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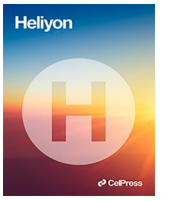

Research article

Dihydromyricetin regulates RIPK3-CaMKII to prevent necroptosis in high glucose-stimulated cardiomyocytes

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ABSTRACT

Background: Diabetic cardiomyopathy is one common cardiovascular complication without effective treatments. Dihydromyricetin (DHY), a natural dihydroflavonol compound extracted from *Ampelopsis grossedentata*, possesses versatile pharmacologically important effects. In our current research, we planned to evaluate the impact and probable DHY mechanisms in high glucose (HG)-induced cardiomyocytes.

Methods: Primary cardiomyocytes were pretreated with different concentrations of DHY (0, 20, 40, 80, 160, and 320 μM) for various time (0, 1, 2, 4, 12, and 24 h). They were then stimulated for 48 h with 5.5 mmol/L normal glucose (NG) and 33.3 mmol/L high glucose (HG). Cell viability, adenosine-triphosphate (ATP) levels, and lactate dehydrogenase (LDH) release of cardiomyocytes were detected. JC-1 staining was employed to measure the mitochondrial membrane potential. MitoSOX staining and dihydroethidium (DHE) staining were applied to evaluate the oxidative stress levels. TDT mediated dUTP nick end labeling (TUNEL) was used to measure apoptotic levels. Expressions of calcium/calmodulin-dependent protein kinase II (CaMKII), phospholamban (PLB), optic atrophy 1 (OPA1), dynamin-related protein 1 (DRP1), caspase 3, mixed kinase lineage domain like protein (MLKL), receptor interacting protein kinase 3 (RIPK3), and receptor interacting protein kinase 1 (RIPK1) were detected by immunofluorescence and/or Western blot.

Results: DHY improved cell viability, enhanced ATP level, and decreased LDH content in HG-stimulated cardiomyocytes, suggesting DHY attenuating cell injury. DHY reduced number of TUNEL positive cells, inhibited RIPK3 and cleaved-caspase 3 expression, implying DHY alleviated necroptosis in HG-stimulated cardiomyocytes. DHY diminished JC-1 monomers, DHE and MitoSOX fluorescence intensity as well as DRP1 expression but increased JC-1 aggregates intensity and OPA1 expression, indicating that DHY attenuated oxidative stress in HG-stimulated cardiomyocytes. DHY also attenuated CaMKII activity by suppressed PLB phosphorylation and inhibited CaMKII oxidation in HG-stimulated cardiomyocytes.

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Conclusions: HG-induced cardiomyocytes injury was alleviated wherein DHY attenuated necroptosis, repressed ROS production, and inhibited CaMKII oxidation, suggesting that DHY may serve as potential agent to prevent and treat diabetic cardiomyopathy.

1. Introduction

Diabetes mellitus has been a chronic metabolic disorder worldwide. Persistent hyperglycemia in diabetes patients usually lead to several complications. DCM (diabetic cardiomyopathy) is a prevalent cardiovascular problem, which can induce myocardial hypertrophy, myocardial fibrosis, cardiac microvascular abnormality, myocardial systolic and diastolic disfunction, and ultimately heart failure [1]. However, there are no special treatments for DCM except lifestyle changes or hypoglycemic drugs which may be of no avail in some cases. Therefore, research is needed to explore the exact mechanisms for DCM and determine effective treatment strategies to reduce the morbidity and mortality of diabetic patients.

Dihydromyricetin (DHY), a natural dihydroflavonol compound isolated from *Ampelopsis grossedentata*, has versatile pharmacological effects including scavenging free radicals, anti-oxidation, anti-inflammation, anti-thrombosis, anti-microorganism, liver protection, anti-cancer and so on [2,3] Previous studies have showed that DHY prevented DCM by activating autophagy and inhibiting miR-34a [4]. DHY also attenuated DCM by inhibiting oxidative stress and reducing apoptosis in the myocardium [5]. Our recent research found that DHY alleviated DCM by activating sirtuin 3 to inhibit oxidative stress and inflammation [6]. Nonetheless, detailed DHY mechanism against DCM is not completely understood.

Decreased cardiac compliance, serious insulin resistance, disordered glucose and lipid metabolism, abnormal autophagy and apoptosis, increased oxidative stress, impaired mitochondrial dysfunction and excessive inflammation are possibly involved in DCM [7]. Persistent hyperglycemia may damage the structure and function of the heart, cause myocardial hypertrophy, mitochondrial dysfunction, endoplasmic reticulum stress, fibroblast proliferation and finally facilitate the development of DCM [8]. In addition, after long-term hyperglycemia stimulation combined with the corresponding cell death receptor, synthesis of adenosine triphosphate (ATP) was weakened, mitochondrial membrane potential was reduced, and mitochondrial inner membrane permeability was damaged, resulting in a large release of cell contents, which eventually led to local inflammatory cell infiltration and activation [9]. Long-term hyperglycemia also induced too much ROS production to initiate mitochondrial damage and inflammatory reaction, and finally lead to cardiac dysfunction and induce DCM. Studies have shown that necroptosis is closely associated with the development of DCM. Necroptosis is programmed cell death facilitated via RIPK3 and RIPK1. RIPK1 binds to RIPK3 for recruiting and phosphorylating the MLKL protein to induce necroptosis. Among them, the signal pathway of necroptosis is primarily regulated by RIPK3 [10]. And increased expression of RIPK3 is one of the important signs of necroptosis [11]. Our recent studies have found that DHY down-regulated streptozotocin-induced necroptosis and inhibited inflammation as well as oxidative stress by activating sirtuin3 [6]. However, more clarification is needed whether DHY inhibited necroptosis in normal glucose (HG)-induced cardiomyocytes injury.

CaMKII is a multifunctional serine/threonine kinase, that has been identified as key regulatory protein in the processes of intercellular coupling, Ca^{2+} handling, cell death, inflammation and mitochondrial function [12]. In addition, CaMKII is activated by binding with Ca^{2+}/calmodulin and involves in various PTMs such as O-GlcNAcylation, S-nitrosylation, oxidation, and autophosphorylation [13]. CaMKII can also be activated by phosphorylation at Thr 287 site and oxidation at Met 281/282 site, which opens mPTP (mitochondrial permeability transition pore) [14]. Furthermore, CaMKII has role in arrhythmia, myocardial infarction, heart failure, ischemia/reperfusion injury, myocardial fibrosis, and myocardial hypertrophy [15]. Briefly, continued over-activation of CaMKII leads to heart damage. Our previous studies have confirmed that necroptosis required CaMKII activation in DCM via a RIPK3-mediated manner [16]. However, whether DHY attenuated high glucose-induced cardiomyocytes necroptosis by regulating CaMKII needs remains unknown.

Therefore, our present research aimed to explore DHY impact in HG-induced cardiomyocytes and clarify possible involvement of necroptosis and CaMKII for providing novel strategies in DCM clinical treatment and prevention.

2. Materials and methods

2.1. Neonatal cardiomyocyte culture and treatment

Sprague-Dawley rats of 1–3 days were taken to extract Neonatal cardiomyocytes. Rats were euthanized after soaking in iodophor and rubbing with alcohol. Heart was instantly excised. Blood was diluted with pre-cooled PBS (Beyotime, Shanghai, China). Subsequently, the hearts were cut into pieces and 0.25% trypsin was used to completely digest them in 3 min each of 10–12 cycles at 37 °C. Except for the first time, digested supernatants were taken in DMEM (Dulbecco's modified Eagle's medium, Gibco, Carlsbad, CA, USA) with 10% fetal bovine serum (FBS, Gibco, Carlsbad, CA, USA) for terminating the digestion. All supernatants were filtered via the 200-mesh cell sieve followed by two-time centrifugation for 5 min at 1200 rpm. The content was re-suspended in DMEM having 10% FBS in a culture dish. After culturing for 4 h at 37 °C in 5% CO_2 incubator, suspended cardiomyocytes (10^5-10^6/mL) were re-placed for 48 h into a new culture dish. DHY was used to pre-treat cardiomyocytes [17,18]. Stimulations were then made for 48 h with 5.5 mmol/L normal glucose (NG) or 33.3 mmol/L HG. NIH Guidelines for Care and Use of Laboratory Animals were strictly complied regarding the detailed protocols. The Animal Ethical Committee of Nantong University approved the designed protocols (No. NTU-20220401).

2.2. Cell viability

Cell Counting Kit-8 (CCK-8) cytotoxicity assay was employed to assess the viability of cardiomyocytes (C0038, Beyotime, Shanghai, China) [18]. Briefly, cardiomyocytes were seeded in 96-well plate in DMEM. After DHY were pretreated for 4 h, and stimulation for further 48 h with NG or HG, each well was added with 10 μL of CCK-8 reagent followed by incubation of 1 h in the dark at 37 °C. An automatic micro-plate reader was used to measure absorbance at 450 nm (Bio-Tek, Winooski, VT, USA). Relative optical density of every sample represented the cell number. It was normalized to the NG group in triplicate.

2.3. Lactate dehydrogenase (LDH) measurement

LDH levels from cell culture medium were identified as per the instruction of commercially available LDH-Cytotoxic Assay Kit (C0017) [19,20]. Cardiomyocytes were plated with density of about 80 %. After DHY pretreatment for 4 h, and stimulating with NG or HG for further 48 h, centrifugation of cells was made for 5 min at 400 g. The 120 μL supernatant was carefully transported to 96-well plate. It was then added with 60 μL test solution. Then plate was placed in dark for 30 min at 25 °C. Relative level of LDH release was determined by taking absorbance at 490 nm. It was presented via the ratio of the value in control group in triplicate.

2.4. ATP measurement

ATP level in cardiomyocytes was assessed as per the instruction of the commercial ATP Assay Kit–Firefly Luciferase Method (S0026, Beyotime, Shanghai, China) [19,20]. Hundred μL of ATP assay reagent was added into the medium after the cardiomyocytes were treated and equilibrated for 10 min at room temperature. Then, a microplate-reader was used to obtain the luminescence intensity (BioTek, Winooski,VT, USA). ATP relative levels were calculated via ratio of the value in control group in triplicate.

2.5. Detection of mitochondrial membrane potential (Δψm)

Δψm, considered as the fluorescence intensity, was measured as per recommendations of JC-1 kit (C2006) [19,20]. JC-1 staining solution was used to incubate cardiomyocytes at 37 °C in the dark for 20 min. Then, 4′, 6-diamidino-2-phenylindole (DAPI) was added for staining the nuclei in short while. The laser confocal microscope was employed to assess the fluorescence intensity (Leica, Wetzlar, Germany). Δψm was assessed by the relative intensive of green fluorescence for J-monomers from the red fluorescence for J-aggregates.

2.6. Measurement of ROS

Levels of ROS were evaluated through MitoSOX and DHE staining [19,20]. The 2 μM DHE was used to stain cardiomyocytes in the dark at 37 °C for 30 min to detect superoxide generation. Mitochondrial superoxide levels were detected by Mito-tracker (100 nM, Beyotime, Shanghai, China) and MitoSOX (5 μM, YEASEN, Shanghai, China) staining for 20 min at 37 °C without light. Then, DAPI was incubated for 5 min for nuclei staining. Laser confocal microscope was used to assess the fluorescence.

2.7. Immunofluorescent staining

Cardiomyocytes were washed two times by PBS and fixed via immunofluorescent fixation solution for 20 min, followed by 60 min incubation with blocking solution having 0.5% Triton X-100 (Beyotime, Shanghai, China). Subsequently, incubation of cardiomyocytes was made overnight at 4 °C with primary anti-RIPK3 (1:200, Novusbio, Littleton, CO, USA), DRP1 or OPA1 (1:50, Cell Signaling Technology, Danvers, MA, USA) antibodies. Afterward, IgG conjugated with Alexa Fluor 488 or Cy3 was used to incubate the cells for 2 h (1:500, Beyotime, Shanghai, China). Nuclei were stained by adding DAPI. At the end, laser confocal microscopy was employed to assess the protein expression, considered as the fluorescence intensity.

2.8. TUNEL staining

After washing two times by PBS, the cardiomyocytes were fixed for 30 min through fixation solution, followed by incubation for 5 min with blocking solution having 0.5% Triton X-100 away from light. Staining of the cells in each well was made for 1 h through 50 μL TUNEL detection solution containing 45 μL fluorescein-dUTP solution and 5 μL terminal deoxynucleotidyl transferase (TdT) in the dark as per the recommendations of One-Step TUNEL apoptosis assay kit (C1086) [19,20]. Afterward, DAPI was used to stain the nuclei. Apoptotic positive cardiomyocytes with green was seen and photographed by laser confocal microscopy.

2.9. Western blot

Lysis buffer was used to harvest the protein samples from cardiomyocytes (Beyotime, Shanghai, China) and phenylmethanesulfonyl fluoride (PMSF, 100:1 in volume). The proteins (about 50 μg) were then separated by SDS-PAGE, followed by transferring to a polyvinylidene fluoride (PVDF) membrane (Millipore, Kenilworth, NJ, USA). Next, being blocked for 2 h with skim milk (5%) at room temperature, membranes' incubation was made overnight at 4 °C with relevant primary anti-RIPK3 (1:1000, Novusbio, Littleton, CO,

USA); RIPK1, MLKL, *p*-MLKL, caspase 3 and cleaved caspase 3 (1:1000, Cell Signaling Technology, Danvers, MA, USA); phospholamban (PLB, 1:1000), phosphorylation-PLB Thr 17 (1:200, Santa Cruz Biotechnology, Santa Cruz, CA, USA); phosphorylation-PLB Ser 16 (1:800, Merck KGaA, Darmstadt, Germany); ox-CaMKII (CaMKII oxidation, 1:1000, Millipore, Kenilworth, NJ, USA); *p*-CaMKII (1:2000, Thermo Fisher Scientific, Rockford, IL, USA); CaMKII (1:1000, Abcam, Cambridge, UK); GAPDH (1:5000, Sigma-Aldrich, St. Louis, MO, USA) and β-tubulin (1:3000, CMCTAG, Milwaukee, WI, USA) antibodies. Afterward, HRP-conjugated IgG was employed to incubate the membranes (1:5000, ZSbio, Beijing, China) for another 2 h. Protein bands were visualized by the enhanced chemiluminescence (ECL, Thermo Fisher Scientific, Rockford, IL, USA).

2.10. Statistical analysis

All data were presented as the mean ± standard error of the mean (SEM), and statistically analyzed by Bonferroni post-hoc test with Stata 15.0 and one-way ANOVA. *P*-value of <0.05 had the statistical significance.

3. Results

3.1. DHY attenuates cell injury in HG-stimulated cardiomyocytes

We evaluated the impact on cell viability and injury to clarify DHY roles in HG-induced cardiomyocytes. CCK-8 assay depicted that cardiomyocytes viability was reduced by HG stimulation ($P < 0.001$), which was improved by DHY at 80, 160, and 320 μM ($P = 0.002$, 0.009 and 0.044 correspondingly, Fig. 1A). Nonetheless, no significant differences were found among above three concentrations of DMY, and 80 μM of DHY was chosen for further experiments. Additionally, cell viability was improved by 80 μM DMY pretreatment for 4, 12, and 24 h ($P < 0.001$, Fig. 1B), while no significant differences among above three pretreated time of DMY. Therefore, 4 h of DHY pretreatment was chosen for further experiments. It is noting that no significant difference was observed on LDH content in medium after DHY stimulation, implying that DHY has no toxicity on cardiomyocytes (Fig. 1C). Further experiments showed that high glucose stimulation reduced the viability of cardiomyocytes ($P = 0.009$), elevated the LDH content in the culture medium ($P = 0.003$), while decreased the ATP level ($P = 0.006$). DHY treatment markedly improved cell viability ($P = 0.023$), decreased LDH content ($P = 0.009$), and enhanced ATP level ($P = 0.038$), suggesting DHY attenuating cell injury in HG-stimulated cardiomyocytes (Fig. 1D–F).

3.2. DHY alleviates necroptosis in HG-stimulated cardiomyocytes

Both Western blot and immunofluorescence staining verified that the expression of RIPK3 was augmented in cardiomyocytes after

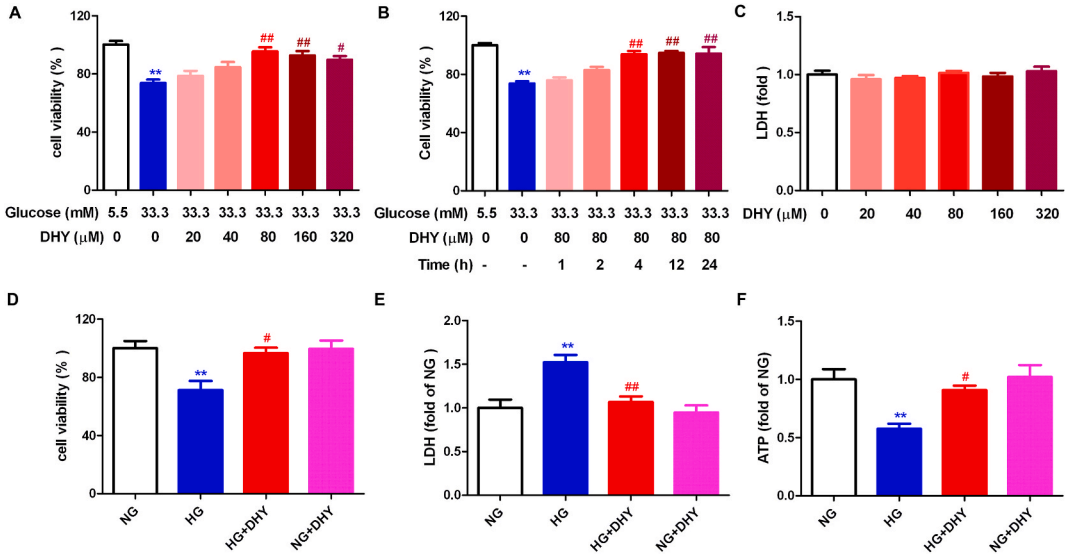

Fig. 1. The effects of DHY on cell viability, LDH and ATP in cardiomyocytes with high glucose stimulation. (**A-B**) After pre-administration with different concentrations of DHY (0, 20 μM, 40 μM, 80 μM, 160 μM and 320 μM) for different time (0, 1 h, 2 h, 4 h, 12 h and 24 h), the cardiomyocytes were stimulated with normal glucose (NG, 5.5 mmol/L) or high glucose (HG, 33.3 mmol/L) for 48 h. Cell viability was evaluated using CCK-8. Data are expressed as the means ± SEM. $^{**}P < 0.01$ verses NG, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ verses HG without DHY, n = 6. (C) After pre-administration with different concentrations of DHY (0, 20 μM, 40 μM, 80 μM, 160 μM and 320 μM) for 52 h, lactate dehydrogenase (LDH) release in the medium was detected. (**D**)After pre-administration with DHY (80 μM) for 4 h, the cardiomyocytes were stimulated with NG or HG for 48 h. Cell viability was evaluated using CCK-8. **(E)** LDH release in the medium was detected. **(F)** Adenosine triphosphate (ATP) level in the cardiomyocytes was measured. Data are expressed as the means ± SEM. $^{**}P < 0.01$ verses NG, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ verses HG, n = 6.

high-glucose stimulation ($P < 0.001$), that was reduced by treating with DHY ($P = 0.002$, Fig. 2A–B). Interestingly, DHY showed no significant effects on MLKL phosphorylation or RIPK1 expression (Fig. 2C–D). The data suggested that RIPK3, however not MLKL or RIPK1, had role of DHY protection for HG-induced necroptosis.

Moreover, necroptosis is manifested by the apoptosis [19,20]. TUNEL staining exhibited that positive cells number and cleaved-caspase 3 expression were enhanced by HG stimulation ($P < 0.001$), that was restored by DHY treatment ($P < 0.001$, Fig. 2E–F). All above results suggested that necroptosis was significantly alleviated by DHY in cardiomyocytes with HG stimulation.

3.3. DHY inhibits oxidative stress in HG-stimulated cardiomyocytes

High glucose augments ROS generation and impairs mitochondrial function and structure, as reflected by reduction in mitochondrial membrane potential (Δψm) [21,22]. JC-1 staining found that the green fluorescence intensity (GFI) of the JC-1 monomers, representing depolarized Δψm, was increased after HG stimulation. While JC-1 aggregates' red fluorescence intensity (RFI), representing normal membrane potential, was reduced after HG stimulation. DHY diminished the GFI however increased the RFI (Fig. 3A). It suggested that DHY improved Δψm after high-glucose stimulation. The decreased Δψm could bring excessive ROS production and serious cell damage. MitoSOX and DHE staining depicted that higher fluorescence intensity of MitoSOX and DHE was observed after HG stimulation, that was attenuated after DHY treatment (Fig. 3B–C). Collectively, oxidative stress was inhibited by DHY in HG-stimulated cardiomyocytes.

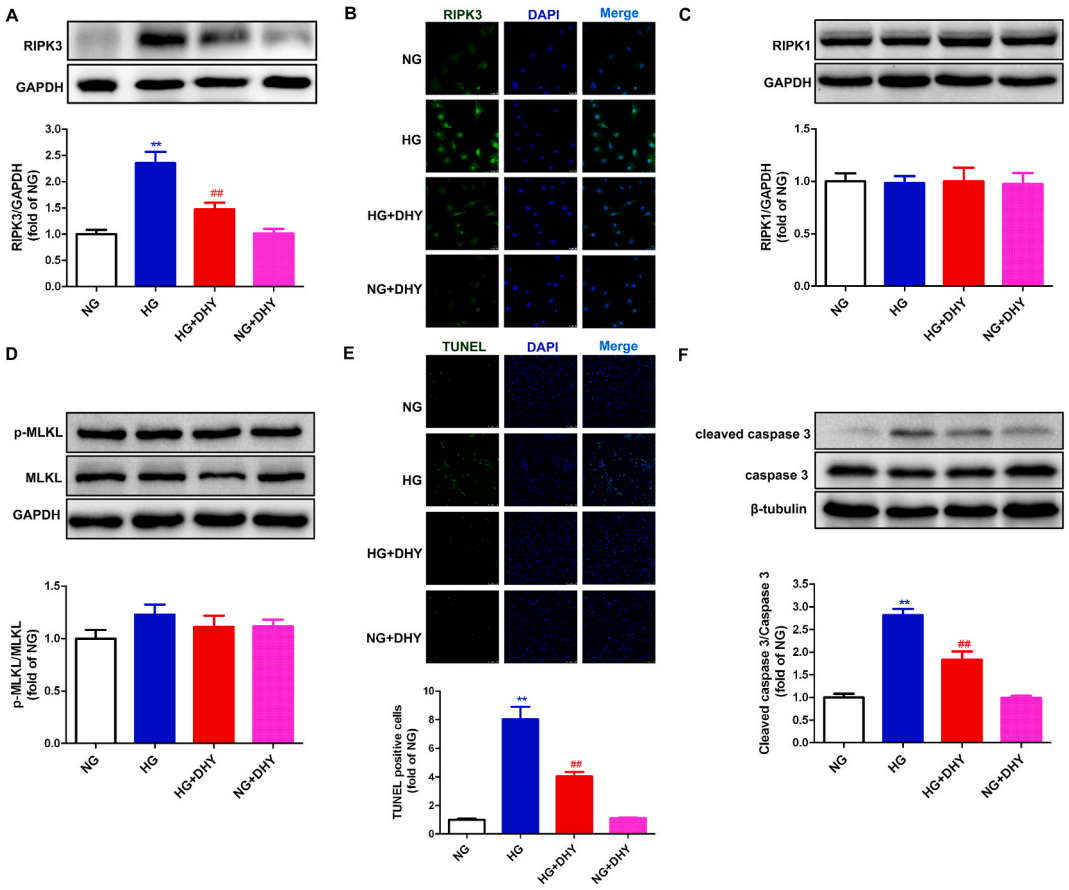

Fig. 2. The effects of DHY on necroptosis in cardiomyocytes with high glucose stimulation. After pre-administration with DHY (80 μM) for 4 h, the cardiomyocytes were stimulated with normal glucose (NG, 5.5 mmol/L) or high glucose (HG, 33.3 mmol/L) for 48 h. **(A)** Expression of RIPK3 protein was quantified by Western blot. GAPDH was used as a loading control. **(B)** RIPK3 was immunofluorescence stained using Alexa Fluor 488 (Green) conjugated IgG. The nuclei were stained using DAPI (Blue). Bar = 25 μm. **(C, D)** Expression of RIPK1 and MLKL protein were detected by Western blot. GAPDH was used as a loading control. **(E)** Cell apoptosis was assessed by TUNEL (green) staining. The nuclei were stained using DAPI (Blue). Bar = 75 μm. **(F)** Expression of caspase 3 and cleaved-caspase 3 proteins were quantified by Western blot. β-tubulin was used as a loading control. Data are expressed as the means ± SEM. $^{**}P < 0.01$ verses NG; $^{\#\#}P < 0.01$ verses HG, n = 6. Uncropped versions of blots (A, C, D, F) have been given as Supplement file. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

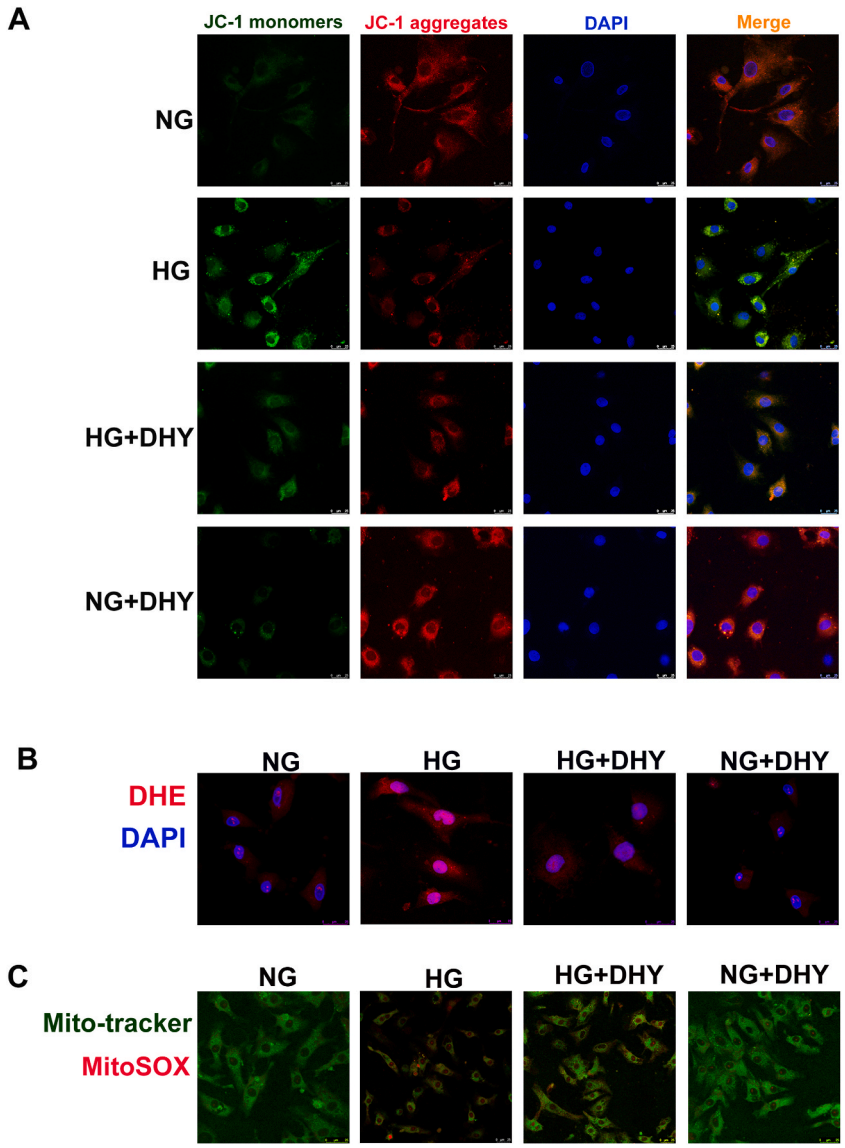

Fig. 3. The effects of DHY on oxidative stress in cardiomyocytes with high glucose stimulation. After pre-administration with DHY (80 μM) for 4 h, the cardiomyocytes were stimulated with normal glucose (NG, 5.5 mmol/L) or high glucose (HG, 33.3 mmol/L) for 48 h. **(A)** Mitochondrial permeability potential (Δψm) was measured by JC-1 staining. Bar = 25 μm. **(B)** Superoxide production in cardiomyocytes was detected with DHE fluorescent probe. Bar = 25 μm. **(C)** Mitochondrial ROS production was detected using MitoSOX. Mitochondrial localization was confirmed by co-localization with Mito-tracker Green. Bar = 25 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3.4. DHY decreases DRP1 but elevated OPA1 expression in high glucose-stimulated cardiomyocytes

OPA1 is the mitochondrial fusion-associated protein, while DRP1 is fission-associated. They are imperative for mitochondrial physiological function and structure [23]. We observed that HG elevated DRP1 but suppressed OPA1 expression, that was reinstated through DHY (Fig. 4). The data demonstrated that DHY balanced mitochondrial fission and fusion in HG-stimulated cardiomyocytes.

3.5. DHY attenuates CaMKII oxidation and CaMKII activity in HG-stimulated cardiomyocytes

Earlier studies proved that phosphorylation of PLB was a reliable indicator of CaMKII activity. It was often employed in assessing the activity of CaMKII [14]. This research revealed that DHY inhibited phosphorylation of PLB at Thr 17 ($P = 0.006$), however not at Ser 16, after HG stimulation (Fig. 5A), suggested that DHY alleviated the activity of CaMKII with HG stimulation.

Furthermore, CaMKII phosphorylation and oxidation are 2 major CaMKII activation processes [24]. Our study found that oxidation

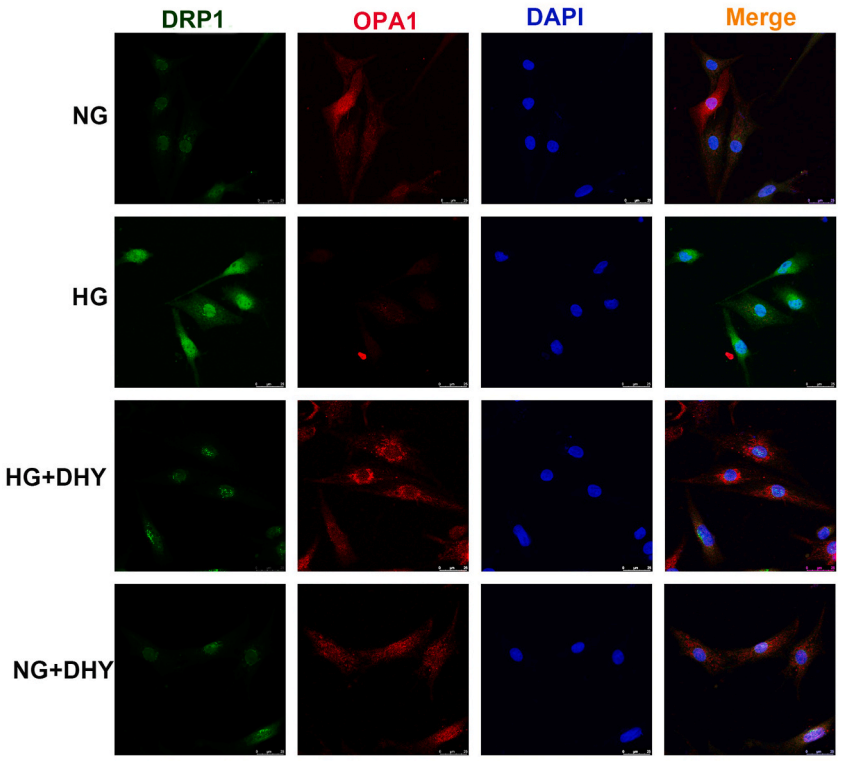

Fig. 4. The effects of DHY on DRP1 and OPA1 in cardiomyocytes with high glucose stimulation. After pre-administration with DHY (80 μM) for 4 h, the cardiomyocytes were stimulated with normal glucose (NG, 5.5 mmol/L) or high glucose (HG, 33.3 mmol/L) for 48 h. DRP1 and OPA1 were immunofluorescence stained with Alexa Fluor 488 (Green) or Cy3 (Red) conjugated IgG respectively. The nuclei were stained using DAPI (Blue). Bar = 25 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

of CaMKII, however not the phosphorylation of CaMKII, was heightened in HG exposed cardiomyocytes, while DHY diminished CaMKII oxidation after high glucose stimulation ($P = 0.002$, Fig. 5B–C). These data demonstrated that DHY reduced CaMKII oxidation and activity in HG-stimulated cardiomyocytes.

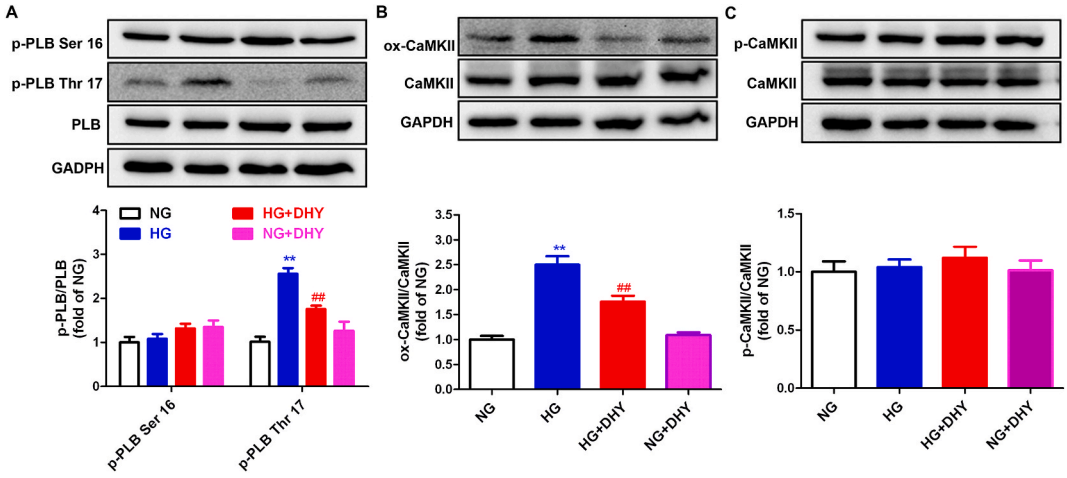

Fig. 5. The effects of DHY on CaMKII activity, CaMKII oxidation and CaMKII phosphorylation in cardiomyocytes with high glucose stimulation. After pre-administration with DHY (80 μM) for 4 h, the cardiomyocytes were stimulated with normal glucose (NG, 5.5 mmol/L) or high glucose (HG, 33.3 mmol/L) for 48 h. **(A–C)** Expression of *p*-PLB Ser 16, *p*-PLB Thr 17, CaMKII oxidation (ox-CaMKII) and CaMKII phosphorylation (*p*-CaMKII) were quantified by Western blot. GAPDH was used as loading controls. Data are expressed as means ± SEM. $^{**}P < 0.01$ verses NG, $^{\#\#}P < 0.01$ verses HG, n = 6. Uncropped versions of blots (A–C) have been given as Supplement file.

4. Discussion

DCM usually impairs cardiac structure, leads to myocardial hypertrophy and fibrosis, and eventually heart failure. This is the most prevalent death cause in diabetes [25]. However, there is no consensus about the treatment besides the lifestyle changes and hypoglycemic drugs with limited effects. Therefore, seeking novel drugs against DCM is a pending topic to be solved urgently. Previous study has shown that DHY attenuated adriamycin-induced myocardial injury in mice [26]. Our research found that DHY repressed angiotensin II-induced rat cardiac fibroblasts proliferation and cardiomyocyte hypertrophy, improved endothelial dysfunction, and suppressed myocardial hypertrophy with transverse aortic constriction in mice [18,27–29]. Our latest study also demonstrated that DHY attenuated diabetic cardiomyopathy via the inhibition of necroptosis, inflammation, and oxidative stress in diabetic mice [6]. Moreover, our present study found that DHY reduced release of LDH however increased ATP production in HG-stimulated cardiomyocytes. Our research provided direct evidence that cell damage was alleviated by DHY in high glucose-stimulated cardiomyocytes, which is beneficial to attenuate DCM and complicated cardiac dysfunction or even failure.

CaMKII has the potential to integrate proapoptotic cytokine, hyperglycemia, Gq-coupled receptors, ROS, and β-adrenergic signals for their sustained activation to promote cardiomyocyte death [30]. Earlier works confirm that CaMKII has role in DCM pathogenesis [14,16]. Moreover, phospholamban (PLB) is a sensitive marker for CaMKII activation [14]. Our these results depict that HG enhanced *p*-PLB Thr 17 expression, suggesting that HG increased the activity of CaMKII. In contrast, DHY treatment alleviated phosphorylation of PLB, helping to reduce myocardial infarction risk, improve calcium circulation disfunction and heart failure in diabetes. Furthermore, excessive CaMKII activation accelerates cardiovascular disease progression and aggravates cardiomyocyte injury. Earlier studies have found that CaMKII can also activate by oxidation and phosphorylation [31]. ROS oxidizes methionine residues on CaMKII regulatory domain for inducing oxidation of CaMKII [32]. In addition, CaMKII can be even self-phosphorylated [33]. This study uncovered that HG elevated ox-CaMKII levels in cardiomyocytes without significant difference in CaMKII phosphorylation, which was restored by DHY treatment. It suggested that the improvement on cell damage by DHY might be ascribed to the alleviated ox-CaMKII expression and activity. These results reflected that CaMKII negative regulation by DHY attenuated the HG-induced cardiomyocytes damage.

Several studies have confirmed that mitochondria are highly susceptible to oxidative damage, which were the main source of ROS production and energy. Nevertheless, mitochondrial disfunction causes ROS overproduction and cell death [34]. Moreover, mitochondrial damage also directly hinders cellular ATP production and storage. Thus, maintaining the normal mitochondrial structure and function is a critical for cell survival [35]. Additionally, dynamic balance between fission and fusion are crucial to maintain function and structure of mitochondria. Previous studies have identified that DRP1 is the mitochondrial fission core component, which is gathered into circular structure to mediate mitochondrial fission by mitochondrial cleavage alongside mitochondrial tubules [36]. OPA1 maintains fused mitochondrial network, and drives inner membrane fusion at the mitochondrial inner membrane [37]. These findings revealed that high glucose reduced membrane potential of mitochondria, suppressed ATP production, enhanced DRP1 but decreased OPA1 expression and up-regulated intracellular ROS levels. On the other hand, DHY significantly restored mitochondrial membrane potential, ATP production, oxidative stress as well as cell damage. These results indicated that DHY suppressed oxidative stress and improved mitochondrial function, which could be the dominant mechanism for alleviating HG-induced cardiomyocytes injury.

Accumulated evidence has suggested that necroptosis was associated with the pathogenesis of many diseases like tumors, neurodegenerative diseases, and ischemic cerebrovascular and cardiovascular diseases [38]. RIPK3 binds to RIPK1 via RIPK-homo-interaction motif domain for forming the necrosome and promote necroptosis [39]. Earlier works have revealed that RIPK1 and RIPK3 expressions were increased in palmitic acid (PA)-stimulated cardiomyocytes. Moreover, knockdown of RIPK3 and RIPK1 by siRNA alleviated the PA-induced cardiac hypertrophy and inhibited necroptosis [40]. MLKL, a downstream RIPK3 substrate, oligomerization and translocation to the plasma membrane after RIPK1 binds with RIPK3 for forming necrosomes, eventually leading to necroptosis [41]. One study confirmed that knockdown of MLKL significantly prevented necroptosis [42]. Furthermore, our previous study showed that CaMKII is an alternative substrate for RIPK3-induced necroptosis [14]. Another study also demonstrated that RIPK3 promoted myocardial necroptosis by CaMKII oxidation and phosphorylation in glycated end products-triggered cardiomyocytes injury [43]. This work demonstrated that cleaved-caspase 3 and RIPK3 expressions were enhanced in high glucose-induced cardiomyocytes with aggravated necroptosis. It indicated that HG-induced cardiomyocytes injury has the involvement of necroptosis. Nonetheless, the expression of RIPK 1 and MLKL showed no significant changes, suggesting RIPK1 and MLKL might be not involved, which is similar to our previous study in HG-stimulated cardiomyocytes [14]. But another group demonstrated that high glucose (35 mM) stimulation for 24 h enhanced MLKL, PIPK3, and RIPK1 expression in cardiomyocytes [44]. Our previous work showed that high glucose (25.5 mM) stimulation for 48 h increased RIPK3, and RIPK1, however not MLKL, expression in cardiomyocytes with-nonspecific control siRNA transfection [20]. These inconsistent alterations on RIPK1, RIPK3 and MLKL after high glucose stimulation may be attributable to differences in cell source, glucose dosage, stimulation time and cell state. More importantly, our study confirmed that DHY treatment diminished TUNEL-positive cell numbers, RIPK3 and cleaved-caspase3 expression in high glucose-induced cardiomyocytes. Our current results also implied that DHY significantly inhibited CaMKII activation and oxidation in cardiomyocytes with high glucose stimulation. As well as we know, RIPK3-CaMKII signal pathway-mediated necroptosis is critical in cardiovascular diseases [45]. These above results indicated that DHY regulated RIPK3-CaMKII signal pathway to prevent necroptosis in HG-stimulated cardiomyocytes, which might be novel therapeutic methodology in alleviating the diabetic cardiomyopathy.

However, there is one limitation in our study. The outcomes of current work are based on *in vitro* experiment and lack the DCM animal model *in vivo*. Actually, diabetes-induced toxicity is multifaceted, and cannot be mimicked by high glucose-induced toxicity. Some *in vivo* evaluation will be beneficial to mimic the actual disease condition infurtherstudies.

Collectively, DHY inhibited oxidation of CaMKII, repressed ROS production, and attenuated necroptosis for alleviating HG-induced

cardiomyocytes injury, suggesting that DHY may serve as potential agent to prevent and treat diabetic cardiomyopathy.

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Data availability statement

The data that support the findings of this study are available from the corresponding author (Yun Chen or Guoliang Meng) upon reasonable request.

CRediT authorship contribution statement

Linlin Sun: Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Yujiao Xiao:** Writing – original draft, Conceptualization, Data curation, Formal analysis, Investigation, Methodology. **Wenqing San:** Software, Methodology, Data curation, Conceptualization. **Yun Chen:** Writing – review & editing, Supervision, Conceptualization. **Guoliang Meng:** Writing – review & editing, Supervision, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e28921.

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