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LCZ696 Ameliorates Tachycardia-Induced Cardiac Calcium Dyshomeostasis in the SERCA2*α*-Dependent Pathway

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The incidence, prevalence, and economic burden of heart failure have continued to increase worldwide. It remains unclear whether LCZ696 can ameliorate calcium reuptake in the sarcoplasmic reticulum via the sarcoplasmic endoplasmic reticulum calcium ion-ATPase 2*α* (SERCA2*α*)-dependent pathway during cardiac diastole. We investigated whether LCZ696 could ameliorate tachycardia-induced myocardial injury by modulating cardiac SERCA2*α* levels. A tachycardia-induced myocardial injury model was established by daily intraperitoneal administration of 60 mg/kg isoprenaline (ISO) for 2 weeks. LCZ696 was orally administered for the following 4 weeks. SERCA2*α* and calcium ion (Ca^{2+})-related protein expression was assessed by quantitative real-time polymerase chain reaction (qRT-PCR) and western blotting. For additional *in vitro* studies, HL-1 cardiomyocytes were used. A SERCA2*α* overexpression vector was constructed and transfected into HL-1 cells. The expression of SERCA2*α* and Ca^{2+}-related proteins were also measured using qRT-PCR and western blotting. Our *in vivo* results demonstrated that myocardial injury was successfully induced by intraperitoneal administration of ISO. The expression of both SERCA2*α*- and Ca^{2+}-related proteins was impaired. Oral administration of LCZ696 increased the expression of SERCA2*α*, alleviated Ca^{2+}-related protein impairment and cardiac Ca^{2+} dyshomeostasis, and ameliorated myocardial injury. These results were compared with our *in vitro* findings. Ca^{2+}-related proteins are affected by the overexpression of SERCA2*α*. LCZ696 improved tachycardia-induced myocardial injury by increasing SERCA2*α* expression, which reversed the development of heart failure in ISO-induced mice. These results provide new insights into how sustained LCZ696 treatment in heart failure improves cardiac function through intracellular Ca^{2+}-regulatory mechanisms.

Keywords: calcium cycling; myocardial injury; sacubitril/valsartan (LCZ696); SERCA2*α*; tachycardia
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Introduction

Cardiac calcium ion (Ca^{2+}) homeostasis plays an important role in the development of heart failure (HF), which is characterized by structural and/or functional abnormalities in the heart and elevated natriuretic peptide levels (Ponikowski et al. 2016; Becher et al. 2022). Although recent advances have improved the therapeutic efficacy of cardiovascular disease treatments, the incidence, prevalence, and economic burden of HF continue to increase worldwide. More than 8.9 million patients in China and an estimated 64 million people worldwide have been reported to experience HF (Becher et al. 2022). Cardiac Ca^{2+} dyshomeostasis, characterized by abnormal Ca^{2+} cycling and reduced myocardial contractility, results in cardiac dysfunction and remodeling (Piacentino et al. 2003; Njegic et al. 2020). A previous study demonstrated that long-term tachycardia led to decreased expression of sarcoplasmic endoplasmic reticulum Ca^{2+}-ATPase 2*α* (SERCA2*α*) and induced cardiac Ca^{2+} dyshomeostasis, culminating in myocardial injury and HF (Zhihao et al. 2020).

SERCA2*α* is the predominant isoform in the heart and is crucial for maintaining a balanced Ca^{2+} intracellular concentration during the myocardial contraction-relaxation

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cycle (Sitsel et al. 2019). SERCA2α regulates calcium reuptake in the sarcoplasmic reticulum (SR) to initiate diastole. The importance of SERCA2α in myocardial injury has been studied extensively. A decrease in the expression of SERCA2α has been reported following myocardial injury (Kawase et al. 2011). A study indicated that SERCA2α overexpression by transgenic or viral methods contributes to cardioprotection after myocardial injury and therapeutic approaches increase the expression of SERCA2α, ameliorating tachycardia-induced myocardial injury (Gong et al. 2016).

The angiotensin receptor-neprilysin inhibitor (ARNI) has been considered an important pharmaceutical tool for treating HF in the last decade. A breakthrough in the field of ARNI is LCZ696 (sacubitril/valsartan), a combination of the neprilysin inhibitor sacubitril and the angiotensin receptor blocker valsartan (Jhund and McMurray 2016). LCZ696 ameliorates the development of HF by decreasing sympathetic activity, reversing cardiac remodeling, and inhibiting myocardial fibrosis (Miyoshi et al. 2019). According to 2022 AHA/ACC/HFSA guidelines, ARNI is a new treatment strategy for HF (Heidenreich et al. 2022). Experimental studies and clinical trials have demonstrated that ARNI can improve cardiac function and reduce heart failure-related mortality and rehospitalization rates (Solomon et al. 2012; Gaziano et al. 2020). ARNI is increasingly being used to treat various diseases such as HF, hypertension, chronic atrial fibrillation, diabetes, and chronic kidney disease (Kang et al. 2020; Jackson et al. 2021; Armentaro et al. 2022; Yang et al. 2022). A previous study reported that LCZ696 reduced SR Ca^{2+} leakage and improved cardiac systolic function in human end-stage HF patients (Eiringhaus et al. 2020). Nonetheless, it remains unclear whether LCZ696 can ameliorate calcium reuptake in the SR via the SERCA2α-dependent pathway during cardiac diastole. Therefore, this study aimed to investigate whether LCZ696 ameliorates tachycardia-induced myocardial injury and modulates cardiac Ca^{2+} dysfunction by regulating SERCA2α expression.

Materials and Methods

All procedures involving animals were approved by the Ethics Committee of Soochow University and were performed in compliance with the guidelines of the Soochow University Laboratory Animal Center and Animal Care and Use Committee of Soochow University.

Animal experiments and groups

Twenty mice (6-8 week-old males, weighing 25-35 g) from the Institute of Cancer Research were randomized into three groups: control (n = 6), tachycardia (n = 7), and LCZ696 (n = 7). The mice were fed and drank freely at 25°C and 50% humidity. Before the experiments, the animals were given a week to acclimatize. Tachycardia-induced myocardial injury was induced by intraperitoneal administration of 60 mg/kg of isoprenaline (ISO) (MB2028, Dalian Meilun Biotechnology, Liaoning, China) once a day during the first 2 weeks. After establishing tachycardia-induced myocardial injury, mice were orally administered 60 mg/kg of LCZ696 (MB5069-1, Dalian Meilun Biotechnology) or an equal volume of 0.9% saline once a day for the following 4 weeks. None of the mice died during the experiments. Mice were housed in ventilated cages. At the end of the experiment, the mice were weighed and euthanized via intraperitoneal administration of sodium pentobarbital (100 mg/kg). Blood was collected from the heart and stored at −80°C for further analysis after centrifugation at 1,500 × *g* at 4°C for 5 min. After disinfection, the thorax was opened rapidly, and the heart was resected, photographed, and weighed. The ratio of heart to body weight was also calculated. Myocardial tissues were half fixed in 4% paraformaldehyde, half frozen in liquid nitrogen, and stored at −80°C for further experiments. Strict aseptic conditions were maintained during all the procedures. The treatments received in the different groups were as follows: (1) intraperitoneal administration of saline once a day for the first 2 weeks and oral administration of saline once a day for the following 4 weeks (control group); (2) intraperitoneal administration of 60 mg/kg ISO once a day for the first 2 weeks and oral administration of saline once a day for the following 4 weeks (tachycardia group); and (3) intraperitoneal administration of 60 mg/kg ISO once a day for the first 2 weeks and oral administration of 60 mg/kg LCZ696 once a day for the following 4 weeks (LCZ696 group).

Enzyme-linked immunosorbent assay (ELISA)

Blood samples were collected; after coagulation for 2 h at room temperature (22-26°C), the blood samples were centrifuged at 1,000 × *g* for 10 min, and the serum was collected and stored at −20°C until analysis. ELISA kits were used to quantify the plasma levels of brain natriuretic peptide (BNP) (EM3376M, Weiao Biotechnology, Shanghai, China), interleukin (IL)-1β (EM3184M, Weiao Biotechnology), and IL-6 (ab222503, Abcam, Cambridge, UK). Following the protocol provided with the kit, the sample was added, incubated, washed with the enzyme, incubated, and colored. Briefly, 50 μL of the standard sample was added to the blank well, and 50-μL of the samples was tested in the sample well, followed by incubation at 37°C for 40 min. The plates were washed 4-6 times with 350 μL of the diluted wash solution. Subsequently, 100 μL of the diluted solution of biotinylated antibody was added to the blank well, and 100 μL of the working solution of biotinylated antibody was added to the standard. The plates were sealed using Microseal and mixed at 37°C for 30 min. The plate was washed, and the streptavidin-biotin-peroxidase complex (SABC) complex working solution was added at 37°C for 20 min. Afterward, 3,3',5,5'-tetramethylbenzidine (TMB) color reagents were added and incubated at 37°C for 20 min. Finally, the termination solution was added, and the absorbance was measured at 450 nm using a spectrophotometer (Shimadzu UV Spectrophotometer UV-1800; Shimadzu

Corp., Kyoto, Japan).

Histological study

Harvested tissue samples were dehydrated using gradient ethanol after being fixed in ice-cold 4% paraformaldehyde. Before the histological assessment, heart tissues were embedded in paraffin and sectioned into 4-μm slices. Hematoxylin and eosin (HE) staining was performed according to commercial protocols (C0107, Beyotime Institute of Biotechnology, Shanghai, China). Briefly, after deparaffinization and rehydration, the tissue sections were stained with HE solution for 5 min, followed by dehydration with graded alcohol and clearing in xylene. Finally, the stained slides were sealed with neutral resin (E675007, Sangon Biotech, Shanghai, China). Images were captured and analyzed using an inverted fluorescence microscope (Nikon Eclipse Ni, Nikon, Tokyo, Japan) and AxioVision Rel. 4.5 software (Zeiss GmbH, Oberkochen, Germany).

To assess the degree of fibrosis, Masson's trichrome staining was performed according to commercial protocols (BP-DL021, Nanjing Senbeijia Biological Technology, Nanjing, China). The degree of fibrosis was evaluated by the ratio of the fibrotic area (characterized by collagen stained blue) to the area of cardiomyocytes (characterized by myocardial tissue stained red) and was measured using AxioVision Rel. 4.5 software (Zeiss GmbH).

Quantitative real-time-polymerase chain reaction (qRT-PCR)

Total RNA was extracted from isolated myocardial tissue using TRIzol reagent. cDNA was synthesized using the IScript cDNA Synthesis Kit (170-8890, Bio-Rad, Hercules, CA, USA). PCR was performed using SYBR Premix EX TaqTM II (DRR081A; Takara Bio, Otsu, Japan). The relative mRNA expression was calculated using the $2^{-\Delta\Delta CT}$ method. The primer sequences used in this study are listed in Table 1 and were used to measure the absorbance at 450 nm.

Western blot analysis

Protein samples were extracted from myocardial tissues using radioimmunoprecipitation assay (RIPA) buffer (P0013C; Beyotime, Beijing, China). Protein samples were separated using polyacrylamide SDS-PAGE, transferred to nitrocellulose blotting membranes, and blocked with 1% bovine serum albumin for 1 h. Western blotting was performed using antibodies against cardiac Troponin I (1:2,500, ab209809, Abcam), SERCA2α (1:2,000, ab3625, Abcam), calmodulin-dependent protein kinase II (CaMKII) (1:1,000, ab181052, Abcam), protein kinase A (PKA) (1:5,000, bs-17184R, BoAoSen Biotechnology, Beijing, China), Phospholamban (PLB) (1:2,000; bs-4490R, BoAoSen Biotechnology), S100A1 (1:1,000; bs-0850R, BoAoSen Biotechnology), histidine-rich calcium-binding protein (HRC) (1:2,000; bs-17387R, BoAoSen Biotechnology), α-tubulin (1:5,000, 66031-1-1 g, Proteintech, Chicago, IL, USA), GAPDH (1:5,000, 60004-1-1 g, Proteintech), Bcl-2 (1:2,000, 3498, CST, Danvers, MA, USA) and Bax (1:2,000, 2772, CST). Anti-mouse and anti-rabbit-HRP secondary antibodies (A0208, Beyotime Institute of Biotechnology; A0216, Beyotime Institute of Biotechnology) were used in this experiment. Immunoreactive bands were visualized using enhanced chemiluminescence, and the results were analyzed using a gel imager system (GenoSens 2000, Shanghai Qin Xiang Scientific Instrument, Shanghai, China). Images were subsequently quantified using the ImageJ software (National Institutes of Health, Bethesda, Maryland, USA).

HL-1 cell culture

In the mouse cardiac muscle cell line, HL-1 cells were maintained and grown in high-glucose Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS; Gibco, Thermo Fisher Scientific, Waltham, MA, USA). The medium was supplemented with 100 g/L streptomycin and 100 U/mL penicillin to avoid contamination. The cells were cultured in a humidified atmosphere of 95% air and 5% CO_2 at 37°C, and the cell culture media were exchanged daily. After achieving 80-90% confluence, the cells were passaged.

Vector construction and transfection

A pCDH-CMV-MCS-EF1-CopGFP-T2A-Puro vector (CG20200600025, Jima Biotechnology, Shanghai, China) and SERCA2α (full-length CDS region) lentivirus plasmid (G0218013, General Biosystems, Chuzhou, China) were

Table 1. Primer sequences used for the real-time-polymerase chain reaction.

Gene	Forward primer (5′-3′)	Reverse primer (5′-3′)
GAPDH	TCTCCTGCGACTTCAACA	TGTAGCCGTATTCATTGTCA
SERCA2	TGCTTAAATGCCCGCTGTTT	CTGCCAGGACCATCTCAGAA
HRC	CCAAGGCCACAGTGATGATG	TCACCAGAGTCACCGTCTTC
PKA	ACTTCCCGTTCCTGGTCAAA	CCTGCTGGTCGATGAGAAGA
PLB	GTGCAATACCTCACTCGCTC	GCAGATCAGCAGCAGACATA
CaMK II	TTCTGAGAGCACCAACACCA	CCTCTGGTTCAAAGGCTGTC
S100A1	GACCCTCATCAATGTGTTCCA	CCCCATCTCCGTTTTCATCC

used in this experiment. SERCA2α was amplified by PCR and inserted into the vector with the NheI (GCTAGC) and HindIII (AAGCTT) restriction sites. A SERCA2α overexpression vector (OE-SERCA2) was successfully constructed. In accordance with the protocol, the OE-SERCA2α vector or SERCA2α empty vector was transfected into HL-1 cells using Lipofectamine 2000 (11668-027, Invitrogen, Waltham, MA, USA) for 48 h. The expression of transient cells was determined, and the efficiency was determined. pCDH-CMV-MCS-EF1-copGFP-T2A-Puro-OE SERCA2α plasmids were co-transfected into 293 T cells. Lentiviral supernatants were harvested 48 and 72 h after co-transfection, and virus titers were determined. A lentiviral suspension containing approximately 4×10^6 lentiviral particles (multiplicity of infection = 10) and polybrene (5 μg/mL) was added to the HL-1 cells during infection. Puromycin (Sangon Biotech) was used to screen infected cells. Puromycin-resistant single-cell clones stably overexpressing SERCA2α were established and verified using qRT-PCR.

Cell viability assessment

Cell viability was determined using the Cell Counting Kit-8 (CCK-8) assay (MA0218-2, Shanghai Meilun Biotechnology, Shanghai, China). Briefly, HL-1 cells were collected and seeded in 96-well plates at a density of 5×10^5 cells/well and incubated overnight. Each well was treated with 10 μL CCK-8 solution, and the cells were incubated for 3 h at 37°C. Microplate readers were used to measure the absorbance at 450 nm.

Flow cytometry assessment

Cellular apoptosis was assessed by flow cytometry. Cells were digested using trypsin without EDTA, and the contents were collected by centrifugation at 4°C for 5 min at 300 × *g*. The cells were resuspended in 250 μL 1 × binding buffer. A commercial apoptosis detection kit was used (40302ES60; Yisheng Biotechnology, Shanghai, China). Briefly, 1×10^5 cells were stained with 1:20 anti-mouse Annexin V-FITC and anti-mouse PI for 30 min on ice. The stained cells were analyzed using a Cytomics FC500 flow cytometer (Beckman Coulter, Miami, FL, USA). The percentage of viable apoptotic cells represented by annexin V-positive and PI-negative cells was calculated as the apoptosis rate. Flow images were analyzed using FlowJo software (V10.7.1; Becton, Dickinson and Company, Franklin Lakes, NJ, USA).

Establishment of ISO-induced myocardial injury model in HL-1 cells

This study included the following three groups in our *in vitro* analysis: (1) the control group (n = 3); (2) the ISO group (n = 3; HL-1 cells cultured with 16 μM ISO without LCZ696 for 48 h); and (3) the ISO + 10 μM group (n = 3, HL-1 cells cultured with 16 μM ISO and 10 μM LCZ696 for 48 h). Apoptosis was assessed by flow cytometric analysis, and the expression of Bax and Bcl 2 was evaluated by western blot analysis, as described above. Cardiac troponin I level was assessed to evaluate the myocardial injury. IL-1β and IL-6 levels in the culture medium were determined using ELISA.

Measurement of intracellular Ca^{2+}

Cytosolic Ca^{2+} levels were measured using a calcium ion fluorescent probe (Fluo-4 AM) (40704ES50, Yisheng Biotechnology). The precultured cells were removed, the culture medium was discarded, and the cells were washed thrice with Hanks' Balanced Salt Solution (HBSS). Fluo-4 AM was added for 10-60 min at 37°C, and the cells were washed three times with HBSS. A 30-min incubation at 37°C was used to ensure complete de-esterification of the intracellular AM esters. Calcium levels within the cells were measured using fluorescence microscopy settings appropriate for excitation at 494 nm and emission at 516 nm.

Therapeutic effect of LCZ696 on Ca^{2+} dyshomeostasis

To evaluate the therapeutic effect of LCZ696 on Ca^{2+} dyshomeostasis, intracellular Ca^{2+} was measured using a calcium ion fluorescent probe, as described above. Cell viability was measured by CCK-8 assay, and the expression of SERCA2α was assessed by qRT-PCR, as mentioned above. The expression of HRC, PKA, S100A1, PLB, and CaMKII was evaluated by western blot analysis and qRT-PCR, as described above.

Therapeutic role of SERCA2α on Ca^{2+} dyshomeostasis

To investigate the therapeutic role of SERCA2α in Ca^{2+} dyshomeostasis, four groups of HL-1 cells were used in this study: (1) control group (n = 3); (2) ISO group (n = 3, HL-1 cells cultured with 16 μM ISO for 48 h); (3) NC group (n = 3, HL-1 cells transfected with SERCA2 empty vector cultured with 16 μM ISO for 48 h); and (4) OE-SERCA2 group (n = 3, HL-1 cells transfected with OE-SERCA2 vector cultured with 16 μM ISO for 48 h). Intracellular Ca^{2+} levels were measured, and the expression of SERCA2α was assessed by qRT-PCR. The expression of HRC, PKA, S100A1, PLB, and CaMKII was evaluated by western blot analysis and qRT-PCR, as described above. The cardiac troponin I level was assessed by western blotting, and intracellular Ca^{2+} levels were quantified by a calcium ion fluorescent probe, as mentioned above.

Statistical analysis

All data are expressed as mean ± standard deviation. IBM SPSS Statistics for Windows, version 21.0 software (IBM Corp., Armonk, NY, USA) was used for the statistical analysis. Graphs were prepared using GraphPad Prism v8.0.2 software (GraphPad Prism, San Diego, CA, USA). Multiple groups were compared using one-way ANOVA with Tukey's post-hoc test. The level of significance was set at $p < 0.05$.

Results

LCZ696 alleviated tachycardia-induced myocardial injury

After tachycardia was induced by the intraperitoneal administration of ISO, the heart rate increased to > 800 beats/min. One mouse each in the tachycardia and one mouse in the LCZ696 groups with a heart rate below 800 beats/min were excluded from this experiment. Eighteen mice from each group were analyzed at the end of the experiment, and 18 mice were analyzed with six mice in each group. The ratios of heart weight to body weight, cardiac troponin I level, BNP level, and cardiac fibrosis were assessed to evaluate the myocardial injury. HE staining was performed to show the changes in structure, and Masson staining was performed to evaluate fibrosis (Fig. 1A). Compared with the control group, intraperitoneal administration of ISO during the first 2 weeks in the tachycardia group significantly increased the ratio of heart weight to body weight at week 6 ($p < 0.05$) (Fig. 1B). In addition, the cardiac troponin I level, BNP level, and myocardial fibrosis were markedly increased in the tachycardia group relative to the control group at week 6 (Fig. 1C-E). This indicated that long-term tachycardia induced myocardial injury, whereas these myocardial injury-related manifestations improved in the LCZ696 group at week 6. The ratios of heart weight to body weight, cardiac troponin I levels, BNP levels, and fibrosis were significantly lower in the LCZ696 group than in the tachycardia group at week 6 ($p < 0.05$) (Fig. 1B-E).

LCZ696 repaired Ca^{2+}-related protein impairment in vivo

Cardiac Ca^{2+}-related proteins, including SERCA2α, HRC, PKA, S100A1, PLB, and CaMKII, were assessed to evaluate Ca^{2+} homeostasis. Our qRT-PCR and western blot results showed that the relative mRNA levels and protein expression of SERCA2α, HRC, PKA, and S100A1 were significantly reduced in the tachycardia group compared with the control group ($p < 0.05$) (Fig. 2A-H), whereas the

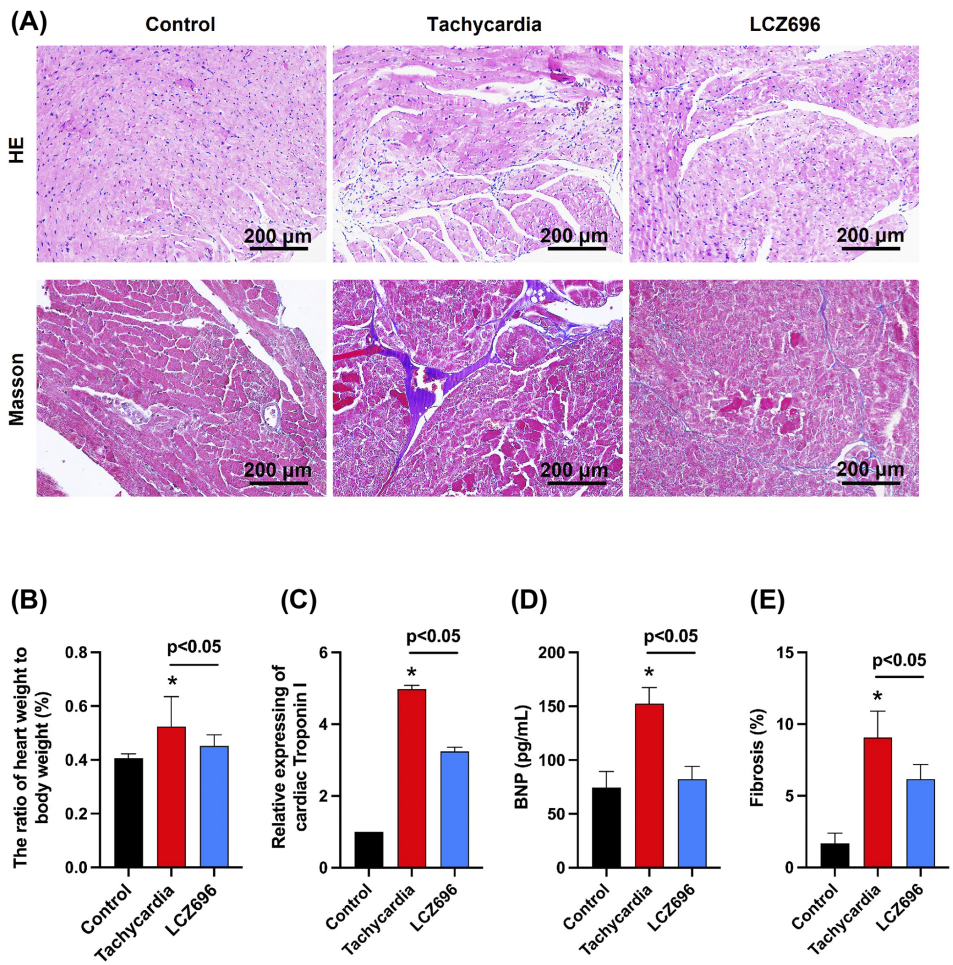

Fig. 1. LCZ696 treatment alleviated tachycardia-induced myocardial injury.
Representative images of hematoxylin and eosin (HE) and Masson's staining (A), cardiac hypertrophy (ratio of heart tissue to body weight) (B), cardiac troponin I relative expression (fold change) (C), plasma brain natriuretic peptide (BNP) levels (D), and fibrosis (%) processed through the HE and Masson's staining (E) in control mice (Control), ISO-induced tachycardia mice (Tachycardia), and ISO-induced LCZ696-treated group (LCZ696). Bars in (A) indicate 200 μm. *$p < 0.05$ vs. control group.

relative mRNA level and protein expression of PLB and CaMKII remarkably increased in the tachycardia group relative to the control group (Fig. 2I-L). These results demonstrate that long-term tachycardia impaired the expression of Ca^{2+}-related proteins. Relative mRNA and protein levels of SERCA2α, HRC, PKA, and S100A1 improved significantly in the LCZ696 group compared to those in the tachycardia group ($p < 0.05$) (Fig. 2A-H). The relative mRNA level and protein expression of PLB and CaMKII notably decreased in the LCZ696 group relative to that in the tachycardia group (Fig. 2I-L). Western blot densitometric analysis is shown in Supplementary Fig. S1A, B. In summary, these results demonstrate that long-term tachycardia changed the expression of Ca^{2+}-related proteins, which might result in cardiac Ca^{2+} dyshomeostasis. LCZ696 treatment reversed these changes, which may contribute to the maintenance of cardiac Ca^{2+} homeostasis.

LCZ696 modulated the expression of Ca^{2+}-related proteins and Ca^{2+} homeostasis in vitro

A cardiomyocyte injury model was established by culturing HL-1 cells in the presence of isoprenaline. Apoptosis and the expression of Bax, Bcl-2, and cardiac troponin I was assessed to evaluate cardiomyocyte injury. The levels of pro-inflammatory cytokines, including IL-1β and IL-6, were determined using ELISA. Apoptosis was analyzed

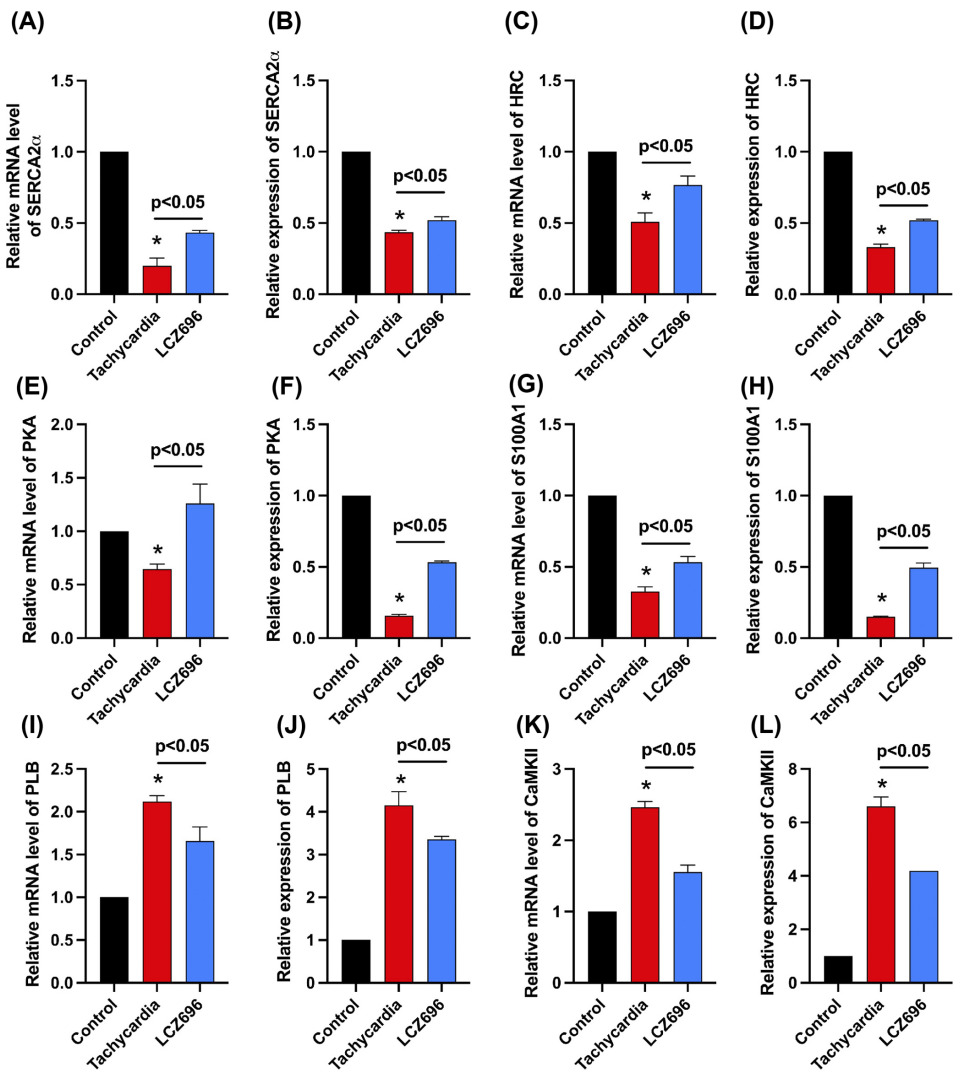

Fig. 2. LCZ696 repaired Ca^{2+}-related protein impairment *in vivo* and promoted Ca^{2+} homeostasis.
The results of qRT-PCR and western blotting showed that relative mRNA levels and protein expression of SERCA2α, histidine-rich calcium-binding protein (HRC), protein kinase A (PKA) and S100A1 were significantly reduced in the tachycardia group compared with the control group, and LCZ696 significantly improved the relative mRNA level and protein expression of SERCA2α, HRC, PKA, and S100A1 in the LCZ696 group relative to the tachycardia group (A-H). Relative mRNA level and protein expression of phospholamban (PLB) and calmodulin-dependent protein kinase II (CaMKII) remarkably increased in the tachycardia group relative to the control group, and LCZ696 notably decreased in the LCZ696 group relative to the tachycardia group, relative to mRNA level and protein expression of PLB and CaMKII (I-L). *$p < 0.05$ vs. control group.

using flow cytometry (Fig. 3A). The relative expression levels of cardiac troponin I, Bax, and Bcl-2 were analyzed using western blotting (Supplementary Fig. S2A, B). Apoptosis was markedly increased in the ISO group compared with that in the control group (Fig. 3B). Moreover, the expression of Bax, which is positively related to apoptosis, increased, whereas the expression of Bcl-2, which is negatively related to apoptosis, decreased in the ISO group compared with the control group (Fig. 3C, D). In addition, cardiac troponin I level was also significantly higher in the ISO group than in the control group ($p < 0.05$) (Fig. 3E). The levels of IL-1β and IL-6 were significantly higher in the ISO group than in the control group ($p < 0.05$) (Fig. 3F, G). This indicated that the administration of ISO induced cardiomyocyte injury and promoted inflammation. A comparison between the ISO and ISO + LCZ696 (10 μM) groups revealed a significant reduction in apoptosis after LCZ696 treatment (10 μM) ($p < 0.05$). This was confirmed by the reduced Bax expression, which is positively related to apoptosis, and the elevated Bcl-2 expression of Bcl-2, which is negatively related to apoptosis, in the ISO + LCZ696 (10 μM) group (Fig. 3B-D). In addition, a noticeable reduction in cardiac troponin I, IL-1β, and IL-6 levels was noted in the ISO + LCZ696 (10 μM) group compared to the ISO group (Fig. 3E-G). Taken together, these findings suggest that LCZ696 treatment significantly ameliorated ISO-induced cardiomyocyte injury and reduced inflammation ($p < 0.05$).

Ca^{2+} levels were determined based on the mean fluorescence intensity of calcium ions recorded using a calcium ion fluorescent probe (Fig. 4A). Cell viability was assessed by the CCK8 assay, and SERCA2α was evaluated by western blotting (Fig. 4B). Our results showed that administration of ISO in the ISO group significantly decreased Ca^{2+} levels compared with the control group, and LCZ696 treatment in the ISO + LCZ696 (10 μM) group alleviated the

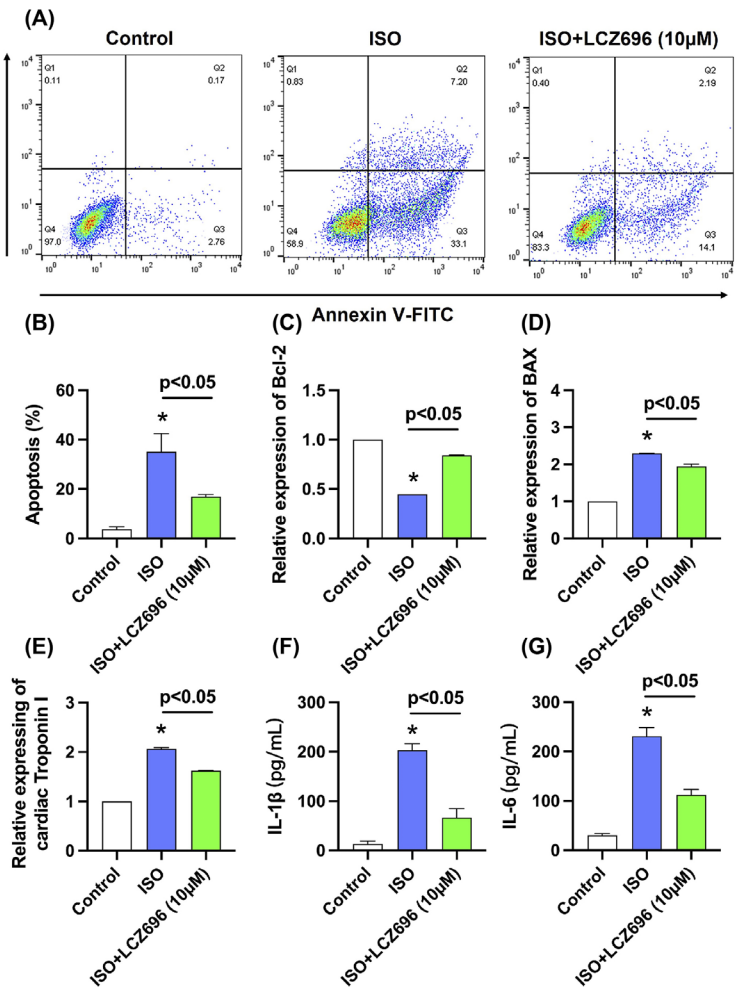

Fig. 3. Establishment of isoprenaline (ISO)-induced cardiomyocyte injury model in HL-1 cells.
Apoptosis was analyzed using flow cytometry (A). Apoptosis was markedly decreased in the ISO + LCZ696 (10 μM) group compared with that in the ISO group (B). The expression of Bcl-2 significantly increased in the ISO + LCZ696 (10 μM) group compared with that in the ISO group (C). The expression of BAX progressively decreased in the ISO + LCZ696 (10 μM) group compared with that in the ISO group (D). Cardiac troponin I expression significantly decreased in the ISO + LCZ696 (10 μM) group compared to that in the ISO group (E). IL-1β and IL-6 levels significantly decreased in ISO + LCZ696 (10 μM) compared with those in the ISO group (F, G). *$p < 0.05$ vs. control group.

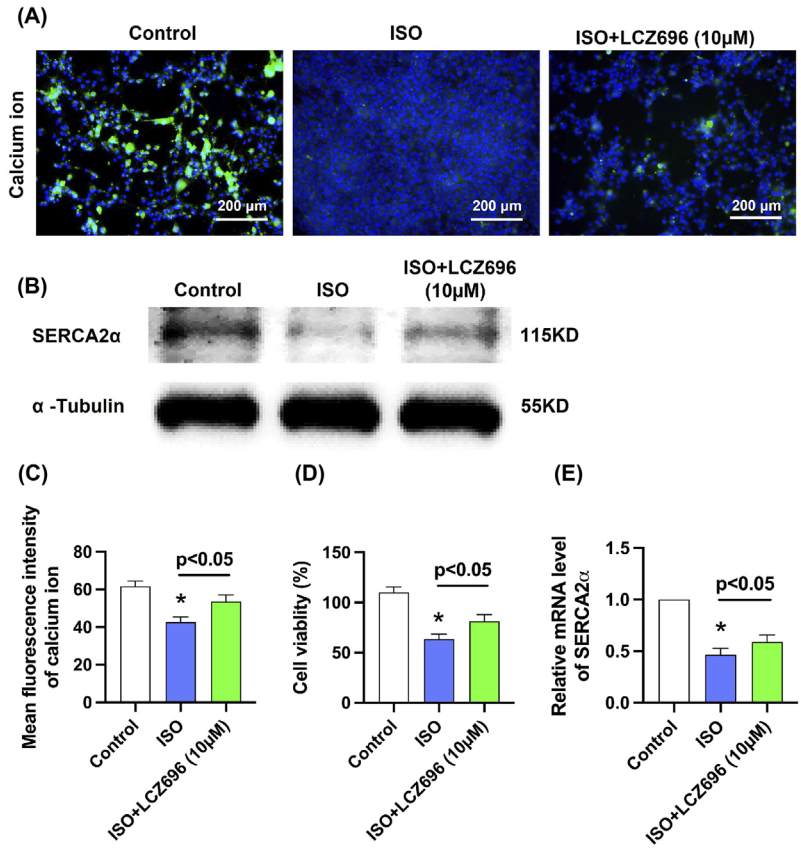

Fig. 4. LCZ696 ameliorated the Ca^{2+} dyshomeostasis.
Calcium ion levels were quantified using fluorescence (A). The expression of SERCA2α was evaluated by western blot analysis (B). The concentration of Ca^{2+} significantly increased in ISO + LCZ696 (10 μM) compared to that in the ISO group (C). Cell viability and relative mRNA levels of SERCA2α increased in ISO + LCZ696 (10 μM) compared to the ISO group (D, E). *p < 0.05 vs. control group.

reduction in Ca^{2+} levels compared with the ISO group ($p < 0.05$) (Fig. 4C). Cell viability was also significantly decreased in the ISO group compared with the control group, and LCZ696 treatment ameliorated this change in the ISO + LCZ696 (10 μM) group ($p < 0.05$) (Fig. 4D). SERCA2α expression was significantly decreased in the ISO group compared to the control group, and the expression of SERCA2α increased in the ISO + LCZ696 (10 μM) group ($p < 0.05$) (Fig. 4E).

To evaluate the therapeutic effect of LCZ696 on ISO-induced Ca^{2+} dyshomeostasis, the expression of HRC, PKA, S100A1, PLB, and CaMKII was detected in HL-1 cells cultured with or without LCZ696 using qRT-PCR and western blotting. Compared to the ISO group, the relative mRNA level of HRC was significantly improved in the ISO + LCZ696 (10 μM) group ($p < 0.05$) (Fig. 5A), whereas no significant difference was observed between the ISO and ISO + LCZ696 (10 μM) groups (Fig. 5B). Compared to the ISO group, the relative mRNA and protein levels of PKA and S100A1 were significantly enhanced in the LCZ696 (10 μM) group ($p < 0.05$), whereas the relative mRNA and protein levels of PLB and CaMKII were significantly reduced (Fig. 5C-J). Western blot results are shown in Fig. 5K. Taken together, our results demonstrate that LCZ696 improved ISO-induced Ca^{2+} dyshomeostasis in HL-1 cells, altering the expression of Ca^{2+}-regulatory proteins.

Overexpression of SERCA2α regulated the expression of Ca^{2+}-related proteins and alleviated myocardial injury

To investigate the therapeutic role of SERCA2α in Ca^{2+} dyshomeostasis, the expression of HRC, PKA, S100A1, PLB, and CaMKII was detected in SERCA2α-overexpressed HL-1 cells using qRT-PCR and western blotting. The results of the densitometric analysis of the western blots are shown in Supplementary Fig. S3A. No significant difference was noted between the ISO and NC groups (HL-1 cells transfected with the SERCA2 empty vector cultured with 16 μM ISO for 48 h) at either the mRNA or protein levels of HRC, PKA, S100A1, PLB, and CaMKII (Fig. 6A-L). Nevertheless, compared with the ISO group, the mRNA level and protein expression of HRC, PKA, and S100A1 were significantly improved in the OE-SERCA2 group ($p < 0.05$) (Fig. 6A-F), whereas the mRNA level and protein expression of PLB and CaMKII were remarkably reduced in the OE-SERCA2 group (Fig. 6G-J). These results illustrate that SERCA2α plays a vital role in regulating the expression of Ca^{2+}-related proteins and maintaining Ca^{2+} homeostasis. In addition, we detected

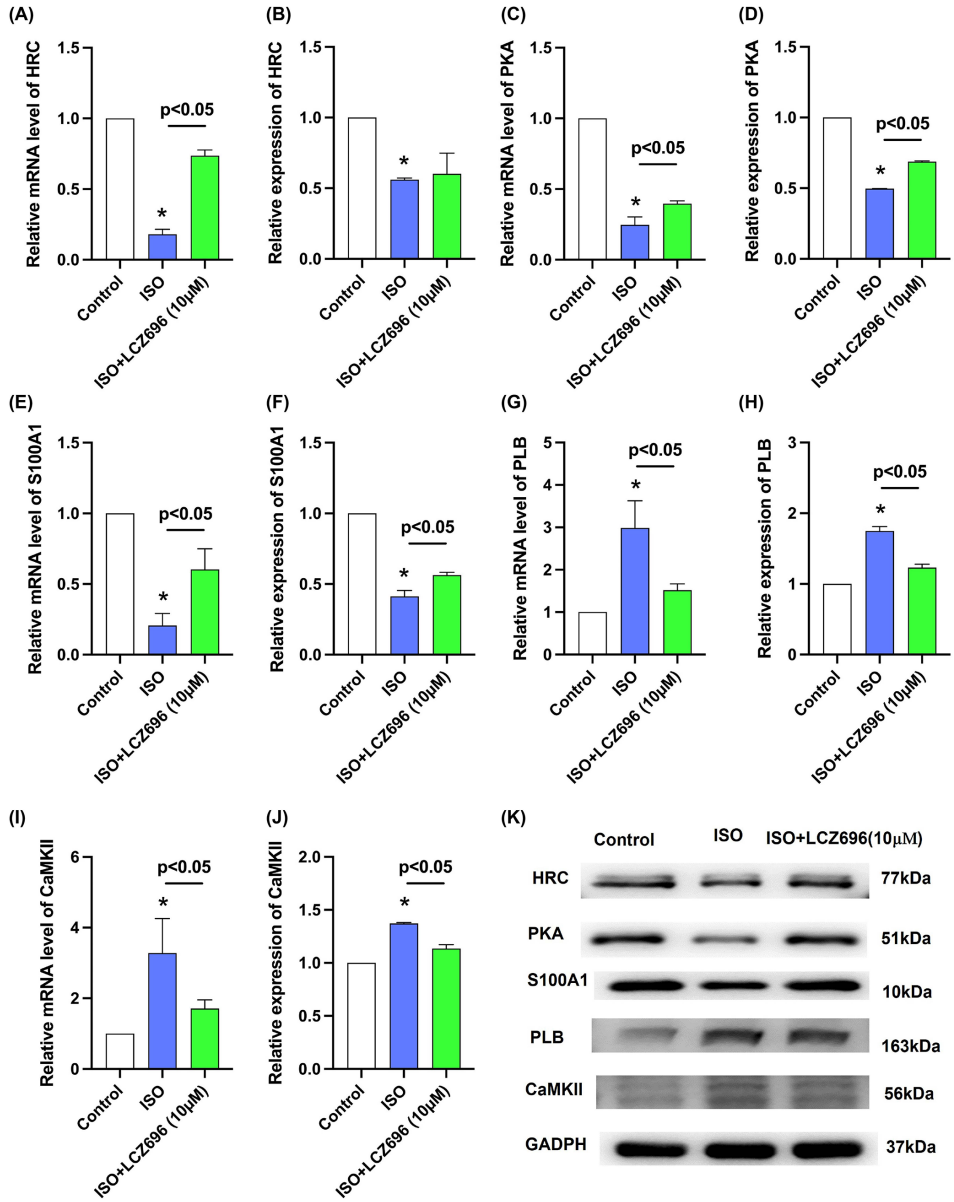

Fig. 5. LCZ696 modulated the expression of Ca^{2+}-related proteins.
The results of qRT-PCR and western blotting showed that relative mRNA and protein levels of histidine-rich calcium-binding protein (HRC), protein kinase A (PKA) and S100A1 were significantly reduced in the ISO group compared with the control group, and LCZ696 significantly improved relative mRNA and protein levels of HRC, PKA, and S100A1 in the ISO + LCZ696 (10 μM) group relative to the ISO group (A-F). The relative mRNA and protein levels of phospholamban (PLB) and calmodulin-dependent protein kinase II (CaMKII) increased remarkably in the ISO group relative to the control group. LCZ696 notably decreased the relative mRNA and protein levels of PLB and CaMKII in the ISO + LCZ696 (10 μM) group relative to the ISO group (G-J). The expressions of HRC, PKA, S100A1, PLB, and CaMKII were evaluated by western blot analysis (K). *p < 0.05 vs. control group.

cardiac troponin I levels and the mean fluorescence intensity of calcium ions. Our results demonstrated that there was no significant difference in cardiac troponin I and Ca^{2+} levels between the ISO and NC groups, while SERCA2α overexpression significantly decreased cardiac troponin I and increased Ca^{2+} levels ($p < 0.05$) (Fig. 6K, L, Supplementary Fig. S3B).

Discussion

In this study, we provide evidence that LCZ696 ameliorates tachycardia-induced myocardial injury by regulating the expression of SERCA2α, modulating the expression of Ca^{2+}-related proteins, and maintaining Ca^{2+} homeostasis. The mechanism by which LCZ696 reduced myocardial injury was at least partly associated with the improvement in the abnormal expression of Ca^{2+}-related proteins, likely

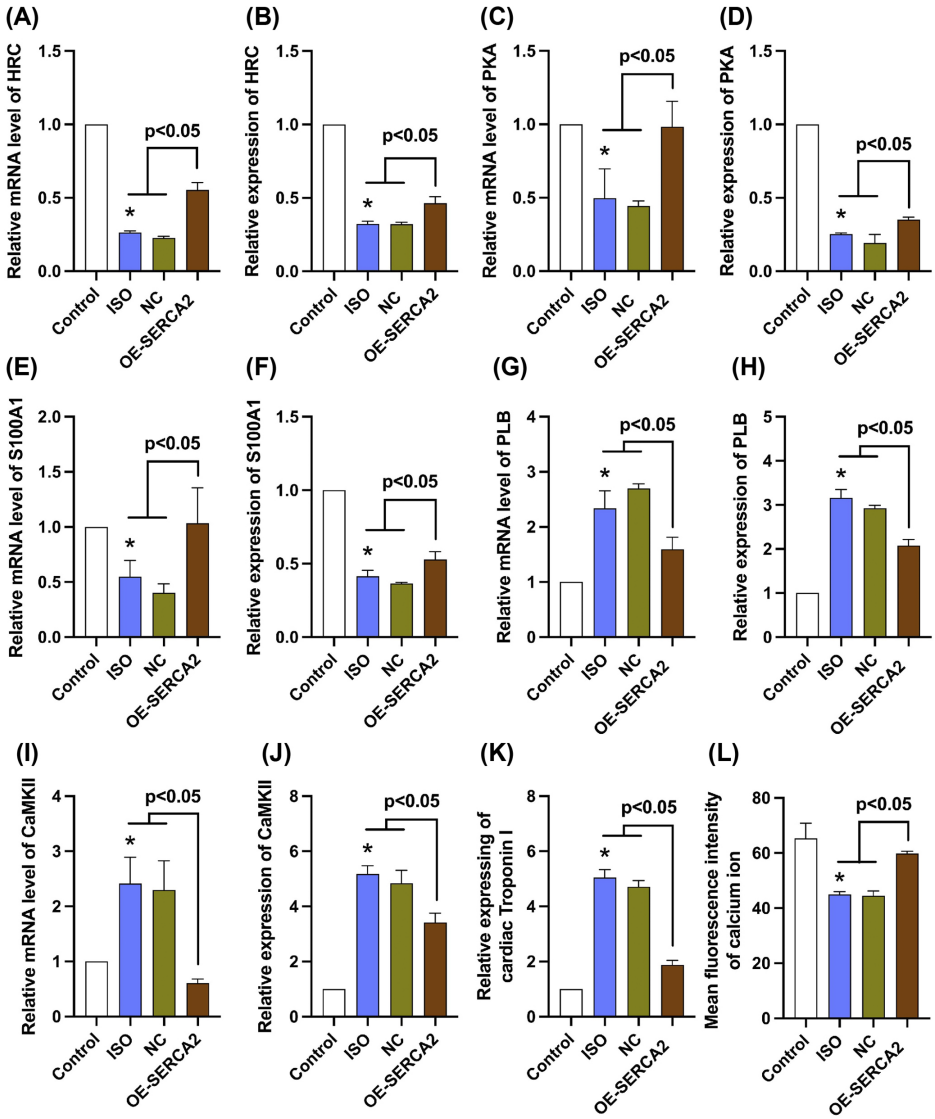

Fig. 6. Overexpression of SERCA2α regulated the expression of Ca^{2+}-related proteins and alleviated myocardial injury. The relative mRNA and protein levels of histidine-rich calcium-binding protein (HRC), protein kinase A (PKA), and S100A1 improved significantly in the OE-SERCA2α group compared to those in the ISO group, whereas no significant difference was noted between the ISO and NC groups (A-F). The relative mRNA and protein levels of phospholamban (PLB) and calmodulin-dependent protein kinase II (CaMKII) were reduced remarkably in the OE-SERCA2α group, whereas no significant difference was observed between the ISO and NC groups (G-J). Cardiac troponin I expression was significantly lower in the OE-SERCA2α group than in the ISO group, whereas no significant difference was noted between the ISO and NC groups (K). The Ca^{2+} concentration was significantly higher in the OE-SERCA2α group than in the ISO group, whereas no significant difference was noted between the ISO and NC groups (L). *$p < 0.05$ vs. control group.

mediated by the increased expression of SERCA2α.

Previous studies have reported that LCZ696 reduces proarrhythmogenic SR Ca^{2+} leakage and improves Ca^{2+} homeostasis under catecholaminergic stress (Eiringhaus et al. 2020). These therapeutic effects contribute to the decreased expression of CaMKII (Chang et al. 2020). Consistent with a previous study, our results showed that oral administration of LCZ696 significantly decreased CaMKII expression. A previous study also demonstrated that CaMKII activation was associated with mitochondrial Ca^{2+} overload, culminating in myocardial death and HF (Joiner et al. 2012). Downregulation of CaMKII can prevent mitochondrial Ca^{2+} overload, ameliorate myocardial injury, and reduce the incidence of arrhythmia (Joiner et al. 2012; Beauverger et al. 2020). Both valsartan and sacubitril have been reported to contribute to the therapeutic effect of LCZ696, as a previous study demonstrated the superior therapeutic efficacy of LCZ696 relative to valsartan in the regulation of CaMKII (Chang et al. 2020). In addition to CaMKII, our results demonstrated that LCZ696 modulated the expression of HRC, PKA, S100A1, and PLB. The HRC plays a key role in SR Ca^{2+} regulation (Arvanitis et al. 2011). HRC has been shown to interact with both triadin and SERCA2α, and SR Ca^{2+} uptake is regulated by HRC in

mouse and human hearts, indicating that it is a regulator of SR Ca^{2+} (Arvanitis et al. 2011). PKA is vital in maintaining cardiac Ca^{2+} homeostasis because reduced PKA expression leads to downregulated Ca^{2+} cycling and decreased cardiomyocyte contractility (Liu et al. 2022). S100A1 is another regulator of Ca^{2+} cycling, and a previous report showed that *S100A1* gene therapy could restore abnormal Ca^{2+} cycling, ameliorate SR dysfunction, and maintain energy homeostasis (Pleger et al. 2011). Moreover, PLB regulates SR Ca^{2+} handling, and its increased expression impairs SR Ca^{2+} cycling (Periasamy et al. 2008). Our *in vivo* results showed that oral administration of LCZ696 improved the expression of HRC, PKA, and S100A1 and reduced the expression of PLB and CaMKII. However, no significant difference was observed in HRC expression between ISO-cultured HL-1 cells with or without LCZ696 treatment. Nonetheless, qRT-PCR results demonstrated higher mRNA levels in HL-1 cells cultured with ISO and 10 μM LCZ696. One possible explanation is that short-term LCZ696 treatment was insufficient to cause increased HRC protein expression, given that changes in mRNA expression precede changes in protein levels. Collectively, our results suggest that oral administration of LCZ696 ameliorated SR dysfunction, enhanced SR Ca^{2+} handling, and improved cardiac Ca^{2+} homeostasis. In addition to regulating cardiac Ca^{2+} homeostasis, LCZ696 improves myocardial injury by alleviating inflammatory cytokine production, and decreasing apoptotic and fibrotic manifestations.

SERCA2α overexpression improved cardiac function in previous *in vitro* and *in vivo* studies (Fargnoli et al. 2013; Gong et al. 2016). Studies have highlighted the importance of SERCA2α in cardiac pathophysiology because of its critical role in preventing the development of HF and in improving both systolic and diastolic functions (Eisner et al. 2013; Zhihao et al. 2020). Various studies have shown that SERCA2α interacts with S100A1 (Kiewitz et al. 2003; Yang et al. 2021), HRC (Arvanitis et al. 2007, 2018), CaMKII (Fischer et al. 2013), PKA (Liu et al. 2022), and PLB (Kranias and Hajjar 2012) proteins. Similarly, these proteins play a role in modulating calcium cycling, leading to improvements in cardiac function. These results suggested that SERCA2α plays a crucial role in calcium regulation. Our *in vitro* study demonstrated that SERCA2α overexpression improved the expression of HRC, PKA and S100A1, and reduced the expression of PLB and CaMKII. These results provide novel evidence that the overexpression of SERCA2α improved anti-arrhythmic protein expression and inhibited proarrhythmic protein expression. Moreover, our *in vitro* and *in vivo* studies showed that LCZ696 might ameliorate the decrease in SERCA2α expression after the administration of ISO. Taken together, our findings provide evidence that oral administration of LCZ696 modulates the expression of SERCA2α. Furthermore, the increased expression of SERCA2α enhances the expression of Ca^{2+}-related proteins, including HRC, PKA, and S100A1. Moreover, it inhibited the expression of Ca^{2+}-related proteins, including PLB and CaMKII. These therapeutic effects contribute to maintaining cardiac Ca^{2+} homeostasis and ameliorating myocardial injury.

In mice with sympathetic hyperactivity-induced HF, angiotensin receptor inhibitors increased cardiac SERCA2α expression and rearranged cardiac Ca^{2+}-handling protein networks, thereby improving ventricular function (Ferreira et al. 2011). Nevertheless, another study showed that a neprilysin inhibitor affected pro-arrhythmogenic SR Ca^{2+} leakage without affecting the expression and effect of SERCA2α (Eiringhaus et al. 2020). These findings suggest that, in our study, the therapeutic effects of LCZ696 on SERCA2α were most likely dependent on angiotensin receptor inhibitors. The effect of neprilysin inhibitors on the expression of SERCA2α and Ca^{2+} hemostasis should be investigated in the future.

This study had several limitations. First, although an interaction between LCZ696 and SERCA2α was observed, the underlying molecular mechanism was not investigated in detail. Second, the potential variation in Ca^{2+}-related protein levels may be influenced by other factors, such as differences in animal species, drug induction, dosage, and duration of drug therapy. Nonetheless, cardiac arrhythmia was not observed in this study. Future studies should explore whether LCZ696 treatment decreases the incidence of cardiac arrhythmia in patients with tachycardia-induced myocardial injury.

In conclusion, our findings provide novel evidence that LCZ696 treatment upregulates SERCA2α expression, restores calcium-related protein levels, and ameliorates tachycardia-induced myocardial injury. Our results further support the application of LCZ696 in clinical practice to prevent HF development.

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Author Contributions

Data curation, formal analysis, investigation, methodology, and writing—original draft, L.L.; supervision and writing—review and editing, S.S.; methodology, Z.Y.; methodology, S.Z.; conceptualization, supervision, funding acquisition, validation, project administration, and writing—review and editing, C.Z. All authors have read and agreed to the published version of the manuscript.

Conflict of Interest

The authors declare no conflict of interest.

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Supplementary Files

Please find supplementary file(s);
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