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# Endoplasmic reticulum stress mediates homocysteine-induced hypertrophy of cardiac cells through activation of cyclic nucleotide phosphodiesterase 1C

**Wentao Sun[1,2], Yang Zhou[1,2], Hongmei Xue[1,2,3], Haitao Hou[1,2], Guowei He[1,2,4,5], and Qin Yang[1,2,*]**

[1]Center for Basic Medical Research & Department of Cardiovascular Surgery, TEDA International Cardiovascular Hospital, Chinese Academy of Medical Sciences & Peking Union Medical College, Tianjin 300457, China, [2]The Institute of Cardiovascular Diseases, Tianjin University, Tianjin 300457, China, [3]Department of Physiology, Hebei Medical University, Shijiazhuang 050017, China, [4]Drug Research and Development Center, Wannan Medical College, Wuhu 241002, China, and [5]Department of Surgery, Oregon Health and Science University, Portland, OR 97239-3098, USA

*Correspondence address. Tel: +86-22-65209089; E-mail: qyanghk@163.com

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**Abstract**

Although the association of elevated homocysteine level with cardiac hypertrophy has been reported, the molecular mechanisms by which homocysteine induces cardiac hypertrophy remain inadequately understood. In this study we aim to uncover the roles of cyclic nucleotide phosphodiesterase 1 (PDE1) and endoplasmic reticulum (ER) stress and their relationship to advance the mechanistic understanding of homocysteine-induced cardiac cell hypertrophy. H9c2 cells and primary neonatal rat cardiomyocytes are exposed to homocysteine with or without ER stress inhibitor TUDCA or PDE1-specific inhibitor Lu AF58027, or transfected with siRNAs targeting PDE1 isoforms prior to homocysteine-exposure. Cell surface area is measured and ultrastructure is examined by transmission electron microscopy. Hypertrophic markers, PDE1 isoforms, and ER stress molecules are detected by q-PCR and western blot analysis. Intracellular cGMP and cAMP are measured by ELISA. The results show that homocysteine causes the enlargement of H9c2 cells, increases the expressions of hypertrophic markers β-MHC and ANP, upregulates PDE1A and PDE1C, promotes the expressions of ER stress molecules, and causes ER dilatation and degranulation. TUDCA and Lu AF58027 downregulate β-MHC and ANP, and alleviate cell enlargement. TUDCA decreases PDE1A and PDE1C levels. Silencing of PDE1C inhibits homocysteine-induced hypertrophy, whereas PDE1A knockdown has minor effect. Both cAMP and cGMP are decreased after homocysteine-exposure, while only cAMP is restored by Lu AF58027 and TUDCA. TUDCA and Lu AF58027 also inhibit cell enlargement, downregulate ANP, β-MHC and PDE1C, and enhance cAMP level in homocysteine-exposed primary cardiomyocytes. ER stress mediates homocysteine-induced hypertrophy of cardiac cells via upregulating PDE1C expression. Cyclic nucleotide, especially cAMP, is the downstream mediator of the ER stress-PDE1C signaling axis in homocysteine-induced cell hypertrophy.

**Key words** endoplasmic reticulum stress, homocysteine, phosphodiesterase, cardiac hypertrophy

## Introduction

Elevated plasma level of homocysteine is currently considered an independent risk factor for cardiovascular diseases. It was reported that excessive homocysteine may induce cardiomyocyte hypertrophy [1–3], myocardial fibrosis [4], myocyte death [5,6], and extracellular matrix protein deposition in the heart [7], suggesting the role of homocysteine in promoting cardiac hypertrophy and remodeling. The findings regarding the correlation between homocysteine concentration and left ventricular mass in humans further demonstrated the significance of homocysteine in inducing and/or aggravating cardiac hypertrophy [8,9]. Nevertheless, the molecular mechanisms by which homocysteine induces cardiac

hypertrophy remain inadequately understood so far. It was reported that homocysteine may promote the binding of myosin enhancer factor 2C (MEF2C) with histone deacetylase1 to inactivate MEF2C, which results in the suppression of anti-hypertrophic miR-133a in cardiomyocytes [10]. By enhancing the expression of Cu-transporting ATPase 1 (ATP7a), homocysteine can lower intracellular copper content and cause subsequent inhibition of cytochrome C oxidase activity, eventually leading to cardiac hypertrophy [1]. In apolipoprotein E-deficient ($ApoE^{-/-}$) mice treated with homocysteine, Zhang and colleagues observed the upregulation of endoplasmic reticulum (ER) stress molecules in myocardium, suggesting the involvement of ER stress in homocysteine-induced myocardial fibrosis/hypertrophy [11]. However, it remains unknown how ER stress mediates hypertrophic changes of cardiac cells under hyperhomocysteinemic conditions.

Cyclic nucleotide phosphodiesterases (PDEs), a superfamily comprised of 11 gene families (PDE1 to PDE11), play critical roles in cardiovascular pathophysiology by catalyzing the hydrolysis of cyclic nucleotides. Alterations in the expression and activity of several PDEs, such as PDE1, 2, 3, 4, 5, and 9, have been observed in angiotensin-II (Ang-II)- and transverse aortic constriction (TAC)-induced cardiac hypertrophy models. For example, in rats infused with Ang-II, enhanced activities of PDE1, 2, 4, and 5, with a resultant increase in the hydrolysis of cyclic guanosine monophosphate (cGMP) and cyclic adenosine monophosphate (cAMP), were found to be associated with the progression of cardiac hypertrophy [12]. Liu and colleagues reported the posttranscriptional regulation of PDE5A by miR-19 a/b-3p in response to Ang-II infusion and its role in cardiac hypertrophy [13]. Both PDE3 inhibitor and gene ablation of PDE3 ameliorated cardiac hypertrophic changes in mice induced by TAC [14], and the anti-hypertrophic effect conferred by PDE5 and PDE9 inhibition was demonstrated to be cGMP-dependent [15,16].

Encoded by three distinct genes PDE1A, 1B, and 1C, the PDE1 family was one of the first identified classes of the PDE superfamily. PDE1 isoforms exhibit species and tissue-specific expression patterns [17–19] and show different specificities in hydrolysis of substrates cAMP and cGMP [20]. The pan-PDE1 inhibitor IC86340 or knockdown of PDE1A were found to prevent phenylephrine-induced pathological myocyte hypertrophy and hypertrophic marker expression in rat ventricular myocytes, along with an increase in cGMP/PKG activity [18], while knockdown of PDE1C showed an anti-hypertrophic effect on mouse cardiac myocytes in a cAMP/PKA-dependent manner [21]. Distinct from other PDE subfamilies, PDE1 is also referred to as $Ca^{2+}$/calmodulin-stimulated PDE [18,20]. Given the well-established role of $Ca^{2+}$ signaling in pathological cardiac remodeling, understanding the role of PDE1 in cardiac diseases is of great interest. However, clarification of the role of PDE1 in cardiovascular pathophysiology has been hampered by the lack of potent PDE1-specific inhibitor, and more experimental evidence is needed to ascertain the significance and mechanisms of PDE1 modulation as well as the contributing role of different isoforms of PDE1 in cardiac cell hypertrophy.

In the present study, by using a novel potent PDE1-specific inhibitor Lu AF58027 and gene silencing techniques, we investigated the role of PDE1 isoforms in hypertrophic changes caused by homocysteine in cardiac cells and further elucidated the relationship between ER stress and PDE1 regulation in homocysteine-induced cell hypertrophy.

## Materials and Methods

### Reagents and chemicals

D,L-homocysteine was purchased from Sigma-Aldrich (St Louis, USA). Lu AF58027 was from Lundbeck A/S (Copenhagen, Denmark). Tauroursodeoxycholic acid (TUDCA) was from Aladdin (Shanghai, China). Bovine serum albumin (BSA), DAPI solution (1 mg/mL), glycerol, 4% paraformaldehyde, and Tween-20 were purchased from Solarbio (Shanghai, China). Pancreatin was from Beyotime (Shanghai, China). Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS), and penicillin-streptomycin (P/S) were purchased from Thermal Fisher Scientific (Waltham, USA).

### Primer and siRNA sequences

Primers used for quantitative polymerase chain reaction (q-PCR) experiments were synthesized by GenePharma (Shanghai, China), and the sequences are as follows: beta-smooth muscle myosin heavy chain (*β-MHC*): 5′-AAGAACCTACTGCGACTG-3′ (forward) and 5′-TCTCCTCTGCCTCATCC-3′ (reverse); atrial natriuretic peptide (*ANP*): 5′-GAAGTCAACCCGTCTC-3′ (forward) and 5′-AATCCTGTCAATCCTACC-3′ (reverse); *PDE1A*: 5′-CCACGCAGCC GACATC-3′ (forward) and 5′-CTCCACTATGAAATCAATGAAACC-3′ (reverse); *PDE1C*: 5′-GCAGCAGAATGGTGACTTG-3′ (forward) and 5′-AAGGTAAGGCGACTTGTGG-3′ (reverse); and *GAPDH*: 5′-GCCATCACTGCCACTC-3′ (forward) and 5′-GGTAGGAA CACGGAAGG-3′ (reverse).

The siRNAs used in this study were also provided by GenePharma, and the sequences are as follows: PDE1A-1 sense 5′-GGAAGCAGUUUAUAUCGAUTT-3′, and antisense 5′-AUCGAUAU AAACUGCUUCCTT-3′; PDE1A-2 sense 5′-GUUGGUUACAGCAA GUACATT-3′, and antisense 5′-UGUACUUGCUGUAACAACTT-3′; PDE1A-3 sense 5′-GGAACCUAGUGAUUGAAAUTT-3′, and antisense 5′-AUUUCAAUCACUAGGUUCCTT-3′; PDE1C-1 sense 5′-GA GAGCAACUCUCUGAAAUTT-3′, and antisense 5′-AUUUCAGAGAG UUGCUCUCTT-3′; PDE1C-2 sense 5′-CUGGAGAUCUUUGCUAU AATT-3′, and antisense 5′-UUAUAGCAAAGAUCUCCAGTT-3′; PDE 1C-3 sense 5′-GGCCACAGAUAUGUCUUGUTT-3′, and antisense 5′-ACAAGACAUAUCUGUGGCCTT-3′; and scrambled control sense 5′-UUCUCCGAACGUGUCACGUTT-3′, and antisense 5′-ACGUGACACGUUCGGAGAATT-3′.

### Cell culture and treatment

H9c2 cardiac cells (ATCC, Manassas, USA) were grown to 60% confluence in 6-well culture plates in low glucose DMEM containing 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37°C in a humidified incubator with 5% $CO_2$. Cells were then treated with inhibitors of ER stress or PDE1 or transfected with siRNAs targeting PDE1 isoforms before exposed to 100 μM homocysteine for 72 h. For pharmacological inhibition, ER stress inhibitor TUDCA (200 μM) or PDE1-specific inhibitor Lu AF58027 (100 or 500 nM) was added to the culture medium 1 h before the application of homocysteine. Cells exposed to vehicle severed as the control.

Primary neonatal rat cardiomyocytes were prepared from 1- to 2-day-old newborn Wistar rats. Briefly, the hearts were washed, minced in DMEM, and incubated with 0.25% pancreatin solution with gentle agitation at 4°C overnight, then digested with 0.8mg/ml collagenase II at 37°C for 10 min. The cell-suspensions were centrifuged at 1500 rpm for 5 min and the precipitated cells were resuspended in DMEM supplemented with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin. The cell suspension was

pre-plated and cultured in 5% $CO_2$ incubator at 37°C for 2 h to separate fibroblasts (attached to the plate) and cardiomyocytes (unattached). The cardiomyocytes in the supernatant were then collected and cultured for another 48 h prior to 72 h-exposure to 100 μM of homocysteine in the presence or absence of TUDCA (200 μM) or Lu AF58027 (500 nM).

### Gene silencing by siRNAs

H9c2 cardiac cells were transfected with specific siRNA targeting PDE1A or PDE1C. Cells transfected with the scrambled control siRNA served as control. To perform transfection, cells were incubated in Opti-MEM® I Reduced Serum Media (Thermo Fisher Scientific) for 24 h. The mixture of siRNA (gene-specific or corresponding scrambled control; 20 μM) and Lipofectamine® 2000 Transfection Reagent (Thermo Fisher Scientific) in a ratio of 5:2 was added with further incubation for 6 h. Culture medium was then changed to normal DMEM and siRNA knockdown efficiency was determined at both mRNA level by q-PCR and protein level by western blot analysis after 24 and 48 h, respectively. For each target, three siRNA sequences were tested and the one with the highest knockdown efficiency was selected for subsequent experiments (Supplementary Figure S1).

### Determination of cell surface area

Morphology of H9c2 cells and primary neonatal rat cardiomyocytes was examined and images were captured using a phase contrast microscope, and the cell surface area was measured by Image J software (National Institute of Mental Health, Bethesda, USA). Five fields in each well were viewed at magnification × 200. The average value was calculated and employed as the cell surface area.

### Determination of cell viability and cytotoxicity

Cell viability was assessed by using the MTT Cell Proliferation kit (Beyotime). Briefly, H9c2 cardiac cells were passaged in 96-well plates and treated with 100 μM homocysteine for 72 h. Then the cells were cultured in fresh culture medium with 0.5 mg/mL of MTT solution for 1 h at 37°C. Subsequently the amount of formazan was dissolved in DMSO and measured with a microplate reader (Tecan, Mannedorf, Switzerland) at 570 nm as an index of cell viability.

Cell cytotoxicity was evaluated by using the LDH Cytotoxicity Assay kit (Beyotime) according to the manufacturer's instructions. Briefly, cells were passaged in 96-well plates and treated with 100 μM homocysteine for 72 h. The release of LDH in the medium (LDHm) and in the cell extract (LDHc) of each well was measured with a microplate reader (Tecan) at 490/620 nm. Cytotoxicity was defined as the ratio of LDHm/(LDHm + LDHc) × 100%.

### Immunofluorescence microscopy

H9c2 cardiac cells were grown on sterilized coverslips. After treatment with homocysteine alone, or in combination with Lu AF58027 or TUDCA, the cells were fixed with 4% paraformaldehyde (15 min), permeabilized with 0.1% Tween-20 for 5 min and then blocked with 2% BSA in PBS for 1h. The cells were then incubated overnight at 4°C with primary antibody targeting α smooth muscle actin (1:500; Abcam, Cambridge, UK), followed by incubation with Alexa Fluor 488-conjugated Rabbit IgG (1:1000; Abcam) for 1 h at room temperature. Nuclear DNA was labeled with DAPI. Images were captured with a fluorescence microscope (Olympus DP73, Tokyo, Japan).

### Transmission electron microscope examination

H9c2 cardiac cells with or without homocysteine exposure were fixed with 2.5% glutaraldehyde in PBS for 2 h, then washed with 0.1 M PBS and postfixed with 1% $OsO_4$ in PBS at 4°C for 1 h. After extensive washes with cold water, the samples were dehydrated in gradient ethanol and embedded in Epon812 for ultrathin sectioning (80 ± 5 nm) by Leica Ultracut R (Wetzler, Germany). After being counterstained with 2% uranyl acetate and lead citrate, the cells were observed under a transmission electron microscope (Hitachi-7500, Tokyo, Japan) to examine their ultrastructure, and images were captured with a CCD imaging system (Megaview-III, Munster, Germany).

### q-PCR

Extraction of total RNA from H9c2 cardiac cells was performed using total RNA reagent (RNAiso Plus, TaKaRa Biotechnology, Dalian, China) according to the manufacturer's instructions. Reverse transcription and PCR amplification were performed in LightCycler 96 (Roche, Basel, Switzerland) employing the TransScript Green Two-Step qRT-PCR SuperMix system (Transgen, Beijing, China) under optimal PCR cycle conditions: 94°C for 30 s, 45 cycles of 5 s at 94°C, 50°C for 15 s, 72°C for 10 s, and melting at 95°C for 10 s, 65°C for 10 s and 97°C for 1 s. *GAPDH* was amplified in parallel as an internal control. q-PCR was performed in triplicate for each gene. The threshold cycle (Ct) values were calculated and statistically evaluated using SPSS version 20 (IBM-SPSS Inc, Armonk, USA). Expression levels of the target messenger RNAs were normalized to that of *GAPDH* and the relative differences were determined using the comparative Ct (ΔΔCt) method, and fold expression was calculated using $2^{-\Delta\Delta Ct}$, where ΔΔCt represents ΔCt values normalized with the mean ΔCt of the control samples.

### Western blot analysis

Whole cell proteins of H9c2 cells and primary neonatal rat cardiomyocytes were extracted using radio-immunoprecipitation assay buffer containing protease inhibitor (Solarbio). Protein concentration was determined by bicinchoninic acid assay. Protein samples (40 μg/lane) were subject to sodium dodecyl sulfate polyacrylamide gel electrophoresis, followed by electrotransfer to a polyvinylidenedifluoride (PVDF) membrane (Thermo Fisher Scientific) for detection of proteins of interest. Details of the procedures were published previously [22,23]. Briefly, PVDF membranes were incubated overnight at 4°C with primary antibodies targeting 1) β-MHC (1:1000; Sigma-Aldrich), ANP (1:500; GeneTex, San Antonio, USA), PDE1A (1:1000; Abcam), PDE1B (1:500; Abcam), and PDE1C (1:1000; Abcam); 2) ER stress molecules including 78-kDa glucose regulated protein (GRP78; 1:2000; Abcam), protein kinase RNA-like ER kinase (PERK; 1:1000; Cell Signaling, Beverly, USA), phosphorylated (Thr980) PERK (1:500; Bioss, Woburn, USA), inositol-requiring enzyme 1 (IRE1; 1:1000; Abcam), phosphorylated (Ser724) IRE1 (1:1000; Abcam), and activating transcription factor 6 (ATF6; 1:1000; Abcam), followed by incubation with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG or horse anti-mouse IgG secondary antibodies (1:3000; Cell Signaling) for 1 h at room temperature. β-Actin (1:2000; Absin, Shanghai, China) was used as an internal loading control. The protein bands were

visualized using an enhanced chemiluminescence kit (Beyotime), followed by documentation using the G:BOX gel doc system (Syngene, Cambridge, UK) and quantification using the Quantity One imaging system version 4.6.6 (Bio-Rad, Hercules, USA).

### Measurements of intracellular cAMP and cGMP

The levels of intracellular cAMP and cGMP were determined by using cAMP and cGMP ELISA kits (R&D Systems, Minneapolis, USA) according to the manufacturer's instructions. In brief, appropriate dilutions of cell lysates of H9c2 or primary neonatal rat cardiomyocytes (100 µL/well) and cAMP or cGMP conjugate (50 µL/well) were added to each well of the 96-well microplate coated with monoclonal antibody specific for cAMP or cGMP. The plate was covered with an adhesive strip and incubated for 2 h at room temperature on the shaker. After aspiration and four times wash to remove excess conjugate and unbound sample, 200 µL of substrate solution was added to each well. After 30 min of incubation in the dark, the color development was stopped by addition of 200 µL stop solution into each well, and the absorbance was read at 450/540nm with a microplate reader (Tecan). The triplicate readings for each sample were averaged. The average optical density values of the samples were converted to pmol of cAMP or cGMP per mg protein as calculated from standard curves after subtraction of the non-specific binding.

### Statistical analysis

Data are expressed as the mean ± standard error of the mean (SEM). One-way analysis of variance was used to assess differences among groups followed by Scheffe post hoc test using SPSS version 20. When two groups were compared, differences were assessed by Student's $t$ test. $P < 0.05$ was considered to be statistically significant.

## Results

### Homocysteine induces hypertrophy of H9c2 cardiac cells

q-PCR and western blot analysis revealed that the mRNA and protein levels of the cardiac hypertrophy markers β-MHC and ANP were upregulated in homocysteine-exposed H9c2 cardiac cells (Figure 1A,B). Consistently, the size of cells was found to be markedly increased. The surface area of homocysteine-exposed cells was significantly larger than that of the control cells (Figure 1C). Exposure to 100 µM homocysteine for 72 h did not pose significant impact on the cell viability and did not show observable cytotoxicity (Figure 1D).

### Inhibition of ER stress suppresses homocysteine-induced hypertrophy of H9c2 cardiac cells

Homocysteine exposure increased the expression of GRP78 and enhanced the phosphorylation of PERK and IRE1, suggesting the induction of ER stress in H9c2 cells (Figure 2A). Transmission electron microscope examination showed dilatation and degranulation of rough ER in homocysteine-exposed cells (Figure 2B). These morphological alterations indicated swelling of the rough ER and loss of the attached ribosomal granules, which may lead to disturbances in protein synthesis and transportation. Inhibition of ER stress with TUDCA reduced the expression/activation of the ER stress molecules, along with an amelioration of the ER dilatation and degranulation (Figure 2A,B).

The increase of cell surface area induced by homocysteine was significantly suppressed by TUDCA (Figure 2C). Both mRNA and protein analyses showed decreased expressions of β-MHC and ANP, in homocysteine-exposed cells that were treated with TUDCA, as compared to cells with homocysteine-exposure alone (Figure 2D).

### PDE1 upregulation is involved in homocysteine-induced hypertrophy of H9c2 cardiac cells

Homocysteine upregulated the protein expression of PDE1 in H9c2 cardiac cells. Among the three isoforms of PDE1, PDE1A and PDE1C were found to be significantly upregulated after homocysteine exposure, while PDE1B remained unaffected (Figure 3A). Inhibition of PDE1 with Lu AF58027, a specific PDE1 inhibitor, exhibited an obvious suppressing effect on homocysteine-induced hypertrophy of H9c2 cells, especially at the higher dose. As shown in Figure 3B, 500 nM of Lu AF58027 significantly decreased the protein levels of β-MHC and ANP in homocysteine-exposed cells, and reduced the cell surface area that was increased by homocysteine (Figure 3C). This anti-hypertrophic effect of Lu AF58027 was associated with a downregulation of PDE1A and PDE1C proteins (Figure 3B).

### Silencing of PDE1C isoform effectively inhibits homocysteine-induced hypertrophy of H9c2 cardiac cells

Transfection of the H9c2 cells with siRNA targeting the PDE1A isoform significantly suppressed the expression of PDE1A but showed no influence on the expression of the PDE1C subtype, and vice versa. The knockdown efficiency and specificity of siPDE1A and siPDE1C on their target genes were shown in Figure 4A. Selective knockdown of the PDE1C isoform significantly inhibited homocysteine-induced increase in cell surface area (Figure 4B), along with a significant downregulation of β-MHC and ANP expressions (Figure 4C) in H9c2 cells. In comparison, successful knockdown of PDE1A showed minor effect on homocysteine-induced increase of cell size, although it markedly inhibited the expression of β-MHC (Figure 4B,C).

### Activation of PDE1C by ER stress mediates homocysteine-induced hypertrophy via cAMP in H9c2 cardiac cells

In H9c2 cells subjected to homocysteine exposure, inhibition of ER stress with TUDCA significantly lowered the protein expressions of PDE1A and PDE1C (Figure 5A). In contrast, the PDE1 inhibitor Lu AF58027 exerted no effect on the upregulation/activation of ER stress molecules induced by homocysteine. The protein level of GRP78 and the phosphorylation level of PERK and IRE1 remained upregulated and did not differ from the levels in homocysteine-exposed cells. Inhibition of PDE1 showed no protection against homocysteine-induced dilatation and degranulation of the rough ER (Figure 5B).

Further measurement of cyclic nucleotides showed that cAMP and cGMP contents were significantly reduced in H9c2 cells after homocysteine exposure. Treatment with the PDE1 inhibitor Lu AF58027 at 500 nM restored the cAMP level in cells exposed to homocysteine. Restoration of the cAMP content was also observed in cells treated with the ER stress inhibitor TUDCA. Neither Lu AF58027 nor TUDCA restored the cGMP content that was decreased by homocysteine (Figure 5C). These results indicated that cyclic nucleotide, especially cAMP, is likely the downstream mediator of

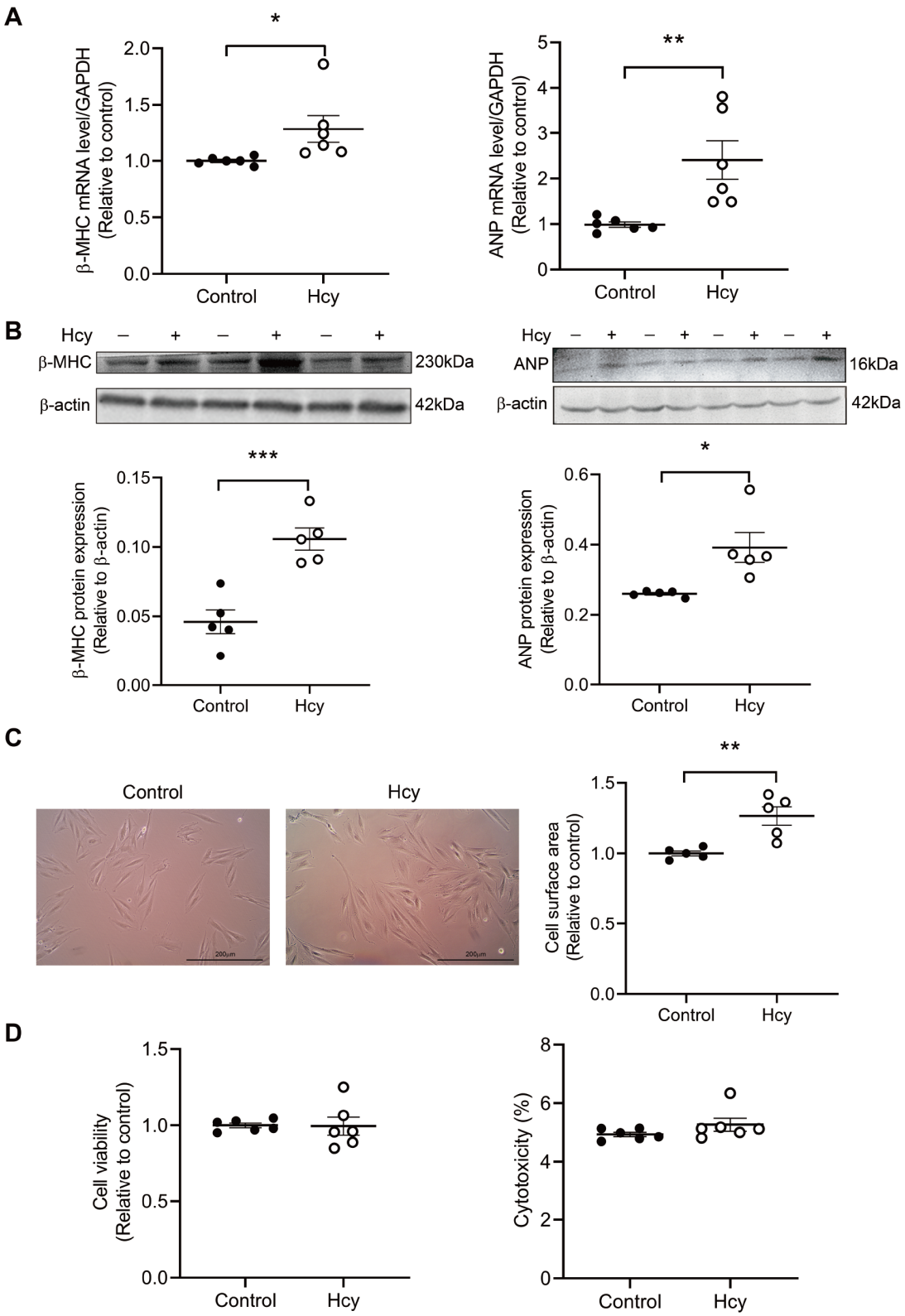


**Figure 1. Hypertrophic effect of homocysteine (Hcy) on H9c2 cardiac cells** Exposure to 100 μM Hcy for 72 h significantly upregulated the expressions of cardiac hypertrophy markers β-MHC and ANP in H9c2 cells at both the mRNA level (A, $n=6$) and protein level (B, $n=5$), and enlarged the cell surface area (C, $n=5$). Such exposure showed no cytotoxicity and barely affected cell viability (D, $n=6$). Data are presented as the mean ± SEM. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.01$ vs control.

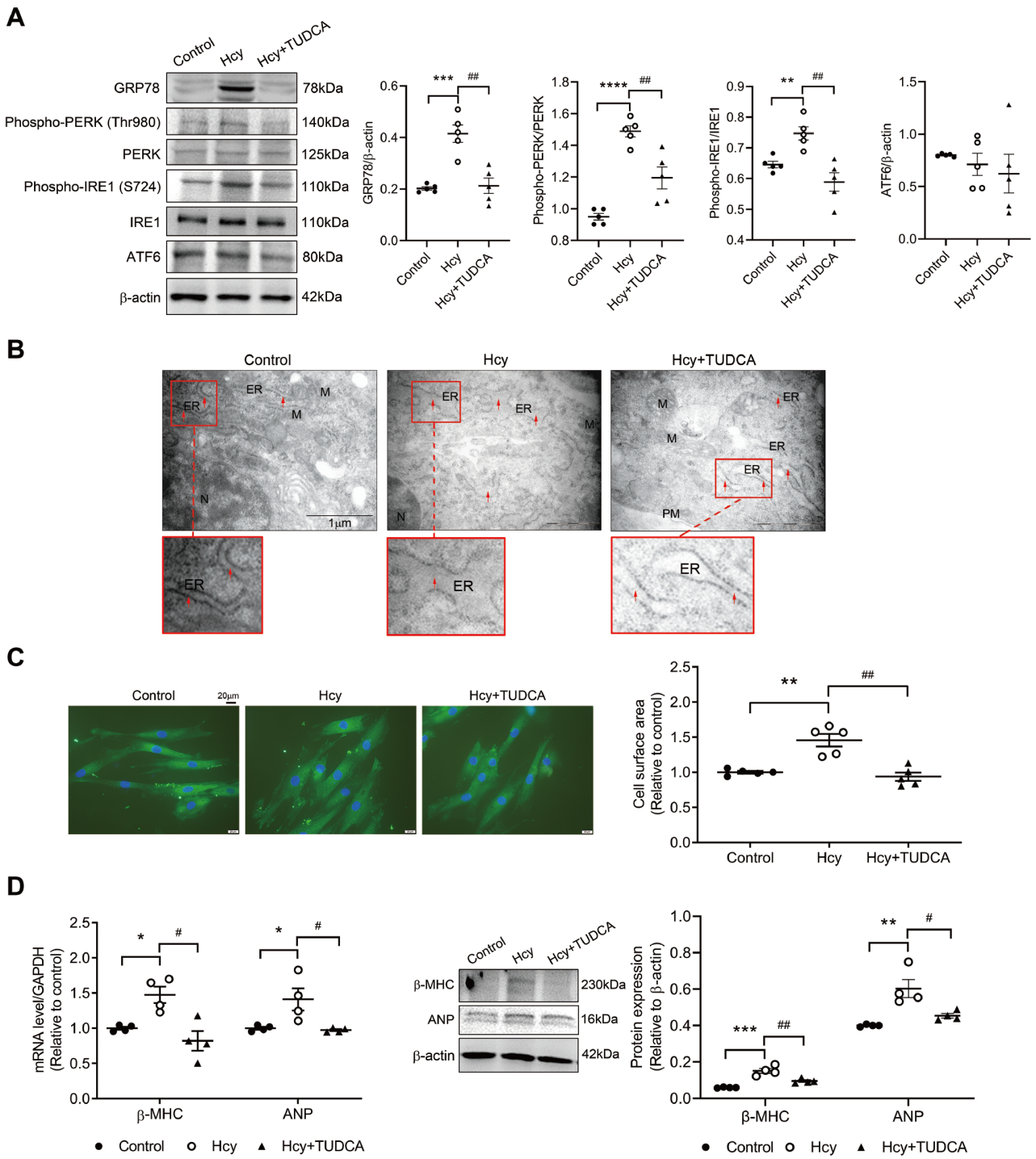


**Figure 2. The role of endoplasmic reticulum (ER) stress in homocysteine (Hcy)-induced hypertrophy of H9c2 cardiac cells** Representative blots and expression levels of ER stress molecules from 5 independent experiments showed that inhibition of ER stress by TUDCA suppressed the expression/activation of ER stress molecules (A). Transmission electron microscopy examination of the H9c2 cardiac cells, showing dilatation and degranulation of rough ER and loss of mitochondrial cristae after Hcy exposure, which could be ameliorated by TUDCA. Red arrows denote the rough ER. M, N, and PM represent mitochondrion, nucleus, and plasma membrane, respectively. Scale bar = 1 μm (B). Representative immunofluorescence microscopy images of control H9c2 cardiac cells and cells treated with Hcy in the presence or absence of the ER stress inhibitor TUDCA. Scale bar = 20 μm. The increase of cell surface area caused by Hcy was reversed by TUDCA, as calculated from 5 independent experiments (C). Inhibition of ER stress with TUDCA suppressed homocysteine-induced upregulation of β-MHC and ANP expression at both the mRNA level and protein level (D, $n = 4$). Data are presented as the mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$ vs control; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs Hcy. TUDCA: tauroursodeoxycholic acid.

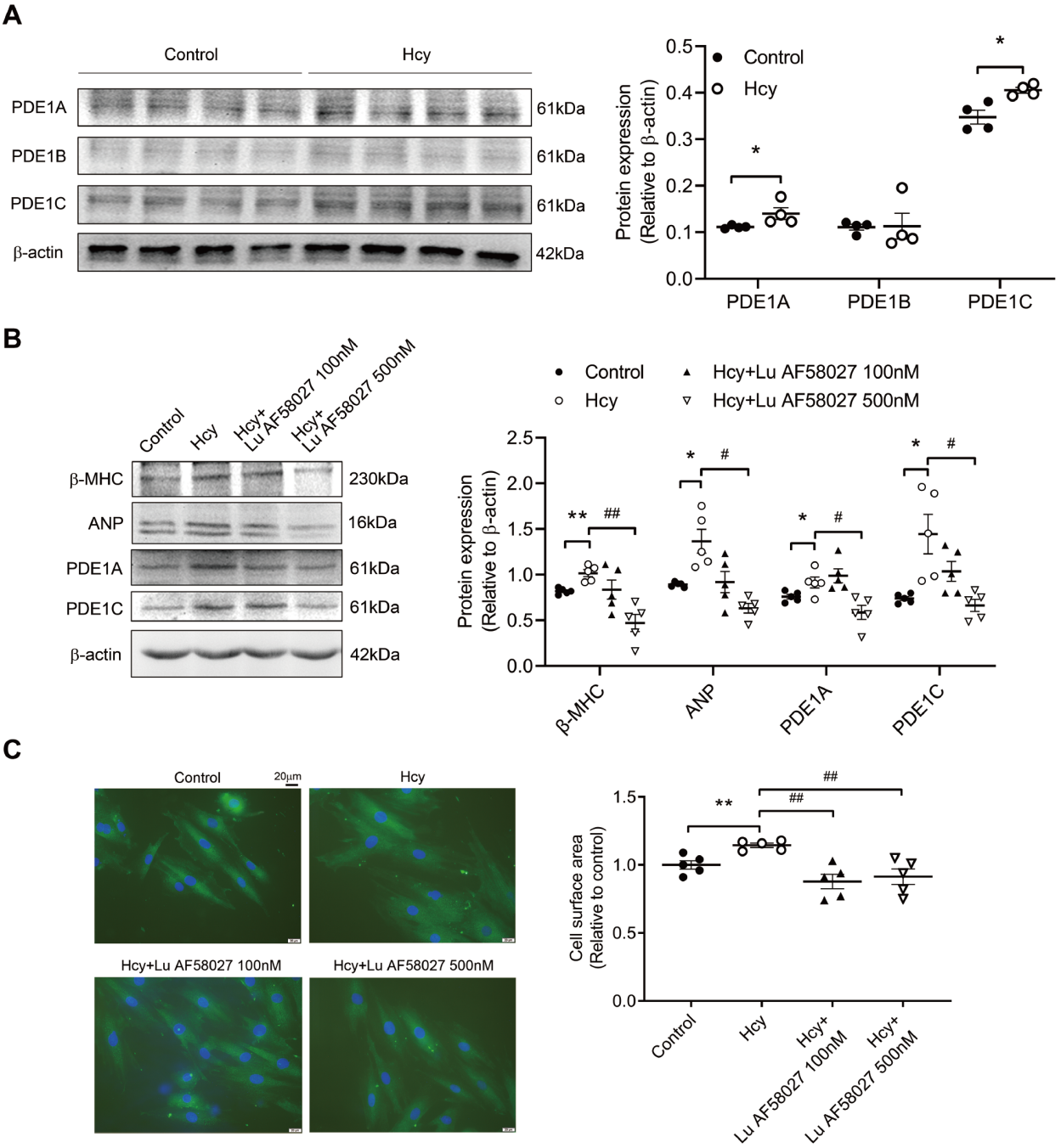


**Figure 3. The role of PDE1 in homocysteine (Hcy)-induced hypertrophy of H9c2 cardiac cells** Western blot analysis showed the upregulation of PDE1A and PDE1C protein expressions after Hcy exposure (A, $n=4$). Inhibition of PDE1 with Lu AF58027 suppressed Hcy-induced expressions of β-MHC and ANP, along with a downregulation of PDE1A and PDE1C proteins (B, $n=5$). Representative immunofluorescence microscopy images of control H9c2 cardiac cells and cells treated with Hcy in the presence or absence of the PDE1 inhibitor Lu AF58027. Scale bar = 20 μm. The increase of cell surface area induced by Hcy was reversed by Lu AF58027, as calculated from 5 independent experiments (C). Data are presented as the mean ± SEM. $^{*}P<0.05$, $^{**}P<0.01$ vs control; $^{\#}P<0.05$, $^{\#\#}P<0.01$ vs Hcy.

ER stress-PDE1 signaling pathway in homocysteine-induced hypertrophy.

## ER stress-PDE1C-cAMP signaling transduction pathway is involved in homocysteine-induced hypertrophy of primary neonatal cardiomyocytes

Primary neonatal rat cardiomyocytes exposed to homocysteine showed significant increase in cell size and upregulation in the expressions of β-MHC and ANP. Both ER stress inhibitor TUDCA and PDE1 inhibitor Lu AF58027 suppressed homocysteine-induced cell enlargement and expressions of β-MHC and ANP (Figure 6A). The anti-hypertrophic effect of Lu AF58027 was accompanied by a decrease of PDE1C and a restoration of cAMP level. Treatment with the ER stress inhibitor TUDCA also decreased the protein level of

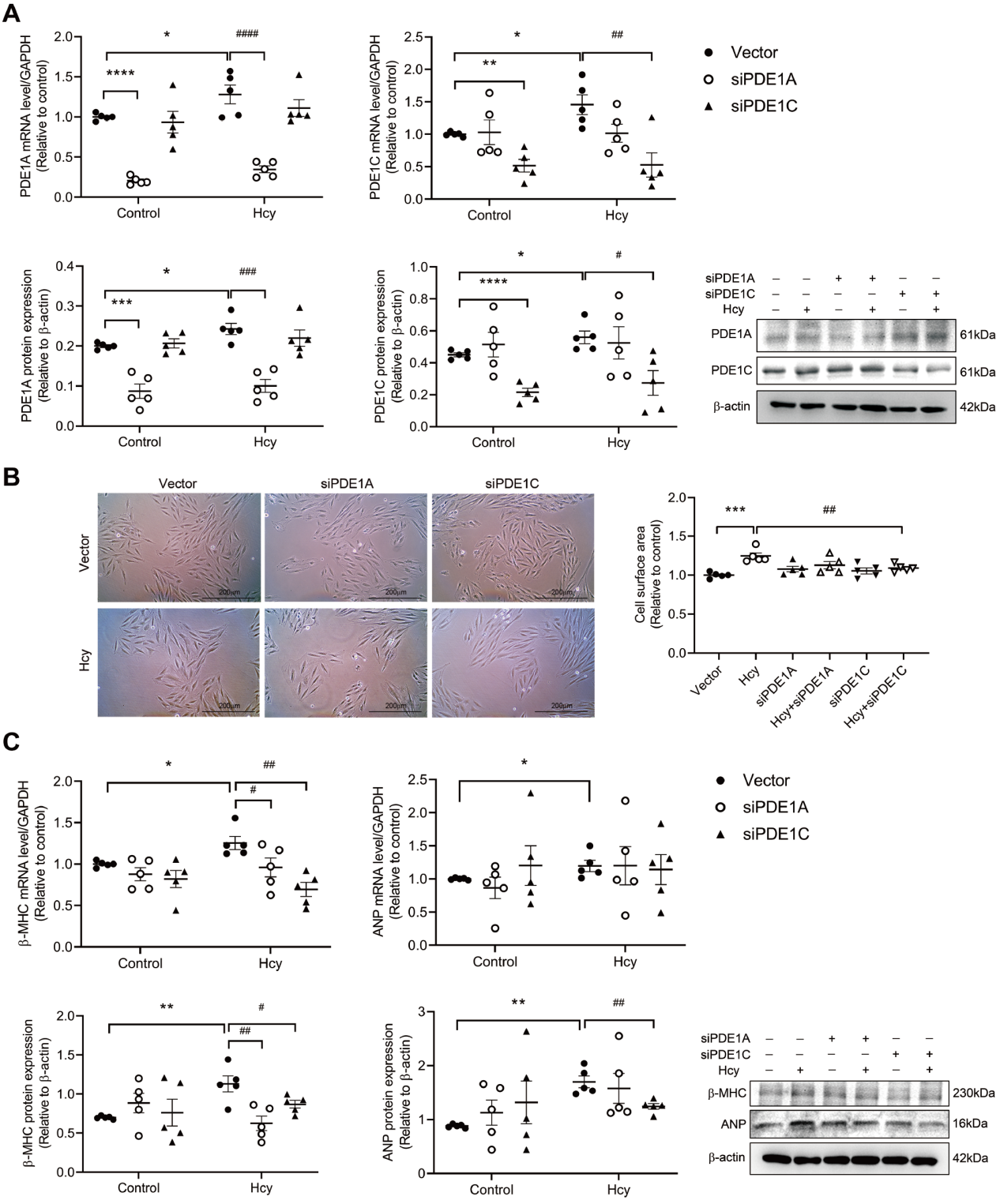


**Figure 4. Effect of silencing of PDE1A and PDE1C isoforms on homocysteine (Hcy)-induced hypertrophy of H9c2 cardiac cells** The knockdown efficiency and specificity of siRNAs (siPDE1A and siPDE1C) for target genes. The PDE1A targeting siRNA (siPDE1A) significantly suppressed the mRNA and protein expressions of PDE1A in both control and Hcy-exposed cells but showed no influence on the expression of the PDE1C isoform, and vice versa (A, $n=5$). Selective knockdown of the PDE1C but not PDE1A isoform in H9c2 cardiac cells significantly suppressed the increase of cell surface area induced by Hcy (B, $n=5$). PDE1C silencing inhibited Hcy-induced expressions of the hypertrophic markers β-MHC and ANP in H9c2 cells, while PDE1A knockdown only decreased the β-MHC level (C, $n=5$). Data are presented as the mean ± SEM. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, $^{****}P<0.0001$ vs control (vector); $^{\#}P<0.05$, $^{\#\#}P<0.01$ vs Hcy (vector).

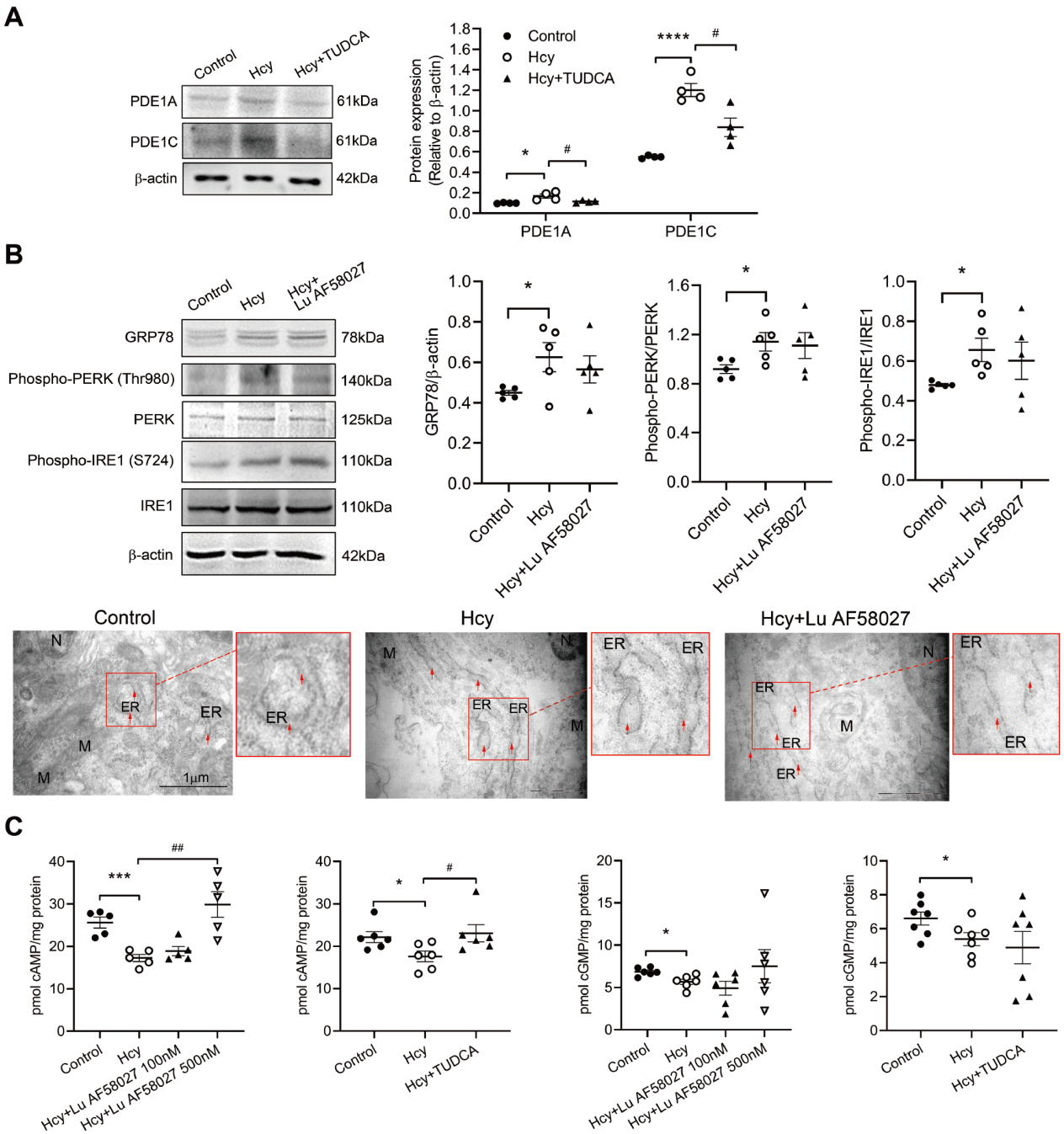


**Figure 5. Association between ER stress and PDE1 in homocysteine (Hcy)-induced hypertrophy of H9c2 cardiac cells** Inhibition of ER stress with TUDCA significantly decreased the protein expressions of PDE1A and PDE1C in Hcy-exposed H9c2 cells (A, $n=4$). Hcy-induced upregulation/activation of ER stress molecules was not affected by the PDE1 inhibitor Lu AF58027 (B, upper panel, $n=5$). Inhibition of PDE1 showed no protection against Hcy-induced dilatation and degranulation of the rough ER (B, lower panel). Hcy-induced decrease of cAMP level was restored by the PDE1 inhibitor Lu AF58027 and the ER stress inhibitor TUDCA. Neither Lu AF58027 nor TUDCA restored the cGMP content (C, $n=5$–7). Data are presented as the mean ± SEM. $^{*}P<0.05$, $^{***}P<0.001$, $^{****}P<0.0001$ vs control; $^{\#}P<0.05$, $^{\#\#}P<0.01$ vs Hcy. TUDCA: tauroursodeoxycholic acid.

PDE1C and increased cAMP content in homocysteine-exposed cardiomyocytes (Figure 6B,C). These results were consistent with the findings of the experiments in H9c2 cardiac cells.

## Discussion

In the present study, by using pharmacological tools and gene silencing techniques, we elucidated the role and relationship of PDE1 and ER stress in homocysteine-induced hypertrophic changes in cardiac cells. Our results demonstrated that: (1) PDE1 activation and ER stress contribute to the development of cell hypertrophy induced by homocysteine; (2) the hypertrophy-promoting effect of PDE1 is primarily achieved by the PDE1C isoform which functions as an effector molecule of ER stress in response to homocysteine stimulation; and (3) cyclic nucleotide, especially cAMP, is the downstream mediator of ER stress-PDE1C signaling axis in homocysteine-induced hypertrophy of cardiac cells.

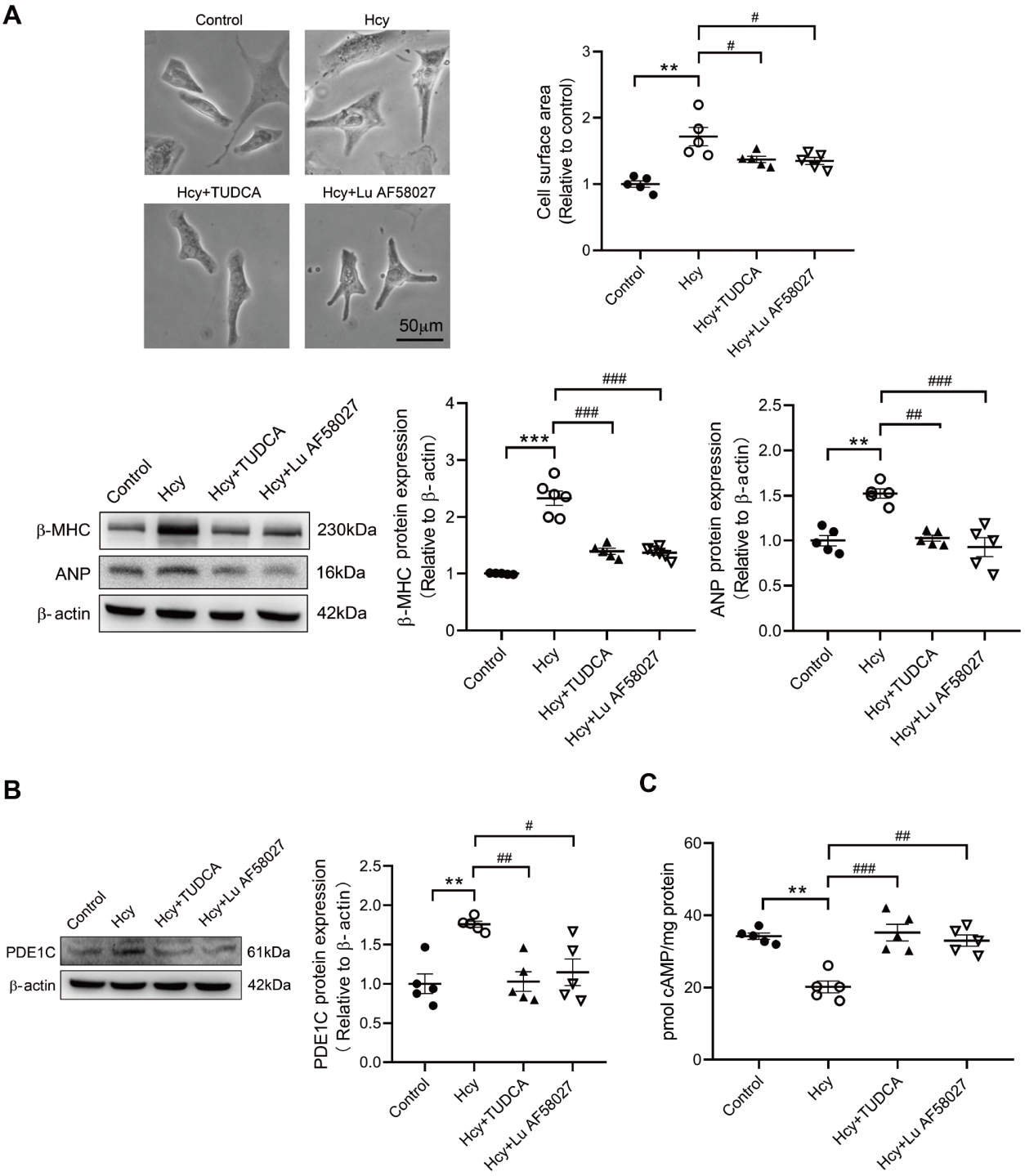


**Figure 6. Association between ER stress and PDE1C in homocysteine (Hcy)-induced hypertrophy of primary neonatal rat cardiomyocytes** ER stress inhibitor TUDCA and PDE1 inhibitor Lu AF58027 suppressed homocysteine-induced cell enlargement and expressions of β-MHC and ANP (A, $n=5$). Both TUDCA and Lu AF58027 inhibited homocysteine-induced expression of PDE1C (B, $n=5$) and elevated the intracellular cAMP content (C, $n=5$). Data are presented as the mean ± SEM. $^{**}P<0.01$, $^{***}P<0.001$ vs control; $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ vs Hcy. TUDCA: tauroursodeoxycholic acid.

To understand the role of PDE1 family in homocysteine-induced hypertrophy of cardiac cells, we first examined the effect of homocysteine on the expressions of PDE1 isoforms. Western blot analysis showed different expression abundances of PDE1 isoforms in H9c2 cells with an order of PDE1C > PDE1A > PDE1B. Homocysteine significantly upregulated the protein expressions of PDE1A and PDE1C, and such upregulation was antagonized by the pretreatment with Lu AF58027. As a specific PDE1 inhibitor, Lu AF58027 was proven to have very low affinity towards other PDE enzymes ($K_i$ values > 10 μM). For the PDE1 family, Lu AF58027

showed a higher potency in inhibiting PDE1C than in inhibiting PDE1A and PDE1B ($K_i$: 0.6, 4, 18 nM for 1C, 1A, and 1B respectively) [24]. In this study, Lu AF58027 was used to treat homocysteine-exposed cells at two concentrations, 100 and 500 nM. At both concentrations, it effectively suppressed the cell enlargement and upregulation of the expressions of hypertrophic markers induced by homocysteine, accompanied by a remarkable downregulation of PDE1C expression. At 500 nM, Lu AF58027 also significantly decreased the protein level of PDE1A. Considering the fact that all PDE1 isoforms are fully (PDE1C) or nearly fully inhibited (PDE1A: 99% and PDE1B: 97%) by Lu AF58027 at the concentration of 500 nM (data from Lundbeck), we further selectively silenced the transcription of PDE1A and PDE1C genes with siRNAs to clarify the significance of each isoform in homocysteine-induced hypertrophy. Measurements of cell surface area and hypertrophic markers β-MHC and ANP supported a significant role of the PDE1C isoform in mediating the hypertrophic effect of homocysteine. In comparison, successful knockdown of PDE1A barely affected the increase in cell size induced by homocysteine, although effective inhibition of β-MHC expression was observed. Taken together, these data suggest that the PDE1 family mediates homocysteine-induced cell hypertrophy, among which the 1C isoform plays a predominant role.

Measurements of cyclic nucleotides showed a significant decrease of both cAMP and cGMP contents in homocysteine-exposed H9c2 cells, while only cAMP content was found to be restored by 500 nM Lu AF58027. Together with the findings of the anti-hypertrophic efficacy of Lu AF58027 at this concentration, these results suggest that cAMP rather than cGMP is the downstream signal molecule of PDE1 in homocysteine-induced hypertrophy of cardiac cells. Since PDE1A and PDE1B isoforms are known to preferentially hydrolyze cGMP, while PDE1C hydrolyzes cAMP and cGMP with a similar high affinity [21,25], the finding of cAMP-mediation somehow also supports the predominant role of the PDE1C isoform in homocysteine-induced hypertrophic changes of H9c2 cells.

Another important finding of the present study is the relationship between ER stress and PDE1 regulation in homocysteine-induced hypertrophy of cardiac cells. Inhibition of ER stress suppressed H9c2 cell enlargement and downregulated the expressions of β-MHC and ANP induced by homocysteine, accompanied by a decrease in PDE1A and PDE1C expressions and an increase in cAMP content. Inhibition of PDE1 during homocysteine exposure exerted no suppressive effect on ER stress, and the expression of ER stress signal molecules remained upregulated. Collectively, these results indicate that PDE1 functions downstream of ER stress to take part in homocysteine-induced hypertrophy. Downregulation of PDE1C expression and restoration of cAMP level achieved by the ER stress inhibitor in primary neonatal cardiomyocytes also support the notion of ER stress-triggered PDE1 activation and its role in the hypertrophic effect of homocysteine. Studies on the relationship between PDEs regulation and ER stress are quite limited, and available evidence is in favor of the view that PDE activation leads to ER stress. It was reported that the PDE5 inhibitor sildenafil ameliorated isoprenaline- and TAC-induced cardiac hypertrophy and dysfunction, and attenuated ER stress in cardiomyocytes [26]. In a recent study, Chang and colleagues [27] demonstrated that cardioprotection conferred by swimming exercise in aged mice is attributed to the suppression of oxidative and ER stress as well as inhibition of PDE5. The ER stress inducer tunicamycin compromises the beneficial effect of swimming exercise without affecting PDE5 expression, while the PDE5 inhibitor sildenafil significantly decreases the levels of the ER stress-related proteins in the hearts of aged mice [27]. Meanwhile, inhibition of PDE4 was reported to attenuate ER stress through the activation of Nrf-2/HO-1 in neurons exposed to oxygen-glucose deprivation [28]. In the present study, we first reported the regulatory effect of ER stress on PDE1 and its downstream signaling molecule cAMP, which enriched our knowledge of the link between ER stress and PDE regulation. We also observed that the downregulation of PDE1A and PDE1C expression by the ER stress inhibitor TUDCA in homocysteine-exposed H9c2 cells is associated with inactivation of both PERK and IRE1 branches of ER stress, as evidenced by decreased phosphorylation of PERK and IRE1. The insignificant change in the level of ER-transmembrane protein ATF6 suggests a minor role of the ATF6 signaling branch in homocysteine-induced H9c2 cell hypertrophy. Future investigations on how PERK and/or IRE1 activation modulates PDE1 expression are warranted to further clarify the signaling cascade linking ER stress to PDE1 dysregulation. We are also aware that although H9c2 cells and primary neonatal cardiomyocytes are suitable and commonly used for in vitro studies of cardiac hypertrophy [29–33], further studies employing i*n vivo* animal model will help further evaluate the significance of ER stress-PDEC1-cAMP signaling axis in the development of cardiac hypertrophy in hyperhomocysteinemia.

In conclusion, this study demonstrated that ER stress mediates homocysteine-induced hypertrophy of cardiac cells via upregulating PDE1C expression. Cyclic nucleotide, especially cAMP, is the downstream mediator of the ER stress-PDE1C signaling axis in homocysteine-induced cell hypertrophy.

## Supplementary Data

Supplementary data is available at *Acta Biochimica et Biophysica Sinica* online.

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## Conflict of Interest

The authors declare that they have no conflict of interest.

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