Lycium barbarum polysaccharide protects cardiomyocytes from hypoxia/reoxygenation injury via activation of SIRT3/CypD signaling

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Background: Myocardial ischemia-reperfusion is a common pathological feature of many heart and vascular diseases, but the molecular mechanism of this process is still unclear, and there is no effective way to protect cardiomyocytes. The aim of this study was to examine the effects and underlying molecular mechanisms of *Lycium barbarum* polysaccharide (LBP) on myocardial ischemia-reperfusion injury in cardiomyocytes.

Methods: The cardiomyocyte cell line H9c2 were used to establish an *in vitro* hypoxia/reoxygenation (H/R) model. After treatment with LBP and/or the SIRT3 inhibitor 3-TYP, cell morphology was observed under the light microscopy. The Cell Counting Kit (CCK)-8 and 5-ethynyl-2'-deoxyuridine (EdU) assay were used to detect cell proliferation, and flow cytometry was performed to assess cell apoptosis. The lysine (166)-acetylation of CypD1 was determined by co-immunoprecipitation assay. Enzyme-linked immunosorbent assay (ELISA) was used to determine the lactate dehydrogenase (LDH) level in the culture medium. Na^+-K^+-ATPase activity, Ca^{2+}-ATPase activity, and nitric oxide (NO) levels were measured.

Results: LBP alleviated cell damage and upregulated STIR3 expression in a dose-dependent manner. Upregulated SIRT3 expression and suppressed acetylation of CypD were also observed in H/R-induced H9c2 cells treated with LBP. Indeed, LBP remarkably reversed the inhibition of proliferation and cell apoptosis in H/R-induced H9c2 cells by activating SIRT3/CypD signaling. Blockade of SIRT3 with SIRT3 inhibitor (3-TYP) inhibited the protective effect of LBP on H9c2 cells. LBP markedly alleviated the H/R-induced increase of LDH release, and the decrease of Na^+-K^+-ATPase activity, Ca^{2+}-ATPase activity, and NO levels. Inhibition of SIRT3 restored the protective effects of LBP.

Conclusions: LPB induced deacetylation of CypD by upregulating SIRT3, thereby protecting mitochondrial function and relieving H/R-induced injury in cardiomyocytes.

Keywords: Myocardial ischemia-reperfusion injury (MIRI); Lycium barbarum polysaccharide (LBP); SIRT3; CypD; mitochondrial damage

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Introduction

Cardiovascular disease often leads to an ischemic myocardium. While therapy such as promoting myocardial blood supply, reducing oxygen consumption, and interventional therapy can restore the myocardial ischemia to a normal perfusion status (1,2), a long duration of secondary myocardial injury may follow, affecting myocardial ultrastructure, energy metabolism, cardiac function, and electrophysiology (3-6). This injury is defined as myocardial ischemia-reperfusion injury (MIRI), which is the leading cause of morbidity and mortality (7). In recent years, several methods have been explored for MIRI therapy, on the basis of mechanisms including reactive oxidative stress (ROS) injury (8), abnormal mitochondrial membrane potential (9), dysregulation of ATPase activity (10), inflammation (11), and cardiovascular injury (12). However, while these therapeutic methods have shown potential effects in animal models and *in vitro* studies, they are not available for clinical applications. Therefore, novel molecular targets are necessary for MIRI therapy.

Mitochondrial dysfunction is a leading cause of MIRI (13). In myocardial ischemia, the main form of energy metabolism in the mitochondria is anaerobic glycolysis, whereby adenosine triphosphate (ATP) levels decrease, leading to intracellular acidosis. In contrast, during MIRI, increased blood oxygen levels lead to ROS formation, thereby undermining oxygen balance and resulting in the opening of mitochondrial permeability transition pores (mPTPs). This results in swelling of the mitochondrial matrix, release of pro-apoptotic factors, and subsequent apoptosis of myocardial cells (14). The function of the mitochondria is regulated by several proteins, which may be potential therapeutic targets for heart disease. The SIRT3/CypD pathway is a crucial regulator of mPTP (15,16). CypD and SIRT3 proteins are localized in the mitochondrial matrix (17). SIRT3 is a key regulator of the deacetylation of CypD. Increased deacetylation of CypD decreases mPTP opening and damages mitochondria (18), and is followed by cell apoptosis (15). Elevated SIRT3 mediates the protective effect of metformin on cell survival after hypoxia/reoxygenation (H/R) injury (7). SIRT3 modulates mPTP by regulating the deacetylation of CypD, thereby inhibiting mPTP opening and decreasing cell apoptosis (19-21).

Lycium barbarum polysaccharide (LBP), the main effective component of *Lycium barbarum*, enhances immunity and oxidation resistance, and inhibits inflammation (22) and cell apoptosis (23). A clinical study shows that LBP has a protective effect on cardiovascular, but its mechanism of action is still unclear, which also limits its clinical application (24). Liu *et al.* (25) suggested that LBP is useful for cardiac hypertrophy therapy (26) and demonstrated that LBP protects against MI injury in H9c2 cells and myocardial ischemia reperfusion in a rat model. Moreover, LBP significantly decreases the myocardial levels of lactate dehydrogenase (LDH) and increases the Na^+-K^+-ATPase and Ca^{2+}-ATPase activity. These factors can be used as indicators of mPTP opening. Another study showed that LBP can alleviate MI by regulating Nrf2-related autophagy (27). In addition, LBP clearly decreases the positivity rate of myocardial Bax protein and myocardial cell apoptosis and increases the positivity rate of Bcl-2 in a dose-dependent manner (28). However, whether the SIRT3/CypD pathway participates in the protective function of LBP in mitochondria in MIRI remains unclear.

This current study analyzed the protective effects of LBP in MIRI cardiomyocytes and explored the underlying mechanisms by analyzing the effects on the SIRT3/CypD pathway and mitochondrial mPTP. The data herein provide insights into potential novel drug targets and therapies for MIRI. We present the following article in accordance with the MDAR reporting checklist (available at https://atm.amegroups.com/article/view/10.21037/atm-22-6081/rc).

Highlight box

Key findings

- LPB induced deacetylation of CypD by upregulating SIRT3, thereby protecting mitochondrial function and relieving H/R-induced injury in cardiomyocytes.

What is known and what is new?

- LBP alleviates H/R-induced H9c2 proliferation inhibition and apoptosis by activating SIRT3/CypD pathway.
- LBP alleviates mitochondrial injuries caused by H/R through activating SIRT3/CypD pathway.

What is the implication, and what should change now?

- LBP may be a potential novel treatment for MIRI and further research in warranted.

Methods

Cell culture

Rat cardiac myoblasts (H9c2) were purchased from Cell Bank (Chinese Academy of Sciences, Shanghai, China) and used to construct an H/R model. The cells were cultured

in Dulbecco's Modified Eagle Medium (DMEM; GIBCO, USA) with 10% fetal bovine serum (GIBCO, USA) at 37 ℃ under 5% CO_2.

Cell treatment

The H9c2 cells were cultured with 0, 0.5, 1.0, 2.0, 3.0, and 4.0 μg/mL LBP (#PB13182, Beijing Pufei Co., Ltd., China), 95% purity via UV (ultraviolet), containing six types of monosaccharides: arabinose, rhamnose, xylose, mannose, galactose and glucose, and small fractions of galacturonic acid and amino acids) for 48 hours and cell viability was assessed (29).

To analyze the protective effects of LBP on H/R, H9c2 cells were randomly divided into the following 5 groups: control, H/R, H/R + LBP 0.5 μg/mL, H/R + LBP 1.0 μg/mL, and H/R + LBP 2.0 μg/mL. For the control group, after routine liquid exchange, the cells were cultured under normal conditions for 12 hours. In the H/R group, cells were cultured under hypoxia conditions for 10 hours, followed by normoxia conditions for 2 hours (30). In the H/R + LBP 0.5, H/R + LBP 1.0, and H/R + LBP 2.0 groups, cells were pre-cultured in 0.5, 1.0, or 2.0 μg/mL LBP for 48 hours.

To verify the role of SIRT3 in the protective function of LBP, cells were incubated with the SIRT3 inhibitor (3-TYP) (99.93%, HY-108331, MedChem Express, Monmouth Junction, NJ, USA) at final concentrations of 0, 0.5, 1.0, 2.0 μM for 10 hours and cell viability was assessed using the Cell Counting Kit (CCK)-8. The effects of 3-TYP on SIRT3 mRNA and protein expressed were analyzed. H9c2 cells were pre-cultured in 0, 0.5, 1.0, and 2.0 μg/mL LBP for 48 hours. Each group of cells was then divided into 3 groups, namely, control, H/R, and H/R + 3-TYP. H/R treatment was performed as described above. 3-TYP was added to the medium at a final concentration of 1 μM to inhibit SIRT3. These 5 groups of cells, control, H/R, H/R + LBP (2.0 μg/mL), H/R + 3-TYP (1 μM), and H/R + LBP + 3-TYP, were then used for follow-up studies.

Quantitative real-time fluorescence reverse-transcription polymerase chain reaction (qRT-PCR)

Total RNA was extracted from H9c2 cells using the Oligotex mRNA Mini kit (Qiagen, Valencia, CA, USA) and reverse-transcribed into cDNA with the PrimerScript RT reagent kit (Takara, Dalian, China) in accordance with the manufacturer's instructions. PCR amplification was performed with a SYBR Premix Ex Taq™ II kit (#640022, TAKARA, Dalian, China) in a ViiATM 7 real-time fluorescence quantitative PCR system. The mRNA expressions of SIRT3 and CypD was normalized to that of β-actin. The relative expressions of SIRT3 and CypD was analyzed using the $2^{-\Delta\Delta CT}$ method. Primer sequence, SIRT3 forward (F): 5'-TGTGGGGTCCGGGAGTATTA-3', reverse (R): 5'-AGTAGTGAGCGACATTGGGC-3'; CypD (F): 5'-AGGCACTTGTGTCCTGCTTT-3', (R): 5'-AGGTTTCCCAGTTGTCGGTC-3'; β-actin (F): 5'-GTGGCGTCCTATAAAACCCG-3', (R): 5'-TAGGGGCCGGGAAAGTTAAG-3'.

Western blot analysis

Cells were lysed with RIPA buffer (Thermo Scientific, Belmont, MA, USA) at 4 ℃ for 30 minutes. The extracted proteins were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes by electroblotting. Membranes were blocked with nonfat milk at 4 ℃ overnight, followed by incubation with the following primary antibodies at 4 ℃ overnight: anti-SIRT3 (1:800, ab189860, Abcam, Cambridge, MA, USA), anti-CypD (1:800, ab231155), anti-proliferating cell nuclear antigen (PCNA; 1:1,000, ab92552), anti-CyclinD1 (1:800, ab156448), anti-CDK4 (1:1,000, ab199728), anti-P21 (1:1,000, ab215971), anti-caspase-3 (1:800, ab184787), anti-Bax (1:1,000, ab182733), anti-Bcl-2 (1:1,000, ab196495), and anti-β-actin (1:800, ab8226). The membranes were then incubated 1.5 hours at 25 ℃ with the secondary antibody (1:2,000, ab216773, Abcam, China). β-actin was used as the internal reference. Target bands of proteins were visualized with an ECL western blotting kit (Millipore, Boston, MA, USA). The relative expressions of proteins was assessed using the $2^{-\Delta\Delta CT}$ method.

Co-immunoprecipitation

Lysine (166)-acetylated CypD1 was examined with Western blot analysis. The cells were lysed via Pierce™ IP Lysis Buffer (#87788, Thermo Scientific) and after centrifugation (13,000 ×g, 10 minutes, 4 ℃), the supernatant was collected. Samples were incubated with acetylated-lysine antibody (#9441, Cell Signaling Technology, Boston, MA, USA) at 4 ℃ for 12 hours. Protein A/G-agarose beads (20 μL of 50% bead slurry) were then added and shaken at 4 ℃ for 3 hours. The immunoprecipitates were washed

3 times with lysis buffer and were detected by Western blot.

CCK-8 assay for cell proliferation

The CCK-8 assay (#C0038, Beyotime, China) was used to detect cell proliferation in accordance with the manufacturer's directions. After H/R treatment, cells in different groups (3,000 cells/well) were seeded in 96-well plates at 37 ℃ under 5% CO_2. At 0, 12, 24, 48, and 72 hours, 5 mg/mL CCK-8 was added to each well and incubated at 37 ℃ for 2 hours. The absorbance at 450 nm was measured with a microplate reader. Experiments were performed in triplicates.

5-ethynyl-2'-deoxyuridine (EdU) assay

The EdU Apollo 567 *in vitro* kit (GV-CA1170, Givei, China) was used to measure cell proliferation. A total of 1×10^4 cells/well were seeded into a 96-well plate and cultured in a 5% CO_2 incubator at 37 ℃. EdU was diluted to 10 μM with culture medium and added to the cell medium. After incubation for 2 hours, the medium was discarded, and cells were washed. The cells were fixed with 4% paraformaldehyde in phosphate buffered saline (PBS) and incubated for 30 minutes at 25 ℃. The cells were washed, permeabilized with 100 μL of Triton X100, and incubated in a decolorizing shaker for 10 minutes. After another washing, cells were incubated with 100 μL of Apollo567 staining reaction solution for 30 minutes at 25 ℃ in the dark. Cells were washed, fixed, and observed under a fluorescence microscope (Olympus, Japan). The excitation wavelength was 550 nm, and the emission wavelength was 565 nm.

Flow cytometry analysis

Cells were seeded into six-well plates (5×10^5 cells/well). After 24 hours treatment, cells were digested with 0.25% trypsin and resuspended in binding buffer, followed by incubation with 5 μL Annexin V-FITC (Annexin V-FITC Apoptosis Detection kit, BD Biosciences, San Jose, CA, USA) and 5 μL propidium iodide (PI, Sigma, USA) at 2–8 ℃ for 5 minutes in the dark. Fluorescence-activated apoptotic cell assays were performed with a flow cytometer and EXPO32 software (COULTER-XL.MCL, Beckman Coulter, Miami, FL, USA).

Mitochondrial function tests

After 24 hours treatment, cells were digested with 0.25% trypsin and washed with PBS. The level of LDH released in the medium was detected using a human LDH enzyme-linked immunosorbent assay (ELISA) kit (ml076593, Mlbio, China) according to the manufacturer's directions. The mitochondria of H9c2 cells were isolated using the Cell Mitochondria Isolation Kit (Beyotime, Shanghai) in accordance with the manufacturer's instructions. Na^+-K^+-ATPase activity was detected using a Na^+-K^+-ATPase assay kit (Nanjing Jiancheng Biochemistry Co., Nanjing, China) and Ca^{2+}-ATPase activity was measured as previously described (28). Nitric oxide (NO) levels were detected with Griess reagent (Beyotime Biotechnology, Shanghai, China) according to the manufacturer's instructions.

Statistical analysis

All measurements were repeated three times for each sample. GraphPad Prism 7.0 was used for statistical analysis. The measurement data were expressed as mean ± standard deviation (SD). One-way analysis of variance (ANOVA) was used for comparison among multiple groups. Tukey's test was used for pairwise comparisons between groups. P value <0.05 was considered statistically significant.

Results

LBP relieves H/R-induced H9c2 cell damage and induces SIRT3 expression

The effects of LBP on H9c2 cells were examined. When the concentration was less than or equal to 2.0 μg/mL, the effects of LBP on cells were not clear. When the concentration of LBP was greater than or equal to 3.0 μg/mL, LBP significantly promoted the growth of H9c2 cells ($P<0.05$; *Figure 1A*). Therefore, in subsequent experiments, the LBP concentrations used were 0.5, 1.0, and 2.0 μg/mL. H/R decreased cell viability, and this was ameliorated by LBP in a dose-dependent manner ($P<0.05$; *Figure 1B*). Moreover, H/R decreased the levels of SIRT3 mRNA and protein expressions, while LBP promoted the SIRT3 mRNA and protein expressions in a dose-dependent manner ($P<0.05$; *Figure 1C-1E*). These results suggested that the mechanism through which LBP alleviates H/R-induced cardiomyocyte damage might be associated with SIRT3.

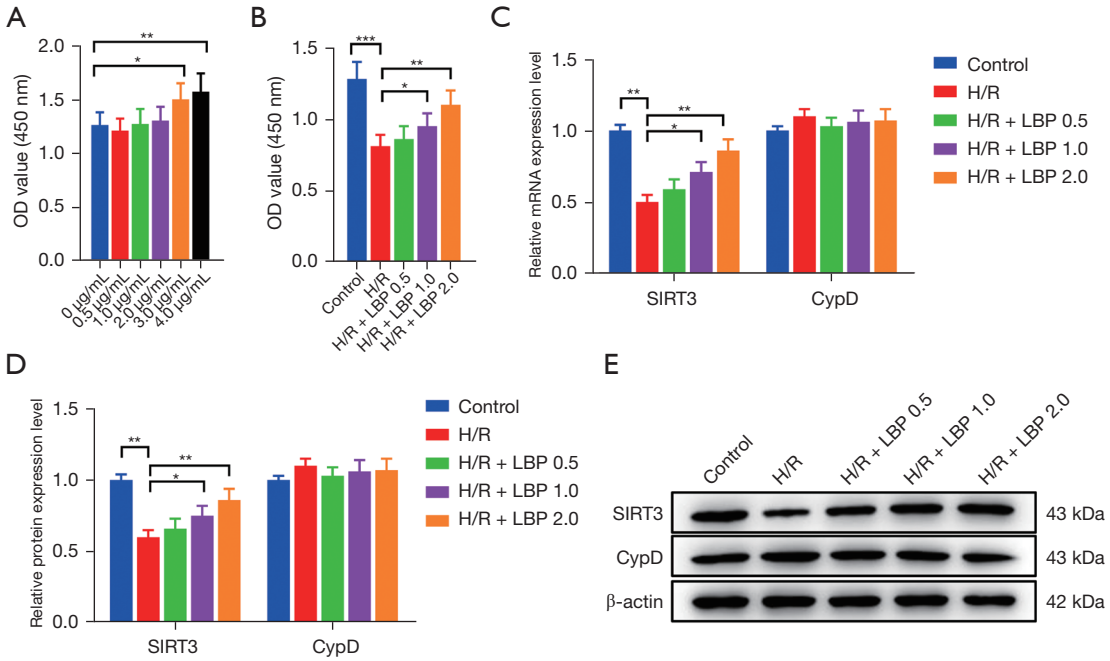

Figure 1 LBP relieves H/R-induced H9c2 cell damage and induces SIRT3 expression. (A) The effects of LBP on the cell viability of H9c2. Cells were cultured in LBP with final concentrations of 0.5, 1.0, 2.0, 3.0, and 4.0 μg/mL for 48 hours. (B) LBP dose-dependently alleviated the cell damage induced by H/R. After the cells were pre-cultured in 0.5, 1.0, and 2.0 μg/mL LBP for 48 hours, the cells underwent H/R. (C) The effects of LBP on the SIRT3 and CypD mRNA expressions were assessed by qRT-PCR. (D,E) The effects of LBP on the SIRT3 and CypD protein expressions were analyzed by Western blot. N=3, mean ± standard deviation; *, P<0.05; **, P<0.01; ***, P<0.001. One-way ANOVA was used for analysis of variance among multiple groups. Tukey's test was used for pairwise comparisons between groups. OD, optical density; LBP, *Lycium barbarum* polysaccharide; H/R, hypoxia/reoxygenation; qRT-PCR, quantitative real time polymerase chain reaction; ANOVA, analysis of variance.

The protection against H/R-induced cell damage by LBP is dependent on SIRT3 expression

To verify that the ability of LBP to alleviate H/R-induced cell damage was associated with SIRT3, 3-TYP was used to inhibit SIRT3 function. Compared with control cells (normal culture), H/R treatment caused morphological damage and decreased the refraction of H9c2 cells. For control H9c2 cells, each concentration of LBP (0.5, 1.0, 2.0 μg/mL) had no significant effect on the morphology. For H9c2 cells of H/R damage, LBP (0.5 μg/mL) at low concentrations had no significant effects. While the cell morphology and growth were best when the LBP concentration was 2.0 μg/mL (*Figure 2*). These results suggested that with increasing LBP concentrations, the morphological damage caused by H/R was gradually relieved. Therefore, 2.0 μg/mL LBP was used in subsequent experiments. Compared with the H/R group, H/R cells treated with 3-TYP (H/R + 3-TYP group) showed greater morphological damage. However, treatment with LBP ameliorated the morphological damaged caused by H/R and 3-TYP (*Figure 2*). These findings suggested that LBP may protect against morphological damage caused by H/R via the induction of SIRT3.

LBP activates SIRT3/CypD signaling in H9c2 cells

H/R induced a decrease in SIRT3 mRNA and protein expressions in H9c2 cells (P<0.01). However, LBP increased the expressions of SIRT3 mRNA and protein in H/R-induced H9c2 cells (P<0.05). The effects of different concentrations of 3-TYP on H9c2 cells were tested. The results showed that when the concentration of 3-TYP was ≤1 μM, the effect of 3-TYP on cell viability was not significant (*Figure 3A*). In addition, 3-TYP had no significant effects on the expression levels of SIRT3 mRNA nor SIRT3 protein (*Figure 3B,3C*). While 3-TYP, a SIRT3 protein inhibitor,

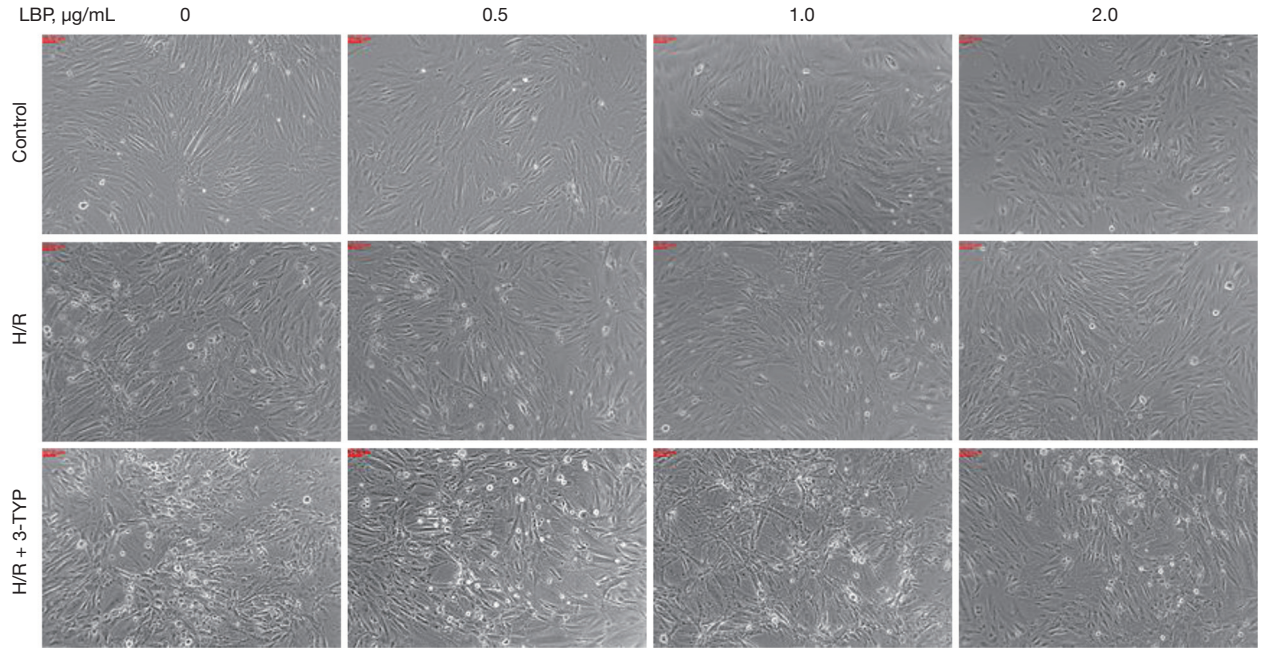

Figure 2 LBP may protect against H/R-induced cell damage through SIRT3. Cell morphology was directly observed by the light microscopy. The cell morphology was slightly irregular in the H/R group compared with the control group. A low concentration of LBP (0.5 μg/mL) had no significant effect on the morphology of H9c2 cells. With increasing LBP concentration, the morphological damage caused by H/R gradually improved. Treatment with 3-TYP (H/R + 3-TYP group) led to greater morphological damage compared to that in the H/R group. Scale bar =50 μm; magnification, ×200. LBP, *Lycium barbarum* polysaccharide; H/R, hypoxia/reoxygenation.

had no significant effect on SIRT3 mRNA, it inhibited the increase in SIRT3 protein induced by LBP under H/R conditions (P<0.05; *Figure 3D-3F*). Although H/R, LBP, and 3-TYP had no clear effects on CypD mRNA and protein expressions (*Figure 3D-3F*), they altered the phosphorylation level of CypD protein. H/R promoted CypD acetylation (P<0.001), whereas LBP inhibited CypD acetylation caused by H/R (P<0.01). The level of CypD protein acetylation in the H/R + LBP + 3-TYP group was significantly higher than that in the H/R + LBP group (P<0.001; *Figure 3G*,*3H*). The use of 3-TYP to inhibit SIRT3 blocked the LBP-induced CypD deacetylation. These results suggested that H/R decreased SIRT3 expression and increased CypD acetylation, whereas LBP treatment reversed the effects of H/R on SIRT3 expression and acetylation of CypD. The protective role of LBP against H/R may involve activation of the SIRT3/CypD pathway.

LBP reverses the H/R-induced H9c2 inhibition of proliferation via the SIRT3/CypD pathway

The CCK-8 and EdU assays showed that the proliferation of H9c2 cells was significantly lower in the H/R group than the control group (P<0.001). Furthermore, 3-TYP enhanced the H/R-induced inhibition of H9c2 proliferation (H/R + 3-TYP *vs.* H/R, P<0.001). LBP treatment significantly elevated the proliferation of H/R-induced H9c2 cells (H/R + LBP *vs.* H/R, P<0.01). Moreover, 3-TYP blocked the protective effects of LBP on the proliferation in H/R-induced cells (*Figure 4A*,*4B*). To validate these results, Western blotting was performed to analyze proliferation biomarkers. In the H/R group, the levels of PCNA, CyclinD1, and CDK4 were distinctly lower compared with those in the control group, while the level of P21 were higher (P<0.01). After treatment with LBP (H/R + LBP group), the PCNA, CyclinD1, and CDK4 expression levels increased, and the P21 level decreased (P<0.001). The protein levels of PCNA, CyclinD1, and CDK4 in the H/R + LBP + 3-TYP group cells were significantly lower than those in H/R + LBP group, whereas the level of P21 were significantly higher (P<0.01; *Figure 4C*,*4D*). LBP alleviated the inhibition of H9c2 cell proliferation induced by H/R, and this function was blocked by 3-TYP. These findings suggested that LBP may act via the SIRT3/CypD pathway

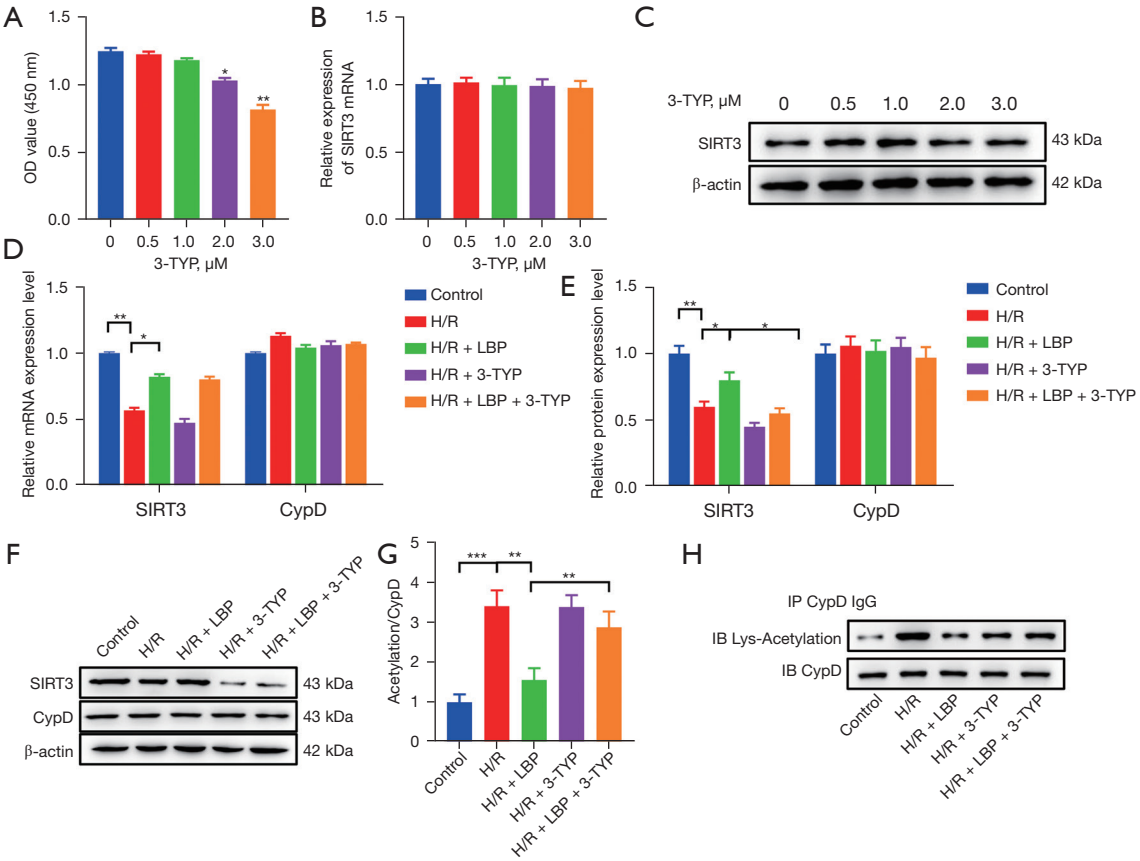

Figure 3 LBP activates SIRT3/CypD signaling in H9c2 cells. (A) The CCK-8 assay was performed to detect the effects of 3-TYP on H9c2 cell proliferation. (B,C) qRT-PCR and Western blotting were used to evaluate the relative mRNA and protein expression of SIRT3, respectively. (D) qRT-PCR was used to detect the relative mRNA expressions of SIRT3 and CypD. (E,F) Western blotting was used to evaluate the relative protein expressions of SIRT3 and CypD. (G,H) Co-immunoprecipitation was used to detect lysine (166)-acetylation of CypD. N=3, mean ± standard deviation; *, P<0.05; **, P<0.01; ***, P<0.001. One-way ANOVA was used for analysis of variance among multiple groups. Tukey's test was used for pairwise comparisons between groups. OD, optical density; LBP, *Lycium barbarum* polysaccharide; H/R, hypoxia/reoxygenation; qRT-PCR, quantitative real time polymerase chain reaction; CCK-8, Cell Counting Kit-8; ANOVA, analysis of variance.

to reverse the H/R-induced inhibition of proliferation in H9c2 cells.

LBP alleviates H/R-induced apoptosis and mitochondrial injury through the SIRT3/CypD pathway

Flow cytometry results showed that H/R treatment enhanced apoptosis in H9c2 cells (P<0.001), whereas LBP treatment decreased the cell apoptosis induced by H/R (P<0.01). The apoptosis rate in the H/R + 3-TYP group was significantly higher than that in the H/R group, and the apoptosis rate in the H/R + LBP + 3-TYP group was significantly higher than that in the H/R + LBP group (P<0.01; *Figure 5A*). Western blot results revealed that in the H/R group, caspase-3 and Bax expressions were higher, while Bcl-2 expression was lower than that in the control group. Furthermore, caspase-3 and Bax protein levels were lower, and Bcl-2 was higher in the H/R + LBP group compared to the H/R group. The protein levels of caspase-3 and Bax in the H/R + LBP + 3-TYP group were significantly higher than those in the H/R + LBP group, whereas the Bcl-2 level was significantly lower than that in the H/R + LBP group (P<0.01; *Figure 5B*). LBP inhibited cardiomyocyte apoptosis induced by H/R. The use of 3-TYP to inhibit SIRT3 aggravated H/R-induced apoptosis and blocked the inhibitory effect of LBP on apoptosis.

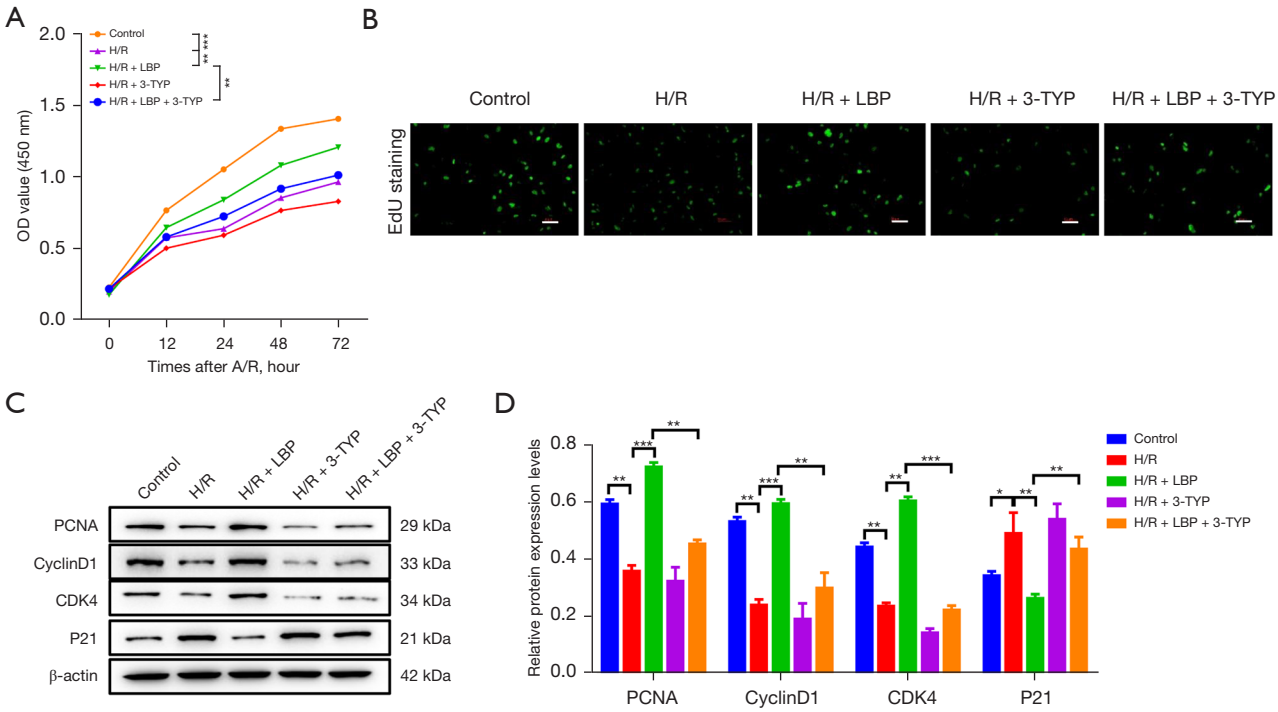

Figure 4 *Lycium barbarum* polysaccharide may act via the SIRT3/CypD pathway to reverse the hypoxia/reoxygenation-induced inhibition of proliferation in H9c2 cells. (A) The CCK-8 assay was performed to assess the proliferation of H9c2 cells. (B) EdU staining was used to determine the proliferation of H9c2 cells (scale bar =50 μm; magnification, ×40) (C,D) Western blotting was used to detect the protein expressions of proliferation biomarkers. N=3, mean ± standard deviation; *, P<0.05; **, P<0.01; ***, P<0.001. One-way ANOVA was used for analysis of variance among multiple groups. Tukey's test was used for pairwise comparisons between groups. LBP, *Lycium barbarum* polysaccharide; H/R, hypoxia/reoxygenation; PCNA, proliferating cell nuclear antigen; CCK-8, Cell Counting Kit-8; ANOVA, analysis of variance.

These findings suggested that LBP might reverse H/R-induced H9c2 apoptosis in a manner dependent on the SIRT3/CypD pathway.

Mitochondrial damage was analyzed by the detection of LDH level. The level of LDH released in the H/R group was significantly higher than that in the control group (P<0.001). The LDH level in the H/R + LBP group was significantly lower than that in the H/R group (P<0.01). In addition, the level of LDH released in the H/R + LBP + 3-TYP group was higher than that in the H/R + LBP group (P<0.05; *Figure 5C*). Compared with that in the control group, the Na^+-K^+-ATPase activity, Ca^{2+}-ATPase activity, and NO levels were lower in the H/R group (P<0.01). LBP significantly promoted the Na^+-K^+-ATPase activity, Ca^{2+}-ATPase activity, and NO levels inhibited by H/R (P<0.01). However, in cells treated with 3-TYP, the effects of LBP on Na^+-K^+-ATPase activity, Ca^{2+}-ATPase activity, and NO levels were reversed (P<0.05; *Figure 5D*). These results suggested that H/R caused cell apoptosis, cytotoxicity, and mPTP opening. LBP alleviated the apoptosis of cells, cytotoxicity, and mPTP opening induced by H/R. Moreover, the SIRT3 inhibitor 3-TYP blocked the anti-apoptotic effects and mPTP opening by LBP. These findings suggested that LBP might alleviate H/R-induced cell apoptosis, cytotoxicity, and mitochondrial edema through the SIRT3/CypD pathway.

Discussion

SIRT3 catalyzes the de-acetylation of CyPD in mPTP, and the acetylation of CyPD is correlated with mPTP formation (31). The SIRT3/CypD pathway participates in mPTP opening and apoptosis of MIRI cells (19-21). LBP is a protective factor against oxygen glucose deprivation/reperfusion (32). LBP protects against MIRI via decreasing

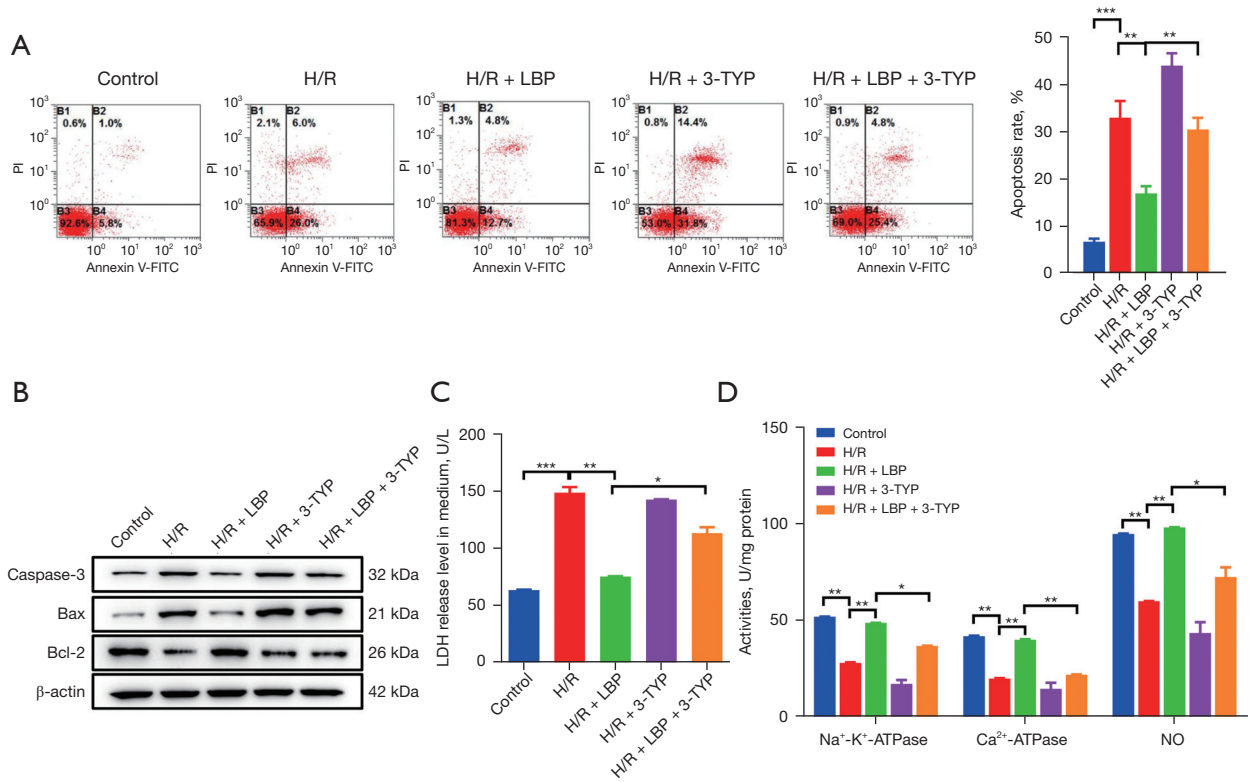

Figure 5 LBP may alleviate H/R-induced apoptosis and mitochondrial injury through the SIRT3/CypD pathway. (A) Flow cytometry was used to detect the apoptotic rate of H9c2 cells. (B) Western blotting was used to analyze the protein expressions of apoptotic factors, including caspase-3, Bcl-2, and Bax. (C) ELISA was used to evaluate the level of LDH released. (D) The effects of LBP and the SIRT3 inhibitor 3-TYP on Na^+-K^+-ATPase activity, Ca^{2+}-ATPase activity, and NO levels. N=3, mean ± standard deviation; *, P<0.05; **, P<0.01; ***, P<0.001. One-way ANOVA was used for analysis of variance among multiple groups. Tukey's test was used for pairwise comparisons between groups. LBP, *Lycium barbarum* polysaccharide; H/R, hypoxia/reoxygenation; ELISA, enzyme-linked immunosorbent assay; LDH, lactate dehydrogenase; NO, nitric oxide; ANOVA, analysis of variance.

LDH level and increasing Na^+-K^+-ATPase and Ca^{2+}-ATPase activity (28). Therefore, we speculated that the SIRT3/CypD pathway might participate in the protective function of LBP against MIRI. However, the underlying mechanisms were unclear.

At present, there is no clinically effective drug for the treatment of H/R injury. In recent years, studies have found that traditional Chinese medicine monomers, including LBP, have good effects on alleviating H/R damage, such as ginsenoside Rb1 (33), baicalin (34), and puerarin (35). This study demonstrated that LBP distinctly reversed the decrease in SIRT3 level and the increase in CypD acetylation induced by H/R. These results suggested that LBP may protect against MIRI damage in H9c2 cells through activation of the SIRT3/CypD pathway. Similar results have been reported in previous studies. Zhai *et al.* (36) found that melatonin increases the expression and activity of SIRT3, and activation of the SIRT3 pathway protects against H/R injury in the MIRI mouse model. Wang and colleagues (37) demonstrated that SIRT3 is activated in H/R-induced H9c2 cardiomyocytes treated with curcumin. To further examine the protective effect of LBP on H/R cells, 3-TYP was used to inhibit the function of the SIRT3 protein. 3-TYP treatment aggravated the cell damage induced by H/R, and the protective effects of LBP on cells under H/R conditions were blocked. Literature reports have shown that activating SIRT3 is of great significance for alleviating cardiomyocyte damage. Feng *et al.* (38) demonstrated that melatonin decreases morphological changes by activating SIRT3 in H9c2 cells. Zheng and

colleagues (39) showed that autophagy in MIRI is regulated by SIRT3, and LBP protects against ischemia/reperfusion injury in rodent retina through antioxidant activity and inhibition of apoptosis (40). Indeed, LBP has been reported to decrease the apoptosis induced by H/R (41). The study by Zhu *et al.* also revealed that LBP can reduce the apoptosis of retinal vascular endothelial induced by high-speed glucose (42). Together with the above-mentioned literature, the results of our current study showed that the mechanisms by which LBP alleviates H/R injury in cardiomyocytes is inseparable from the SIRT3/CypD pathway. LBP may promote the deacetylation of CypD by promoting the expression of SIRT3 protein, thereby protecting cardiomyocytes.

To further analyze the mechanisms by which LBP regulates the SIRT3/CypD pathway to alleviate the H/R damage to cardiomyocytes, the influence on mPTP, cell proliferation, and cell apoptosis were further analyzed. mPTP opening is regulated by the SIRT3/CypD pathway and is a key mechanism through which H/R induces cell damage and apoptosis. H/R-induced SIRT3 inhibition causes CypD acetylation, which opens mPTP, thus leading to mitochondrial edema, which in turn leads to apoptosis (41,43). This current investigation showed that H/R caused the release of LDH and decreased Na^+-K^+-ATPase activity, Ca^{2+}-ATPase activity, and NO levels, whereas LBP inhibited the changes in the above indicators. Using 3-TYP to inhibit SIRT3 reversed the inhibitory effects of LBP on LDH, as well as the LBP-mediated restoration of Na^+-K^+-ATPase activity, Ca^{2+}-ATPase activity, and NO levels. In glutamate-induced nerve cell damage, LBP inhibits cell apoptosis by alleviating mitochondrial dysfunction (44). In an H_2O_2-induced human trophoblast oxidative stress injury model, LBP protects mitochondria against damage by decreasing the level of ROS in the cells, thereby inhibiting cell damage (45). In addition, in Leydig MLTC-1 cells induced by cisplatin, the effects of LBP on inhibiting apoptosis and restoring cell viability are inseparable from its protective function in the mitochondria (46). These findings suggested that H/R causes mPTP opening and decreases the levels of Na^+-K^+-ATPase activity, Ca^{2+}-ATPase activity, and NO levels, thus leading to mitochondrial edema by inhibiting the SIRT3/CypD pathway. LBP may restore mPTP function by increasing the expression of SIRT3, thereby alleviating cell apoptosis caused by mitochondrial damage. However, while 3-TYP targets SIRT3, it can also inhibit other sirtuins, and this may have affected the experimental results. Further research in this area is warranted to verify these results. In addition, in order to clarify the role of SIRT3 in the protection of cardiomyocytes H/R by LBP, gene knockout cells should be used for follow-up studies. Moreover, the regulation of LBP on SIRT3/CypD pathway, the protective effect on mitochondria, and the effect on MIRI injury still need further *in vivo* experiments to confirm. These are our next research direction.

Conclusions

In conclusion, LBP activated SIRT3/CypD signaling in H9c2 cells under H/R injury to relieve the morphological and mitochondrial damage caused by H/R, as well as promote proliferation and decrease H/R-induced apoptosis. LBP prevented the decrease of SIRT3 and consequently, inhibited the acetylation of CypD1, thereby reducing H/R injury. These findings suggested that LBP may be a potential novel treatment for MIRI and further research in warranted.

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Footnote

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Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://atm.amegroups.com/article/view/10.21037/atm-22-6081/coif). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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