducible factor (HIF)-1α (8). In our previous study, we observed that ER stress could be triggered in cardiomyocyte hypertrophy induced by Nampt (9). But the precise mechanism of ER stress in cardiomyocyte hypertrophy induced by Nampt is still poorly understood.

On the other hand, transient receptor potential channel 1 (TRPC1) plays an essential role in regulating cardiomyocyte hypertrophy. It's a member of the TRPC family of ion channels which comprises 6 subtypes (TRPC1 to TRPC6) (10). Activation of TRPC1 is involved in the process of cardiac hypertrophy, and of particular note, it acts as a unique sensor for a wide range of hypertrophic stimuli (11). Evidence has indicated that TRPC1 is up regulated in pressure overload-induced cardiac hypertrophy in mice by initiating Ca^{2+}-dependent signaling pathways (12).

Although both TRPC1 and ER stress are important in cardiac hypertrophy, whether TRPC1 interacts with ER stress in Nampt-induced cardiac hypertrophy remains to be elucidated. Therefore, in our present study, we aimed to investigate the relationship between TRPC1 and ER stress in the cardiomyocyte hypertrophy induced by Nampt.

Materials and Methods

Cell culture and treatments

H9c2 cells were obtained from American Type and Culture Collection (ATCC) and cultured in high glucose DMEM containing 10% fetal bovine serum (FBS, BI, Israel) and 100U/ml penicillin-streptomycin(13). The cells were grown on P60 plates with 2ml medium in the incubator at 37℃ under an environment of 5% CO_2. H9c2 cells were starved in serum free medium for 6h before exposure to 100ng/ml Nampt for 4h, 12h, 24h, 36h, 48h, or 72h. Thapsigargin (0.1μM,Enzo, USA) was used as the agonist and pravastatin (10μM) was used as an inhibitor of ER stress to study the relationship between ER stress and TRPC1 in H9c2 cells (14, 15).

Forced transient expression of TRPC1

TRPC1 over-expression plasmid (kindly from Addgene, ID24408) was transient transfected into H9c2 cells using the cationic lipid, Lipofectamine3000™ (Invitrogen, USA), according to the manufacturer's instruction. After effective over-expression of TRPC1, the level of brain natriuretic peptide (BNP) was assessed by real-time RT-PCR.

RNA interference

TRPC1 specific targeting small inhibitory RNA (shRNA) plasmid(TRPC1-shRNA) was constructed and transient transfected into H9c2 cells using Lipofectamine3000™ (Invitrogen, USA). After effective knock down of TRPC1, the expression of BNP, glucose-regulated protein 78 (GRP78), activating transcription factor 6 (ATF6) and CHOP was analyzed by real-time RT-PCR respectively.

Real-time polymerase chain reaction assays (Real time RT-PCR)

Total RNA was extracted using TRIzol reagent. cDNA was synthesized using oligo (dT) 12-18 primer (Invitrogen) and Superscript II reverse transcriptase (Invitrogen). Then the gene expression was measured on CFX96™ Real-Time PCR Detection System (BIO-RAD, USA) with fluorescence dye SYBRGreen (SYBRGreen Supermix kit, Bio-Rad, USA) according to our previous report (16). Normalization of gene expression was assessed by comparing the expression of β-actin for the corresponding sample. Primers were used as follows: ANP F: 5'-ACCAAGGGCTTCTTCCTCT-3' and R:5'-TTCTACCGGCATCTTCTCC-3'; BNPF:5'-GCTCTTCTTTCCCCAGCTCT-3', and R:5'-ACTGTGGCAAGTTTGTGCTG-3'; GRP78 F:5'-CCCCAGATTGAAGTCACCTTTGAG-3' and R:CAGGCGGTTTTGGTCAT-TG; CHOP F:5'-AGCAGAGGTCACAAGCACCT-3' and R:5'-CTCCTTCATGCGCT-GTTTCC-3'; ATF6 F:5'-GCAGGTGTATTACGCTTCGC-3' and R: 5'-TGTGGTCTTGTTATGGGTGG-3', TRPC1 F:5'-AGCCTCTTGACAAACGAGGA-3' and R:5'TGACAT-CTGTCCGAACCAAA-3'; β-actin F: 5'-ACTATCGGCAATGAGCGGTTC-3'and R:5'-ATG- CCACAGGATTCCATACCC-3'.

Western Blot Analysis

Western blot was performed as described previously (16). Total proteins were lysed by RIPA lysis buffer with the protease inhibitor cocktail (Roche, USA). Cell lysates (25ug) were separated by 10% denaturing SDS-PAGE and then transferred to PVDF membrane (Millipore, USA) using a MiniProtein III system (Bio-Rad, USA). The primary antibody was rabbit anti-mouse TRPC1 (Santa Cruz, USA) or mouse anti β-actin(Santa Cruz, USA). Signals were visualized using enhanced chemiluminescence (ECL) plus substrate (Pierce, USA). The density was determined by the Bio-Rad Quantity One 1-D analysis software (Bio-Rad, USA).

Cell Surface Area Determination

H9c2 cells were cultured on collagen type I-coated coverslips in DMEM-Highmedium containing 1% bovine serum albumin (BSA) and treated with different concentrations of stimuli. After treatment, the cells were rinsed with PBS, fixed with 4% formaldehyde and permeabilized with 0.1% Triton X-100. Then the cells were incubated with 10μM phalloidine(Sigma, USA) for 40 min at 37℃. Finally, 4, 6-diamino-2-phenylindole (DAPI, 1:450) was used to stain the cell nuclei. Eclipse TE2000-U fluorescent microscope system (Nikon, Japan) was applied to visualize the cells, followed by determination of cell surface area with Image-J software (National Institutes of Health).

Statistical analysis

Data were expressed as means ± SEM. Group means were compared by *ANOVA* using the statistical software program SPSS 10.0 for Windows (Chicago,IL, USA), and P value<0.05 was considered statistically significant in all cases.

Results

The expression of TRPC1 is increased in Nampt-induced cardiomyocyte hypertrophy

To evaluate whether cardiomyocyte hypertrophy could be induced by Nampt stimulation, the hypertrophic markers,ANP and BNP, were detected by real time RT-PCR and the cell surface area was detected by F-actin staining. The data showed that, the expression of ANP were up regulated markedly after treated with Nampt for 24h(4.8-fold), 48h (8.4-fold) and 72h (3.0-fold)respectively, compared with those in the controls without any treatment. And BNP levels were elevated about 1.8-fold, 1.9-fold and 1.7-fold respectively (Fig. 1A).The cell surface areas were increased approximate1.3-fold, 1.8-fold and 1.6-fold after Nampt treatment for 24h, 48h and 72h, compared with those of the controls (Fig. 1B, C). The data suggest that Nampt could induce cardiomyocyte hypertrophy in H9c2 cells.

Then we detected mRNA expression of TRPC1 in Nampt-induced cardiomyocyte hypertrophy. As shown in Fig. 1D, the gene expression of TRPC1 was increased significantly in cells treated with Nampt for 24h (6.2-fold), 36h (12.0-fold), 48h (9.3-fold) and 72h (11.5-fold) respectively, compared with those in the controls without any treatment. Moreover, we examined whether Nampt stimulation affected the protein expression of TRPC1 by Westernblot. As shown in Fig. 1E, the protein level of TRPC1 in H9c2 cells was approximately increased 3.2-fold at 24h, 5.8-fold at 48h and 3.3-fold

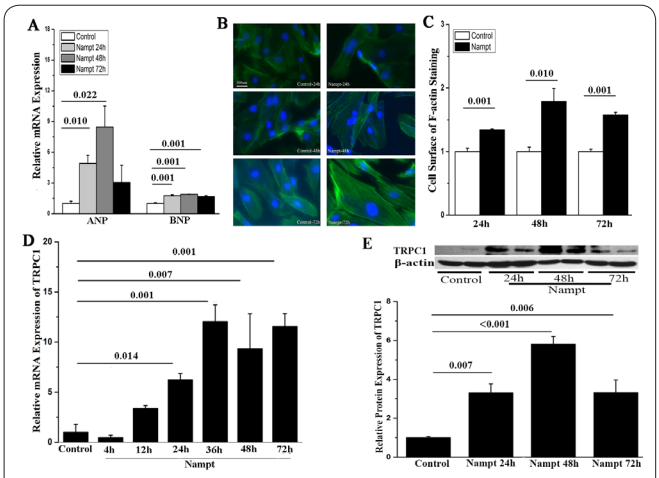

Figure 1. TRPC1 expression in Nampt-induced cardiomyocyte hypertrophy was increased (n=3). A. The mRNA expression of ANP and BNP was induced by 100ng/mL of Nampt after treated for 24h, 48h, or 72 h. **B.** Observation of cytoskeleton F-actin staining by fluorescence microscope, the green color indicated F-actin staining and the blue indicated the cell nucleus stained by DAPI (magnification 200X). **C.** Statistics of cell surface indicated that the cell surface areas enlargement was induced in H9c2 cells by Nampt treatment. **D.** The gene expression of TRPC1 was induced by 100ng/mL of Nampt treated for 24 h, 36 h, 48 h, or 72 h. **E.** The protein expression of TRPC1 was induced by 100ng/mL of Nampt treated for 24 h, 48 h, or 72 h.

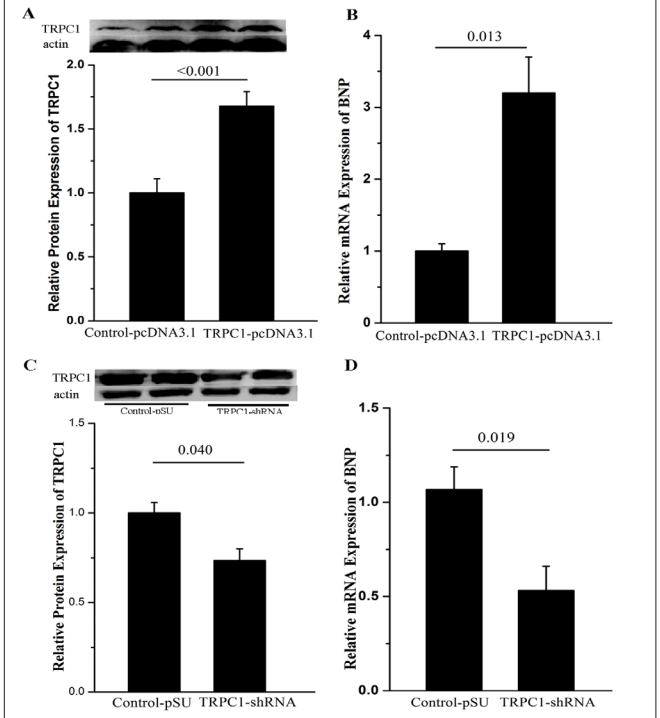

Figure 2. Knockdown of TRPC1 alleviated Nampt-induced cardiomyocyte hypertrophy (n=3). A. TRPC1 expression was induced by transfection of TRPC1 over-expressing plasmid. **B.** Cardiomyocyte hypertrophy was induced by forced expression of TRPC1 significantly. **C.** The level of TRPC1 was inhibited significantly by transfection of TRPC1-shRNA plasmid. **D.** Knockdown of TRPC1 reduced Nampt induced BNP level in H9c2 cells.

at 72h, compared with those of the controls without any treatment.

Knockdown of TRPC1 alleviates Nampt-induced cardiomyocyte hypertrophy

In order to confirm the role of TRPC1 in Nampt-induced cardiomyocyte hypertrophy, the TRPC1 expressing plasmid was transfected into H9c2 cells, which gained enhanced TRPC1 protein expression by nearly 1.7-fold (Fig. 2A). As demonstrated in Fig. 2B, the transcription of the fetal gene BNP was elevated about 3.2-fold in H9c2 cells transfected with TRPC1 expressing plasmid.

Next, TRPC1-shRNA expressing plasmid was transfected to H9c2 cells before Nampt stimulation. The results showed that genetic silencing of TRPC1 expression inhibited Nampt-induced BNP expression by almost 50% (Fig. 2C, D). Taken together, these data revealed a necessary role of TRPC1 in Nampt-indcued pathological cardiomyocyte hypertrophy.

ER stress is activated and interacts with TRPC1 in Nampt-induced cardiomyocytehypertrophy

To investigate the potential mechanism of TRPC1 in Nampt-induced cardiomyocyte hypertrophy, we measured the expression of ER stress markers. As shown in Fig. 3A, ER stress was triggered by Nampt stimulation. Moreover, the Nampt-induced ER stress was inhibited by almost 70% in H9c2 cells pre-treated with pravastatin, and was further increased about 5-fold in H9c2 cells pre-treated with thapsigargin, compared with the cells without pre-treatment (Fig. 3B). Pre-treatment of pravastatin also inhibited the mRNA expression of ANP and BNP nearly to 50%, whereas thapsigargin elevated the Nampt-induced expression of ANP and BNP about 1.5- and 1.9-fold respectively, compared with those in the controls without pre-treatment (Fig. 3C).Furthermore, TRPC1 expression was inhibited upto 50% with pravastatin pre-treatment, whereas was increased by nearly 2.8- fold with thapsigargin pre-treatment (Fig. 3D).

To verify whether TRPC1 mediates cardiomyocyte hypertrophy induced by Nampt via ER stress pathways, we transfected TRPC1-shRNA into H9c2 cells to knockdown TRPC1 expression. The results demonstrated that, gene silencing of TRPC1 reduced the GRP78, ATF6 and CHOP expressionby up to20%, 45% and 60% respectively in H9c2 cells exposed to Nampt (Fig. 3E). These findings provide evidence that the effect of TRPC1 is crosstalked with ER stress in Nampt-induced cardiomyocyte hypertrophy.

We then evaluated the morphologic changes of H9c2 cells after TRPC1 was forced expressed or knocked down. We determined the cell surface area by F-actin staining. As shown in Fig. 3F, TRPC1 over-expression increased the cell surface area by nearly 1.5-fold in H9c2 cells with or without Nampt treatment. And gene silencing of TRPC1 significantly reduced the cell surface area by about 20% in H9c2 cells in the presence of Nampt (Fig. 3G).

Collectively, these results indicated that TRPC1 could play a pivotal role in cardiomyocyte hypertrophy induced by Namptin an ER stress-dependent pathway.

Discussion

In the present study, the crosstalk between TRPC1 and ER stress in Nampt-induced cardiomyocyte hypertrophy was investigated. There are three major findings:

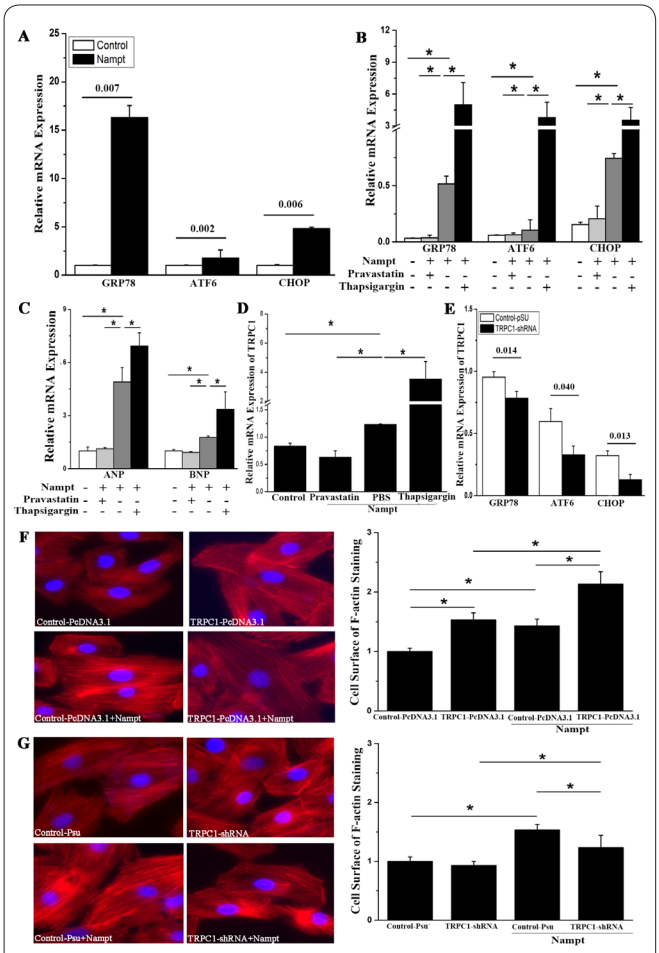

Figure 3. ER stress was activated and associated with TRPC1 in Nampt-induced cardiomyocyte hypertrophy (n=3). A. Levels of ER stress markers (GRP78, ATF6 and CHOP) were elevated in Nampt-induced cardiomyocyte hypertrophy. **B.** The level of ER stress markers in Nampt-induced cardiomyocyte were suppressed by pravastatin but further promoted by thapsigargin. **C.** Pre-treatment with pravastatin partially inhibited the cardiomyocyte hypertrophy induced by Nampt in H9c2 cells, whereas thapsigargin promoted the Nampt-induced cardiomyocyte hypertrophy. **D.** The Nampt-induced expression of TRPC1 was significantly suppressed by pravastatin but promoted by thapsigargin. **E.** Knockdown of TRPC1 expression reduced the levels of GRP78, ATF6 and CHOP in H9c2 cells treated with Nampt. **F, G.** Cell surface areas were increased after forced expression of TRPC1 but decreased after knockdown of TRPC1 expression (the red color indicated F-actin staining and the blue indicated the cell nucleus stained by DAPI, magnification 300X).* $P<0.05$.

(1) TRPC1 expression is increased in hypertrophic cardiomyocytes induced by Nampt in a time-dependent manner; (2) Knockdown of TRPC1 results in the inhibition of cardiomyocyte hypertrophy induced by Nampt, whereas over expression of TRPC1 leads to the promotion of cardiomyocyte hypertrophy. (3) Knockdown of TRPC1 may relieve prolonged ER stress activated by Nampt, which plays a protective role against cardiac hypertrophy. Our data suggest that ER stress may interact with TRPC1 up regulation induced by Nampt.

Evidence has shown that Nampt participates in some pathophysiological cardiovascular processes. In patients with coronary atherosclerosis or patients with acute myocardial infarction,circulating Nampt levels areelevated (17, 18). Nampt heterozygous $^{(+/-)}$mice are protected against is oproterenol or angiotensin II-induced hypertrophy (1). And inhibition of Nampt reduces neutrophil-mediated injury during mice myocardial infarction *in vivo* (19). However, contrary to the above reports, Lim *et al* have reported that Nampt has a cardio-protective effect against cardiomyocytes apoptosis in the ischemia-reperfusion injury (20). Our present study confirmed that cardiomyocyte hypertrophy could be induced by Nampt in a time-dependent manner. The possible reason for this disparity between our data and Lim's report (20) might be explained by different models of diseases. The model they used was ischemia-reperfusion injury and our study model was cardiomyocyte hypertrophy. In addition, the mechanism between cardiac apoptosis and hypertrophy is largely different.

It has been demonstrated that Jun N-terminal kinase 1 (JNK1), p38, and extracellular signal-regulated kinases (ERK) signaling pathways are involved in Nampt-induced cardiomyocyte hypertrophy (1). Nampt expression isincreased mainly through the Ang II type-1 receptor (AT1-R) -Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway in the process of Ang II-induced cardiomyocyte hypertrophy (5). In skeletal muscle cells, Namptregulates glucose metabolism via the Ca^{2+}-mediated adenosine 5'-monophosphate (AMP)-activated protein kinase (AMPK)-p38 mitogen-activated protein kinases (MAPK) pathway (21). To briefly conclude, cardiomyocyte hypertrophy induced by Nampt is related to Ca^{2+} signaling closely. Our results also illustrated that Nampt-induced cardiomyocyte hypertrophy was related to TRPC1, one member of TRPC family of ion channels permeable to Ca^{2+}. The expression of TRPC1 increased along with the Nampt stimulation time prolongation. Moreover, gene silencing of TRPC1 in H9c2 cells treated with Nampt inhibited the up regulation of BNP expression, while forced expression of TRPC1 induced the expression of BNP. These results indicated that enhanced TRPC1 expression contributed to the development of Nampt-related cardiomyocyte hypertrophy.

TRPC1 has been recognized as astore-operated Ca^{2+}channels (SOC). It is primarily located in the membranes of the ER and the plasma, and has been linked to ER Ca^{2+} homeostasis (22). Store-operated Ca^{2+} entry (SOCE) refers to refilling of internal Ca^{2+} stores in the endoplasmic/sarcoplasmic reticulum (ER/SR) after depletion. Recent studies have shown that SOCE contributes to the activation of Ca^{2+}/calmodulin (CaN)-calcineurin pathway, and nuclear translocation of the Ca^{2+}-senstivie transcription factor, nuclear factor of activated T cells (NFAT), which ultimately leads to cardiac hypertrophy (23).

ER is also a crucial organelle in maintaining Ca^{2+}homeostasis. Disturbance of Ca^{2+} homeostasis could trigger ER stress. As a pathway responsible for inducing cardiac hypertrophy, ER stress can directly induce protein synthesis in cardiomyocytes and stimulate the Ca^{2+}/CaN pathway by increasing cytosolic Ca^{2+} (24). Both TRPC1 and ER stress are involved in the Ca^{2+}/CaN pathway in cardiomyocyte hypertrophy, although, the association between TRPC1 and ER stress remains to be clarified. Therefore,we used thapsigargin as the agonist and pravastatin as an inhibitor of ER stress to study the relationship between TRPC1 and ER stress.

Our data showed that ER stress in hypertrophic car-

diomyocytes induced by Nampt was suppressed by pravastatin, but further promoted by thapsigargin. More interestingly, TRPC1 expression induced by Nampt was also inhibited by pravastatin and triggered by thapsigargin. Pravastatin is a water-soluble active drug, and the effects of reducing ER stress may be independent of its cholesterol-lowering activity (15). Thapsigargin is a specific inhibitor of the fusion of autophagosomes with lysosomes, the last step in the autophagic process. The inhibition of the autophagy in turn induces ER stress (14). Our present results demonstrated that TRPC1 might participate in cardiomyocyte hypertrophy via ER stress. To confirm this finding, we transfected TRPC1-shRNA to H9c2 cells to knockdown TRPC1 expression, and observed that the expression of ER stress markers (such as GRP78, ATF6 and CHOP) was decreased significantly.The underlying mechanism might be that, TRPC1 exerts a vital effect in maintaining ER Ca^{2+} homeostasis and alternation of its function leads to prolonged activation of the ER stress.

In conclusion, our results clarify that TRPC1 expression in cardiomyocyte hypertrophy induced by Nampt is in a time-dependent manner, and ER stress participates in the Nampt-induced TRPC1 expression. We propose that knockdown of TRPC1 expression might be a potential therapeutic strategy for cardiomyocyte hypertrophy.

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