Luteolin suppresses lipopolysaccharide-induced cardiomyocyte hypertrophy and autophagy in vitro

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Abstract. Luteolin (LTL) serves essential roles in a wide variety of biological processes. Lipopolysaccharide (LPS) can lead to myocardial hypertrophy and autophagy. However, the roles of LTL on LPS-induced cardiomyocyte hypertrophy and autophagy in rat cardiomyocytes have not yet been fully elucidated. In the present study, the morphology of cultured rat cardiomyocytes was observed under an inverted microscope. Cell viability was detected by MTT assay. α-Actinin and microtubule-associated protein 1 light chain 3 (LC3) expression levels were measured by immunofluorescence assay. In addition, the expression levels of atrial natriuretic peptide/brain natriuretic peptide (ANP/BNP), LC3, and autophagy- and Wnt signaling pathway-associated genes were analyzed by reverse transcription-quantitative polymerase chain reaction or western blot assays. The results indicated that LTL increased the cell viability of cardiomyocytes treated with LPS. LTL decreased the expression of cardiac hypertrophy associated markers (ANP and BNP). LTL decreased α-actinin and LC3 expression levels in LPS-treated cardiomyocytes. It was also demonstrated that LTL suppressed the mRNA and protein expression levels of LPS-mediated autophagy and Wnt signaling pathway-associated genes. In addition, it was demonstrated that silencing of β-catenin inhibited LPS-induced cardiomyocyte hypertrophy and the formation of autophagosomes. Thus, the present study suggested that LTL protected against LPS-induced cardiomyocyte hypertrophy and autophagy in rat cardiomyocytes.

Introduction

Myocyte hypertrophy is one of the most important adaptive responses of the heart (1,2). The cardiomyocyte hypertrophy is characterized by enlarged myocardial cells and the accumulation of sarcomeric proteins (3,4). During this process, the myocardium irreversibly enlarges and expands, which is accompanied with apparent cardiac failure (5).

Lipopolysaccharide (LPS), a pathogen-associated molecular pattern, which can be found in the outer membrane of gram-negative bacteria (6), and can induce strong immune reaction (7). Previous studies have demonstrated that the mechanism of LPS-induced cardiac dysfunction is the activation of inflammation, or necrosis or cardiomyocyte apoptosis (8-10). LPS may couple with the Toll-like receptor 4, leading to cytoplasmic accumulation, nuclear translocation of β-catenin, target gene transcription and expression of myocardial hypertrophy genes (11,12).

Autophagy is a highly conserved lysosomal degradation pathway, of which there are three types: Chaperone-mediated autophagy, macroautophagy and microautophagy (13). Previous studies have demonstrated that autophagy is associated with many physiological and pathological processes; for example, cell development, immunity, infection, aging, cell survival, death and metabolism (14). Autophagy serves an important role in many critical human diseases, for instance, metabolic disorders, muscle atrophy, cancer, neurodegenerative and cardiovascular diseases (15-17). However, excessive autophagy can induce pathological diseases and autophagic cell death (18). A previous study has demonstrated that the suppression of excessive autophagy can relieve acute myocardial injury as well as accelerating rat model survival of LPS-induced cardiomyocyte contractile dysfunction (19).

Luteolin (LTL) is a type of natural flavonoids, and belongs to weak acid tetrahydroxy flavonoids (20). It has been demonstrated that flavonoids have a certain therapeutic effect on cardiovascular disease, by protecting the damaged myocardium (17,18,21,22). Moreover, LTL has a series of pharmacological effects, including anti-inflammatory, antioxidative, antitumor and antiviral (23-25). However, the effect of LTL on LPS-induced cardiomyocyte hypertrophy and the formation of autophagosomes are not fully understood.

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In the present study, the effect of LTL on LPS-induced viability of rat cardiomyocytes, and the effect of LTL on LPS-induced cardiomyocyte hypertrophy and the formation of autophagosomes was investigated. The regulatory effect of LTL on the expression level of β-catenin, was also investigated. In addition, β-catenin expression levels were silenced using small interference (si)RNAs in cardiomyocytes, and this effect on LPS-induced cardiomyocyte hypertrophy and formation of autophagosome was investigated.

Materials and methods

Source of drugs. LTL powder was obtained from Hangzhou Tiancao Technology Co., Ltd. (Hangzhou, China); the purity was >98%. LPS freeze-dried powder was purchased from Beijing Solarbio Science & Technology Co., Ltd. (Beijing, China); the purity was >99%. The two powders were dissolved in DMSO and the solution was filtered with a 0.22 µm membrane.

Cell culture. All animal experiments in the present study were approved by the animal ethics committee of Jiangsu Jiankang Vocational College. Hearts from Sprague Dawley (Nanjing Better Biotechnology Co., Ltd., Nanjing, China; https://www.biomart.cn/46372/index.htm) rats were used in single-cell cultures, as described previously (26). The rats (12 female, 3 male; age 8-10 weeks; average weight 300 g) had free access to water and food, and were maintained at 25°C, 12-h light/dark and 55-65% humidity. The neonatal rats were produced after the animals mated. Neonatal rats (n=5; age, 1-3 days) were used to isolate cardiomyocytes. The hearts were collected and minced into 1 mm² sections. The pieces were dissociated with a buffer containing trypsin (0.05%) (Hyclone, Logan, UT, USA) and collagenase type II (0.4%) (Beijing Solarbio Science & Technology Co., Ltd) at 37°C for 30 min. The dissociation was stopped by the 5 min incubation of cold horse serum (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) at 37°C. The isolated cells were cultured in Dulbecco's modified Eagle's medium/F12 (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich; Merck KGaA). After three days, 0.1 mM bromodeoxyuridine (Sigma-Aldrich; Merck KGaA) was added into the medium to inhibit the growth of cardiac fibroblasts (27). Cells were cultured in a 90% humidified 37°C incubator with 5% CO₂. Finally, the morphology of the cultured rat cardiomyocytes was observed under inverted optical microscope (Nikon Corporation, Tokyo, Japan).

SiRNA transfection. siRNAs were commercially purchased from Thermofisher Scientific, Inc. The sequences of selected regions to be targeted by siRNAs for β-catenin were: 5'-UGG UUGGCUUUGCUCAACAATT-3' (sense), 5'-UUGGUGAGCAAGGCAACCATT-3' (antisense). Cells (1x10⁵ cells/ml) were transfected with 50 nM scramble siRNA (Negative control, NC) or β-catenin-siRNA by Lipofectamine® 3000 (Invitrogen; Thermo Fisher Scientific, Inc.) following the manufacturer's protocol. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and western blot assays were performed to assess the transfection efficiency. Cells transfected into β-catenin-siRNA were cultured for 24 h and then used for subsequent experiments.

Cell treatment. Cardiomyocytes (2.5x10⁵ cells/ml) were treated with 0.1% PBS (blank control group), 10% FBS (negative group, NC), LPS (100 ng/ml), LTL1 (50 mg/ml) and LPS (100 ng/ml), and LTL 2 (100 mg/ml) and LPS (100 ng/ml) for 8 h at 37°C. Subsequently, cells were used to detect the cell viability, the expression levels of α-actinin and LC3, and autophagy and Wnt signaling pathway-associated gene expression. Cardiomyocytes were treated with PBS, FBS, LPS, β-catenin siRNAs and LPS, and β-catenin siRNAs + LTL1 + LPS.

RT-qPCR assay. Total RNA was extracted from the treated cells using TRIzol® (Invitrogen; Thermo Fisher Scientific, Inc.) following the manufacturer's protocol. First-strand cDNA was synthesized using Revert Aid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific Inc.). The mRNA expression levels were determined by qPCR using SYBR GREEN PCR Master Mix (Applied Biosystem; Thermo Fisher Scientific Inc.) and assayed by ABI 7500 Real-time PCR system (Applied Biosystems; Thermo Fisher Scientific Inc.). The thermocycling conditions were set as follows: 10 min pretreatment at 95°C for 10 min, denaturation at 95°C for 15 sec; annealing at 61°C for 30 sec, extension at 72°C for 30 sec (40 cycles) and finally a 7-min extension at 72°C. The data were analyzed using 2^∆∆Cq method (28). The gene-specific primer sequences are listed in Table I; GAPDH was used as an internal loading control.

Western blot assay. Cells (5x10⁴ cells/ml) were lysed on ice in radioimmunoprecipitation assay buffer (cat. no. 8990; Pierce; Thermo Fisher Scientific, Inc.) buffer containing protease inhibitor cocktail (Thermo Fisher Scientific, Inc.). Pierce bicinechincon Protein Assay kit (Thermo Fisher Scientific, Inc.) was applied to analyze the concentration of proteins. Total protein (30 µg) was separated by 10% SDS-PAGE and then transferred onto polyvinylidene fluoride membranes (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The membranes were blocked with 5% skimmed milk and incubated with primary antibody overnight at 4°C. Next day, the membranes were as required incubated with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1:2,000; cat. no. sc-2004; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) or chicken anti-goat IgG-HRP (1:1,000; cat. no. sc-516086; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) for 2 h at room temperature. The protein expression levels were measured using an enhanced chemiluminescence system (Amersham Pharmacia Biotech; GE Healthcare, Chicago, IL, USA). The results were analyzed by Image Lab Software version 4.1 (Bio-Rad Laboratories, Inc.). Primary antibodies used were as follows: Anti-GAPDH (1:1,000; cat. no. sc-20358; Santa Cruz Biotechnology); anti-LC3 [two forms: LC3I and LC3II (29,30)]; 1:3,000; cat. no. ab64781; Abcam, Cambridge, UK); anti-Atg12 (1:1,000; cat. no. ab155589; Abcam), anti-autophagy-related 4b cysteine peptidase (Atg4b; 1:1,000; cat. no. 5299; Cell Signaling Technology, Inc., Danvers, MA, USA); anti-vacuolar protein sorting-associated protein 34 (Vps34; 1:1,000; cat. no. V9764; Sigma-Aldrich; Merck KGaA); anti-B cell lymphoma 2 interacting protein 1 (Bnip1; 1:300;
Table I. Primer sequences for the reverse transcription-quantitative polymerase chain reaction.

| Gene      | Primer sequences (5'-3') |
|-----------|--------------------------|
| GAPDH     | F: TATGATGATATAAGGCGGTAGT R: TGTATCACAATTCCTGGTCATAC |
| LC3       | F: GAGAAGGCTTCTCGCTGAGAAG R: GTGTTGGCTCAACAAAGGAGAG |
| BNP       | F: TCGGGCGATCTAGCGCTTGAGA R: CGAGGCGAGTGCAAGAAGCCG |
| ANP       | F: TTCTCCATCAAGACGCTTCTGG R: GACCTCTCATTTCTACCGGCA |
| Atg12     | F: CAGAAACAGCCATCCCCAGAG R: GCCCTCCAGGATGTCAATGTA |
| Atg4b     | F: TATGATCTCCTGGTTTCTGTGA R: GTTCCCCCAATAAGCTGGAAG |
| Vps34     | F: TGGAACTGGAATAAGGCGG R: GCATCCCTTGGCGAAACAG |
| Bnip1     | F: GAGAGTGAGGAGTTGTGATGA R: TATGGGACGCCCTAGACATG |
| Wnt2      | F: GAGATGCCAGCCCTGATGAA R: GCCAGCCAGCATGTCCTGAG |
| β-catenin | F: GACCTCACCTGACAGATCCAG R: AGCTGAACAAGAGTCCCAAG |
| GSK3β     | F: CTGGGAGCACTGAGGAGAA R: AAGAGGAGGCTTGGAGAGG |

ANP, atrial natriuretic peptide; Atg, autophagy-related 4b cysteine peptidase; Bnip, B cell lymphoma 2 interacting protein; BNP, brain natriuretic peptide; F, forward; GSK3β, glycogen synthase kinase 3β; LC3, microtubule-associated protein 1 light chain 3; R, reverse; Vps, vacuolar protein sorting-associated protein.

Cat. no. sc-1713; anti-Wnt2 (1:300; cat. no. sc-50361; Santa Cruz Biotechnology, Inc.); anti-glycogen synthase kinase 3β (GSK3β); 1:5,000; cat. no. ab32391; Abcam, Cambridge, UK) anti-phosphorylated-glycogen synthase kinase (p-GSK3β); 1:1,000; cat. no. 5558; Cell Signaling Technology, Inc.) and anti-β-catenin antibody (1:1,000; Abcam, cat. no. ab2982).

Cell viability assay. MTT assay was carried out to determine the cell viability. The treated cardiomyocytes (2x10^3 cells/well) were first seeded into 96-well plates and then cultured in cell incubators for 6 h at 37°C. After being washed, cells were incubated with a fluorescence-conjugated secondary antibody (4 µg/ml; Alexa-Fluor® 594 goat anti-rabbit antibody IgG; A-11037; Thermo Fisher Scientific, Inc.) for 1 h at room temperature. Nuclei were counterstained with 4'-6-diamidino-2-phenylindole (DAPI; 1:1,000; Thermo Fisher Scientific, Inc.) for 15 min at 37°C, and the images were then obtained using a fluorescence microscope (Olympus Corporation, Tokyo, Japan). The cell surface area (green of fluorescence staining) was calculated by formula (axb)/2 (a: The longest distance through the nucleus; b: The shortest distance through the nucleus) and analyzed by Image Pro-Plus 6.1 software (National Institutes of Health, Bethesda, MD, USA).

Statistical analysis. All experiments were performed at least three times. Numerical data are shown as the mean ± standard error. Statistical analysis was performed using SPSS 13.0 (SPSS, Inc., Chicago, IL, USA) and statistical significance was assessed by analysis of variance with Dunnett's post-test comparison. P<0.05 was considered to indicate a statistically significant difference.

Results

LTL increases the viability of cardiomyocytes treated with LPS. To investigate the potential roles of LTL in cardiomyocytes, myocardial cells were isolated from newborn rats. The morphology of the cultured rat cardiomyocytes was observed with a microscope and most cells were typical myocardial cells (Fig. 1A). To determine the effect of LTL on cell viability, cardiomyocytes were treated with 0.1% PBS (blank control group), 10% FBS (negative group, NC), LPS alone (100 ng/ml), LTL1 (50 mg/ml) + LPS (100 ng/ml) and LTL 2 (100 mg/ml) + LPS (100 ng/ml) for 8 h. MTT assay was used to detect cell viability. The results indicated that LPS significantly decreased the viability of rat cardiomyocytes (P<0.001), however, such an effect was reversed by LTL (P=0.0487; Fig. 1B).

LTL suppresses LPS-induced cardiomyocyte hypertrophy and formation of autophagosomes. In order to prove the role of LTL on cardiomyocyte hypertrophy and formation of autophagosomes, cultured cardiomyocytes were treated with PBS, FBS, LPS, LTL1 and LPS, and LTL2 and LPS for 8 h. The expression levels of ANP and BNP were increased by LPS significantly decreased the viability of rat cardiomyocytes (P<0.001), however, such an effect was reversed by LTL (P=0.0487; Fig. 1B).
autophagosomes by densitometry (29,33), it was found that LPS increased LC3 expression levels, which was reversed by LTL, suggesting that LTL suppressed LPS-induced formation of autophagosomes (P<0.05; Fig. 2B).

LTL decreases LPS-mediated autophagy and Wnt signaling pathway-associated genes. In addition, the effects produced by LTL on autophagy-associated genes were further analyzed. Cardiomyocytes were treated with PBS, FBS, LPS, LTL1 + LPS, or LTL2 + LPS for 8 h. Expression levels of LC3 and autophagy-associated genes (Atg12, Atg4b, Vps34 and Bnip1) were measured by RT-qPCR and western blot assays. Results demonstrated that LC3 expression levels were notably upregulated in the LPS group in comparison with that of the NC group, and that LC3II expression levels were downregulated and LC3I expression was upregulated...
in both LTL + LPS groups, compared with the LPS alone group (P<0.05; Fig. 3A and B). However, LPS promoted autophagy-associated gene (Atg12, protein level of Atg4b, Vps34 and Bnip1) expression levels, which was then inhibited by LTL (P<0.05; Fig. 3C and D). LTL exhibited an abnormal effect on the mRNA expression of Atg4b in that low concentrations of LTL demonstrated a promoting effect and high concentrations of LTL demonstrated an inhibitory effect. This finding requires further research.

Wnt/β-catenin signaling pathway serves a conservative signaling pathway, and exists in various organisms (34,35). In addition, Wnt/β-catenin signaling pathway serves a dual role in the process of heart development (36,37). In the present study, the expression levels of Wnt signaling pathway-associated genes were investigated (Wnt2, β-catenin and p-GSK3β) using RT-qPCR and western blot assays. LPS significantly increased the expression levels of Wnt2, β-catenin and p-GSK3β, however, such an increase was inhibited by LTL (P<0.05; Fig. 3E and F).

β-catenin expression is silenced using siRNAs in cardiomyocytes. As shown in previous results, LTL decreased LPS-mediated Wnt signaling pathway-associated genes. The knockdown efficiency of β-catenin in cardiomyocytes using RT-qPCR and western blot assays was established. The results indicated that the mRNA expression levels of β-catenin were decreased in si-β-catenin group compared with that of control group (P<0.05; Fig. 4A). Western blot results demonstrated that in comparison with the NC group, the protein expression levels of β-catenin decreased notably in the si-β-catenin group (P<0.05; Fig. 4B and C).

Silencing of β-catenin inhibits LPS-induced cardiomyocyte hypertrophy and formation of autophagosomes. To further
investigate the exact roles of β-catenin in LPS-induced cardiomyocyte hypertrophy and the formation of autophagosomes in them, cardiomyocytes were treated with PBS, FBS, LPS, β-catenin siRNAs + LPS, and β-catenin siRNAs + LTL1 + LPS for 8 h. Immunofluorescence staining was then performed to measure the expression levels of α-actinin and LC3. The results demonstrated that LPS induced α-actinin expression levels, which was then reversed by silencing β-catenin by siRNA (From 7.5 - 5.5 µm$^2$) and by siβ-catenin with LTL1 co-treatment (From 7.5 - 5.0 µm$^2$). This may suggest that silencing of β-catenin and siβ-catenin with LTL1 co-treatment suppressed LPS-induced cardiomyocyte hypertrophy (P<0.05; Fig. 5A). Similarly, LPS was observed to increase LC3 expression levels, which was then reversed by the silencing of β-catenin and by siβ-catenin with LTL1 co-treatment, suggesting this may have inhibited LPS-induced formation of autophagosomes (P<0.05; Fig. 5B). Though there is no difference in the effects of siRNA with or without LTL1 co-treatment, the inhibitory effects of LTL1 co-treatment with siRNA was slightly stronger compared with siRNA treatment alone. If the concentration of LTL is increased, the inhibitory effects may be more pronounced and this needs to be investigated further.

Discussion

Lipopolysaccharide (LPS) is a category of glycolipids composed of two distinct regions, and LPS can lead to endotoxemia induced by infections with gram-negative bacteria (38-40). Cardiac hypertrophy is an adaptive mechanism of the heart, which can strengthen cardiac output in certain physiological and pathological conditions (41). Previous studies showed that LPS could cause cardiac dysfunction through cardiomyocytes apoptosis, activation of inflammation or necrosis (8-10).

Autophagy, as a cellular degradation system, mainly participates in removing redundancy or damaged organelles, and longe梭 proteins and cell components (42). At first, isolated membrane or phagophore of cytoplasm can form autophagosome (43,44). Subsequently, autophagosomes fuse with lysosomes, forming autolysosomes. The contents are then dissolved by the lysosomal hydrolytic enzymes (45). A previous study also demonstrated that autophagy may occur in particular situations, such as oxidative stress, chemical agent’s induction, nutrient deprivation, immune cell activation, cellular and tissue remodeling (42-46). Previous studies have indicated that autophagy was closely associated with immune processes (46,47). In addition, autophagy can be assessed by some markers, such as LC3, Atg, Bnip1 and Vps34 (48-50). Among them, LC3 is a common autophagy-associated marker (33). Previous studies have also shown that LPS-induced autophagy can participate in a variety of biological processes (51,52). Therefore, LPS was selected in the present study to induce hypertrophy and autophagy in cardiac myocytes.

LTL can reduce the mortality of coronary heart disease and has a protective effect on cardiovascular disease (53). Furthermore, some studies demonstrated that LTL repressed
Figure 5. Silencing of β-catenin inhibits LPS-induced cardiomyocyte hypertrophy and formation of autophagosome. (A) Expression levels of α-actinin (a cardiomyocyte specific marker and a hypertrophic marker gene) were measured by immunofluorescence staining; nuclei were stained with DAPI. The myocyte area was calculated by formula (axb)/2 (a: The longest distance through the nucleus; b: The shortest distance through the nucleus) and analyzed by Image Pro-Plus 6.1 software after anti-α-actinin staining (green). (B) LC3 expression levels were measured by immunofluorescence assay; nuclei were stained with DAPI. By specific labeling of autophagosome membranes (LC3II) and the cytoplasmic labeling (LC3I) and the amount of LC3 was quantified by densitometry. ***P<0.001 vs. NC; **P<0.01 and *P<0.05 vs. LPS. LC3, microtubule-associated protein 1 light chain 3; LPS, lipopolysaccharide; LTL, luteolin; NC, normal control; siRNA, small interfering RNA.

Figure 4. β-Catenin expression is silenced using siRNA in cardiomyocytes. (A) Reverse transcription-quantitative polymerase chain reaction and (B) western blot assays were performed to detect the knockdown efficiency of β-catenin siRNA. (C) Quantification of the western blot analysis from (B). ***P<0.001 and **P<0.01 vs. NC; NC, normal control; siRNA, small interfering RNA.
ischemia/reperfusion-induced myocardial damage in cardiomyocytes (54-55). However, the roles of LTL on LPS-induced cardiomyocyte hypertrophy and the formation of autophagosomes in cardiomyocytes were not entirely clear. The present study results found that LPS significantly decreased the viability of rat cardiomyocytes, and that the cell viability of rat cardiomyocytes was recovered when the cells were treated with LTL. In addition, the role of LTL on cardiomyocyte hypertrophy and the formation of autophagosomes was investigated, and it was found that LPS increased α-actinin and LC3 expression levels that were then reversed by LTL. This suggested that LTL suppressed LPS-induced cardiomyocyte hypertrophy and the formation of autophagosomes. The effects produced by LTL on autophagy-associated genes was also investigated. The present results demonstrated that LPS promoted LC3 and autophagy-associated genes (Atg12, Atg4b, Vps34 and Bnip1) expression levels but this was inhibited by LTL. Therefore, it may be suggested that LTL inhibited the LPS-induced cell viability, cardiomyocyte hypertrophy and the formation of autophagosomes in rat cardiomyocytes, but this needs to be investigated further.

Wnt signaling pathway largely serves essential roles in various biological processes (56-58). β-Catenin is a central mediator in this signaling pathway regulated by Wnt proteins, contributing to the development of various diseases, such as cancer, nephropathy, diabetes, Alzheimer's disease and nerve disease (59-61). Previous studies have demonstrated that Wnt signaling pathway can be activated by silencing GSK3β expression in human adipose stem cells (62). Previous studies have demonstrated that Wnt signaling pathway is involved, for example, in the regulation of cardiomyocyte regeneration, injury, hypertrophy (63-65). Hence, LPS might induce the hypertrophy and autophagy of cardiomyocytes via modulating Wnt signaling pathway. As expected, it was found that LPS induced the hypertrophy and autophagy of cardiomyocytes via stimulating Wnt pathway, however, LTL suppressed this pathway modulated by LPS. In addition, β-catenin silencing notably reduced LPS-induced hypertrophy and the formation of autophagosomes of cardiomyocytes.

In summary, the data obtained in the present study indicated that LTL increased the viability of cardiomyocytes treated by LPS, and that LTL inhibited cardiomyocyte hypertrophy and the formation of autophagosomes of rat cardiomyocytes induced by LPS. LTL decreased Wnt signaling pathway mediated by LPS, and silencing of β-catenin inhibited LPS-induced cardiomyocyte hypertrophy and the formation of autophagosomes. Therefore, it may be concluded that LTL may contribute to LPS-induced cardiomyocyte hypertrophy and autophagy in cardiomyocytes by regulating β-catenin, but this needs to be verified.

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Availability of data and materials

The analyzed data sets generated during the present study are available from the corresponding author on reasonable request.

Authors' contributions

XL designed the study, JL and JW performed the experiments, DZ and JW analyzed the data.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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