SPANDIDOS PUBLICATIONS

MOLECULAR MEDICINE REPORTS 16: 8983-8988, 2017

Dihydromyricetin protects against lipopolysaccharide-induced cardiomyocyte injury through the toll-like receptor-4/nuclear factor-κB pathway

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Received May 1, 2016; Accepted March 24, 2017

DOI: 10.3892/mmr.2017.7742

Abstract. Dihydromyricetin (DHM) is a bioactive flavonoid compound extracted from the stems and leaves of *Ampelopsis grossedentata*. Previous studies have indicated that DHM has antioxidation and antitumor capabilities, while the effect of DHM on lipopolysaccharide (LPS)-induced cardiomyocyte injury has not been clarified. Therefore, the aim of the present study was to investigate the effect of DHM on LPS-induced cardiomyocyte injury. In the present study, cardiomyocytes were randomized to the control (PBS), LPS and DHM + LPS groups. The LPS group was treated with 10 μg/ml LPS for 12 h and the DHM + LPS group was treated with 100 or 25 μM DHM 12 h prior to treatment with LPS. The protective effect of DHM on LPS-induced cardiomyocytes injury was evaluated by Cell Counting kit-8 assay, TUNEL staining, reverse transcription-quantitative polymerase chain reaction and western blot analysis. The results demonstrated that LPS treatment led to cardiomyocyte apoptosis, and these effects were significantly attenuated by DHM. Furthermore, LPS treatment downregulated the expression of B-cell lymphoma 2 apoptosis regulator (Bcl-2) and induced increased expression of Bcl-2-associated X apoptosis regulator (Bax). Additionally, DHM treatment reversed LPS-induced changes in Bcl-2 expression and Bax expression in cardiomyocytes, and rescued cells from apoptosis. In addition, increased mRNA expression levels of tumor necrosis factor-α and interleukin-6 induced by LPS were attenuated following treatment with DHM. Further investigation demonstrated that DHM suppressed the activation of toll-like receptor-4 (TLR4), which is involved in regulating the downstream signaling pathway of nuclear factor-κB (NF-κB). DHM attenuated LPS-induced cardiomyocyte injury, the potential mechanism responsible for this effect may involve inhibition of TLR4 activation and subsequent regulation of the associated downstream signaling pathway of NF-κB. The current study indicates that DHM may protect cardiomyocytes against LPS-induced injury by inhibition of the TLR4/NF-κB signaling pathway. The results of the present study may provide support for the development DHM as a strategy for the treatment of heart failure in septic shock.

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Key words: dihydromyricetin, lipopolysaccharide, cardiomyocytes, apoptosis, inflammation, toll-like receptor-4

Introduction

Lipopolysaccharide (LPS), the bacterial endotoxin, is considered to be the primary factor responsible for multi-organ failure in septic shock (1). The myocardium is one of the organs affected by septic shock, which is a major cause of mortality (2). It has been previously demonstrated that LPS activates caspases in cardiomyocytes, which induces end-stage nuclear apoptosis, cleavage of cardiac myofilament proteins and sarcomere disorganization (3). LPS-induced myocardial dysfunction is mediated by various proinflammatory mediators, including tumor necrosis factor (TNF)-α and interleukin 1 (IL-1), which may be responsible for LPS-induced multiple organ failure, including heart failure (4,5). Toll-like receptor-4 (TLR4), which is a type of pattern recognition receptor, is considered to be involved in the LPS-induced innate immune response (6). The TLR4 predominantly expressed in cardiomyocytes recognizes LPS and triggers the recruitment of adaptors, including myeloid differentiation factor 88 and Toll/IL-1 receptor domain-containing adaptor protein-inducing interferon-β (7). These two pathways result in the activation of nuclear factor-κB (NF-κB), a key transcription factor involved in inflammatory activation (7). Therefore, pharmacological interventions that disrupt the TLR4-induced inflammatory response in cardiomyocytes may be a promising approach for the treatment of certain cardiovascular diseases.

Ampelopsis, a plant of the Vitaceae family and widely used in Chinese traditional medicine for treating liver disorders (8), is widely distributed in tropical and subtropical regions (9). Dihydromyricetin (DHM) is a type of flavonoid compound, which is extracted from the stems and leaves of *Ampelopsis grossedentata* (10). Previous studies have reported that DHM functions as an anti-insulin resistance (11),

antitumor (12), anti-inflammatory (13) and antioxidative (14) agent. DHM is reported to suppress the levels of proinflammatory cytokines and increase the level of anti-inflammatory cytokines in macrophage cells (13). Recently, it has been reported that DHM protects against angiotensin II-induced cardiomyocyte hypertrophy by augmenting nitric oxide production (15). In addition, existing data also indicates that DHM protects against Adriamycin-induced heart injury by protecting myocardial cells from apoptosis (16). Therefore, the current study aimed to investigate whether DHM protects against LPS-induced cardiomyocyte injury and to identify the mechanisms involved.

Materials and methods

H9c2 cardiomyocyte culture. The H9c2 embryonic rat heart-derived cell line was obtained from The Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). DHM was purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany) and was dissolved at a concentration of 50 mM in dimethysulfoxide (Sigma-Aldrich; Merck KGaA) for storage at -20˚C. Cells were cultured in Dulbecco's modified Eagle's medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.), penicillin (100 U/ml) and streptomycin (100 mg/ml; Gibco; Thermo Fisher Scientific, Inc.) in a humidified incubator with an atmosphere of 5% CO_2 at 37˚C. Cells were seeded at a density of $1x10^6$ per well onto 6-well culture plates for mRNA extraction, $5x10^3$ cells per well in 96-well plates for terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) analysis and $1x10^7$ per well onto culture dishes (100 mm) for protein extraction. Cells were cultured in serum-free medium for 8 h at 37˚C and pretreated with DHM (25, 50 and 100 μM) or PBS for 12 h prior to LPS (10 μg/ml; L2630; Sigma-Aldrich; Merck KGaA) stimulation for 12 h at 37˚C.

Cell viability assay. Cell viability was evaluated by Cell Counting kit-8 (CCK-8; Sigma-Aldrich; Merck KGaA) assay according to the manufacturer's instructions. Briefly, 10 μl CCK-8 solution was added to each well of a 96-well plate ($1x10^3$ cells per well) and, following 4 h incubation at 37˚C, the absorbance was measured at 450 nm using the BioTek Synergy HT reader (BioTek Instruments, Inc., Winooski, VT, USA). The effect of DHM on cell viability was expressed as the percentage cell viability compared with the control group, which was set at 100%.

TUNEL staining. TUNEL assays were performed using the ApopTag® Plus Fluorescein *In Situ* Apoptosis Detection kit (EMD Millipore, Billerica, MA, USA) to label apoptotic nuclei, according to the manufacturer's instructions. Briefly, following 12 h pretreatment with DHM, cells were incubated with LPS for 12 h and subsequently fixed on coverslips in 1% paraformaldehyde in PBS (Sinopharm Chemical Reagent Co., Ltd., Shanghai, China) at room temperature for 5 min. After washing with PBS three times, cells were stained with TUNEL reagents (EMD Millipore) and 4', 6-diamidino-2-phenylindole (DAPI; 0.3 mmol/l; Invitrogen; Thermo Fisher Scientific, Inc.) for 1 min, and observed under a fluorescence microscope (BX51; Olympus Corporation, Tokyo, Japan). The index of cell apoptosis was calculated as the percentage of apoptotic nuclei/total number of nuclei (n=10 fields of view).

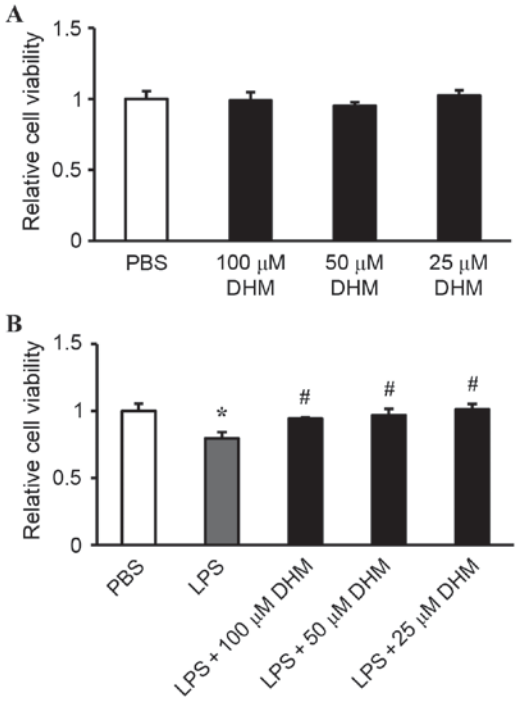

Figure 1. Effect of DHM on cell viability measured by Cell Counting kit-8 assay. (A) Treatment with 100, 50 or 25 μM DHM for 12 h did significantly affect cell viability compared with the PBS control group. (B) Treatment with 100, 50 or 25 μM DHM for 12 h and 10 μg/ml LPS for 12 h increased cell viability compared with the LPS-only group. $^*P<0.05$ vs. PBS group and $^\#P<0.05$ vs. LPS group. DHM, dihydromyricetin; LPS, lipopolysaccharide.

Western blot analysis. Cells in dishes ($1x10^7$ per well) were harvested and lysed in radioimmunoprecipitation assay (RIPA) lysis buffer with shaking for 15 min on ice. RIPA assay lysis buffer contained the following per ml: RIPA (720 μl; Beyotime Institute of Biotechnology, Haimen, China), PMSF (20 μl; 1 mM), cOmplete™ Protease Inhibitor Cocktail (100 μl; Roche; Sigma-Aldrich; Merck KGaA), PhosStop™ (100 μl; Roche; Sigma-Aldrich; Merck KGaA), NaF (50 μl; 1 mM) and Na_3VO_4 (10 μl). The protein concentration was measured using a bicinchoninic acid protein assay kit with a BioTek Synergy HT reader (BioTek Instruments, Inc.). The extracted protein (50 μg) from each sample was separated by 8-12% SDS-PAGE and the proteins were transferred onto polyvinylidene difluoride membranes. The membranes were blocked with 5% non-fat milk powder for 1 h at room temperature and were then incubated with primary antibodies overnight at 4˚C. The primary antibodies used were as follows: B-cell lymphoma 2 apoptosis regulator (Bcl-2; cat. no. 2870; 1:1,000; Cell Signaling Technology, Inc.), Bcl-2-associated X apoptosis regulator (Bax; cat. no. 2772; 1:1,000; Cell Signaling Technology, Inc., Danvers, MA, USA), total (T)-NF-κB p65 (cat. no. 8242; 1:1,000; Cell Signaling Technology, Inc.), phosphorylated (p)-NF-κB p65 (cat. no. 3033; 1:1,000; Cell Signaling Technology, Inc.), TLR4 (cat. no. sc-30002; 1:200; Santa Cruz Biotechnology, Inc., Dallas, Texas, USA) and

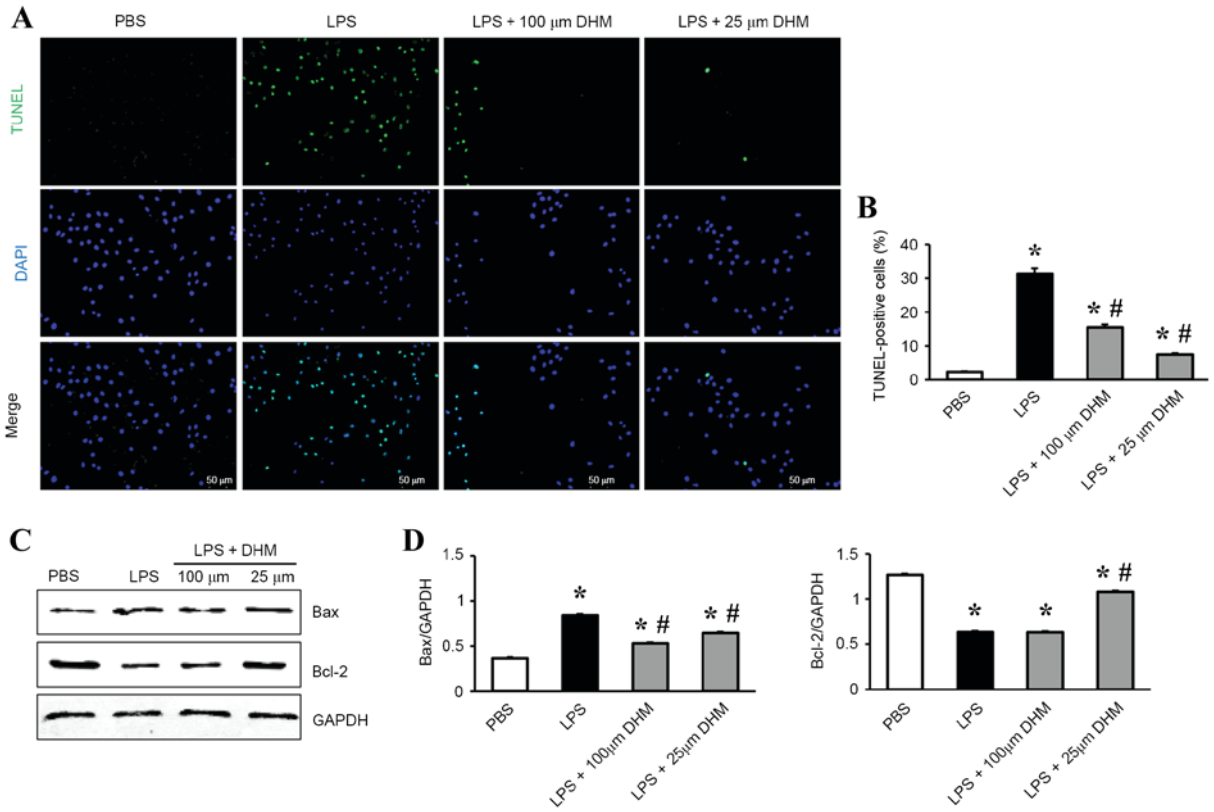

Figure 2. Effect of DHM on cell apoptosis. (A) Representative TUNEL and DAPI-stained images investigating the effect of 100 and 25 μM DHM on cell apoptosis following stimulation with LPS (10 μg/ml) for 12 h. Original magnification, x200. (B) Quantitative results of TUNEL analysis. (C) Representative western blot images investigating the effect of 100 and 25 μM DHM on protein levels of Bax and Bcl-2 in response to LPS. (D) Quantitative results of western blot analysis. *P<0.05 vs. PBS group and #P<0.05 vs. LPS group. DHM, dihydromyricetin; TUNEL, terminal deoxynucleotidyl transferase dUTP nick-end labeling; LPS, lipopolysaccharide; Bax, Bcl-2-associated X apoptosis regulator; Bcl-2, B-cell lymphoma 2 apoptosis regulator.

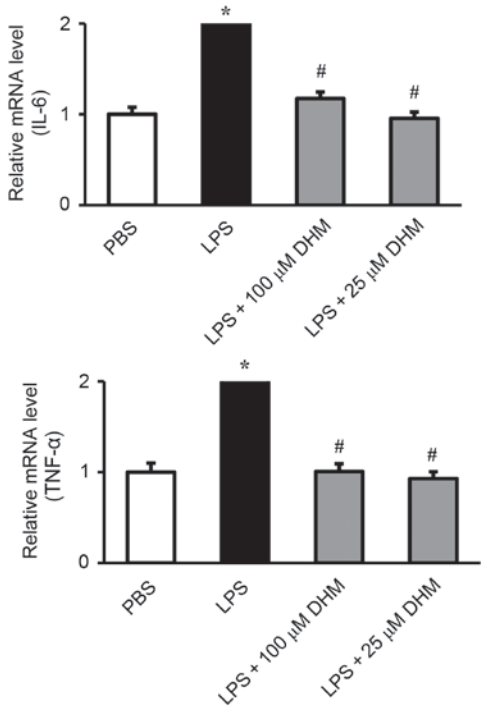

Figure 3. Effect of DHM on the mRNA expression of inflammatory mediators. Reverse transcription-quantitative polymerase chain reaction analysis of the mRNA levels of IL-6 and TNF-α induced by pretreatment with 100 and 25 μM DHM for 12 h followed by 10 μg/ml LPS for 12 h. *P<0.05 vs. PBS group and #P<0.05 vs. LPS group. DHM, dihydromyricetin; IL, interleukin; TNF-α, tumor necrosis factor-α; LPS, lipopolysaccharide.

GAPDH (cat. no. sc-25778; 1:200; Santa Cruz Biotechnology, Inc.). The membranes were subsequently incubated with IRDye 800CW-conjugated secondary antibodies (LI-COR Biosciences; cat. no. 926-32211; 1:200; Lincoln, NE, USA) for 1 h at room temperature. The blots were scanned using an Odyssey® Fc infrared scanner, allowing for simultaneous detection of two targets (phosphorylated and total protein) within the same experiment (LI-COR Biosciences). The specific protein expression levels were normalized against the expression of GAPDH by Quantity One software (version 4.6.2; Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Cells in 6-well plates (1x10^6 per well) were harvested and total RNA was extracted using TRIzol (Thermo Fisher Scientific, Inc.). The yield and purity levels of the RNA were spectrophotometrically estimated using A260/A280 and A230/A260 ratios, obtained via a SmartSpec Plus Spectrophotometer (Bio-Rad Laboratories, Inc.). The RNA (2 mg each sample) was reverse transcribed into cDNA using a Transcriptor First Strand cDNA Synthesis kit (Roche Diagnostics, Basel, Switzerland), according to the manufacturer's protocol. The PCR amplifications were quantified using the LightCycler® 480 SYBR® Green I Master Mix (Roche Diagnostics), according to the manufacturer's protocol, in the LightCycler® 480 Real-Time PCR System (Roche Diagnostics). Briefly, following a 5 min initial

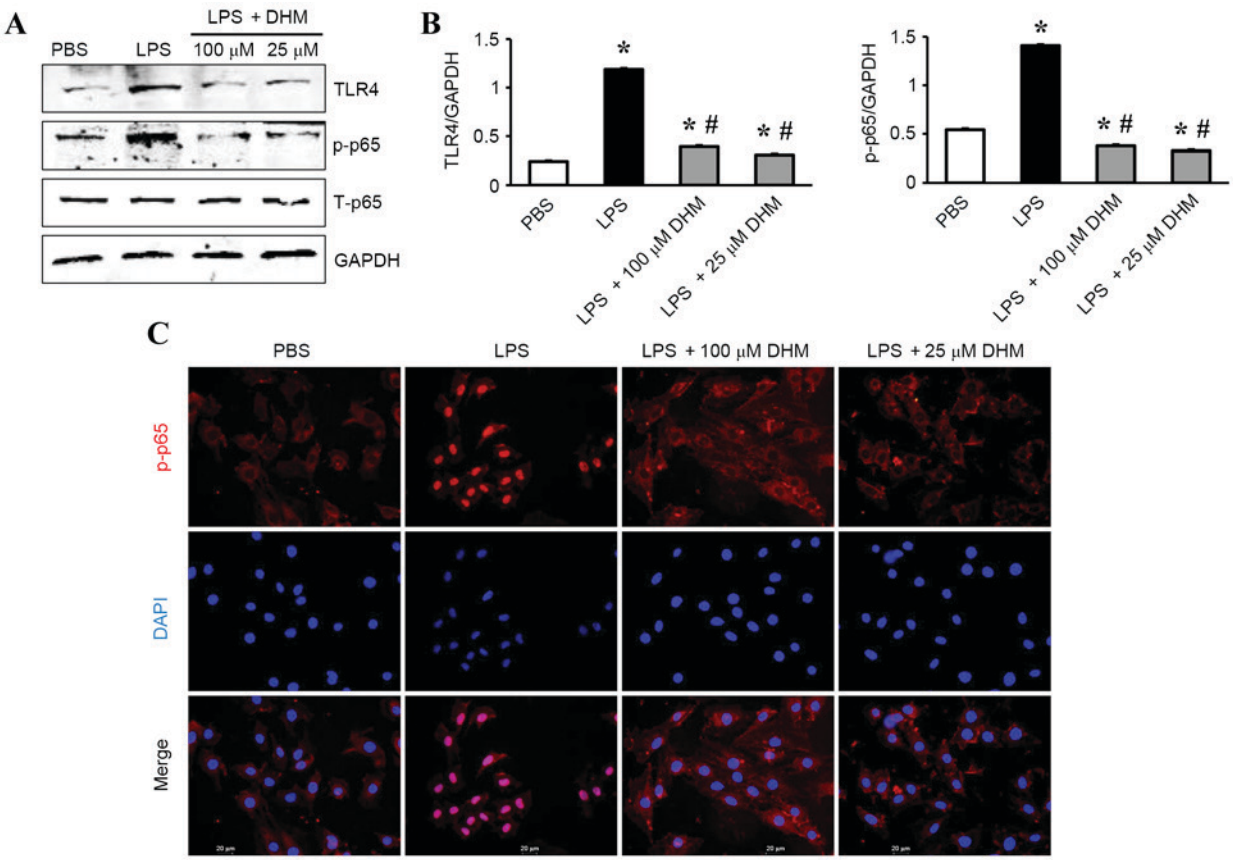

Figure 4. Effect of DHM on the activation of the TLR4/NF-κB pathway. (A) Representative blots and (B) quantitative results of western blot analysis to determine the effect of DHM on the activation of TLR4/NF-κB pathways, including TLR4, p-NF-κB p65 and T-NF-κB p65. (C) Effect of DHM on the LPS-induced nuclear translocation of NF-κB p65. Magnification, x200. *P<0.05 vs. PBS group and #P<0.05 vs. LPS group. DHM, dihydromyricetin; TLR4, toll-like receptor-4; NF-κB, nuclear factor-κB; p-p65, phosphorylated-NF-κB p65; T-p65, total-NF-κB p65; LPS, lipopolysaccharide.

denaturation at 95˚C, a total of 42 primer-extension cycles were carried out. Each cycle consisted of a 10 sec denaturation step at 95˚C, a 20 sec annealing step at 60˚C and a 20 sec incubation at 72˚C for extension. A final extension step was performed at 72˚C for 10 min. The double standard curve was used to quantify the PCR results. Calibrator normalized ratio = (concentration of sample target/concentration of sample reference)/(concentration of calibrator target/concentration of calibrator reference). The results were normalized to the mRNA expression of GAPDH by using the ΔΔCq method (17). The experiment was repeated three times. The sequences of the oligonucleotide primers (Sangon Biotech Co., Ltd., Shanghai, China) were as follows: TNF-α forward, 5'-AGCATGATC CGAGATGTGGAA-3' and reverse, 5'-TAGACAGAAGAG CGTGGTGGC-3'; IL-6 forward, 5'-GTTGCCTTCTTGGGA CTGATG-3', and reverse, 5'-ATACTGGTCTGTTGTGGG TGGT-3'; and GAPDH forward, 5'-GACATGCCGCCTGGA GAAAC-3' and reverse 5'-AGCCCAGGATGCCCTTTAGT-3'.

Immunofluorescence staining. Briefly, the cells ($5x10^3$ per well) were washed with PBS, fixed with 1% paraformaldehyde for 5 min at room temperature, permeabilized in 0.1% Triton X-100 (Amresco, LLC, Solon, OH, USA) in PBS for 5 min at room temperature and blocked with 8% goat serum (Beyotime Institute of Biotechnology) for 1 h at room temperature. Cells were then stained with anti-p-NF-κB p65 (cat. no. BS4135; Bioworld Technology, Inc., St. Louis Park, MN, USA) overnight at a dilution of 1:100 in 1% goat serum at 4˚C. Following 5 washes in PBS, cells were incubated with Alexa Fluor 568 goat anti-rabbit IgG secondary antibody (cat. no. A-11011; 1:200; Invitrogen; Thermo Fisher Scientific, Inc.) for 60 min at room temperature. Following 6 washes in PBS, cells on coverslips were mounted onto glass slides using SlowFade® Gold Antifade Mountant with DAPI (Invitrogen; Thermo Fisher Scientific, Inc.).

Statistical analysis. Data are presented as the mean ± standard error of the mean. Differences among the groups were determined by one way analysis of variance followed by a post-hoc Tukey test. Comparisons between two groups were performed using the unpaired Student's t-test. Statistical analyses were performed using SPSS version 13.0 (SPSS, Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

Effect of DHM on cell viability. The potential cytotoxicity of DHM was examined by CCK-8 assay. H9c2 cells were incubated with varying concentrations of DHM (100, 50 and 25 μM) for 12 h. Cell viability in DHM-treated cells was not significantly different compared with that of control cells treated with PBS, indicating that DHM (100, 50 and 25 μM) did not cause cytotoxicity in H9c2 cells (Fig. 1A). Stimulation

with LPS for 12 h significantly reduced the cell viability, while 100, 50 and 25 μM concentrations of DHM pretreatment attenuated LPS-induced cytotoxicity (Fig. 1B).

DHM attenuates LPS-induced apoptosis in H9c2 cells. TUNEL staining was used to identify the potential protective role of DHM on LPS-induced apoptosis in H9c2 cells. A significant increase in the number of TUNEL-positive nuclei was observed in cells incubated with LPS compared with control cells, and DHM (100 and 25 μM) pretreatment significantly reduced LPS-induced cell apoptosis (Fig. 2A and B). In addition, DHM pretreatment decreased the levels of expression of Bax protein, while increasing the Bcl-2 protein expression levels in H9c2 cells following LPS stimulation (Fig. 2C and D).

DHM inhibits the expression of inflammatory genes induced by LPS in H9c2 cells. The effect of DHM on the induction of IL-6 and TNF-α in response to LPS was measured by RT-qPCR. LPS significantly stimulated the release of IL-6 and TNF-α. Pretreatment with DHM (100 and 25 μM) significantly attenuated the LPS-induced increase in IL-6 and TNF-α (Fig. 3).

DHM reduces the activation of TLR4/NF-κB signaling in response to LPS. The mechanisms underlying the anti-inflammatory effects of DHM on LPS-treated H9c2 cells was investigated by western blot analysis. The results demonstrated that LPS significantly increased the expression level of TLR4 and subsequently increased the levels of p-NF-κB p65 (Fig. 4A and B). While 100 and 25 μM DHM decreased expression of TLR4 and subsequently decreased the levels of p-NF-κB p65 (Fig. 4A and B). In addition, treatment with 100 and 25 μM DHM reduced LPS-induced increases in the nuclear translocation of NF-κB p65 as determined by immunofluorescence staining (Fig. 4C).

Discussion

DHM has been previously demonstrated to be an anti-inflammatory (13) and antioxidative (14) compound. The present study demonstrated a novel role of DHM in the protection of cardiomyocytes from LPS-induced injury. DHM attenuated LPS-induced apoptosis in cardiomyocytes by reducing Bax expression and the upregulation of Bcl-2 expression. DHM also attenuated LPS-induced inflammatory response by the inhibition of the TLR4/NF-κB signaling cascade.

Sepsis is a major consequence of infectious diseases, which causes injury to multiple organs, including injury to the cardiovascular system (18). It has been demonstrated that cardiomyocytes are the major local source of proinflammatory cytokines in the myocardium during sepsis (19). These proinflammatory cytokines, including TNF-α, IL-6 and IL-1β are responsible for LPS-induced cardiac dysfunction and myocardial depression, which may cause cardiomyocyte apoptosis and heart failure (20,21). Therefore, blocking inflammatory signaling may produce beneficial effects in the dysfunctional heart. *Ampelopsis grossedentata* has been used for treating pharyngitis in traditional Chinese medicine for hundreds of years. Hou *et al* (13) reported that DHM, a major bioactive component of *Ampelopsis grossedentata*, suppresses the release of TNF-α, IL-6 and IL-1β in macrophage cells (13). The present study demonstrated that DHM downregulated the expression of TNF-α and IL-6 in LPS-stimulated H9c2 cells, indicating an anti-inflammatory effect of DHM in cardiomyocytes.

Inflammatory mediators cause cardiac cytotoxicity and lead to cardiomyocyte apoptosis, thereby promoting cardiac dysfunction (22). A well-balanced interplay between anti- and proapoptotic Bcl-2 family members is essential for the maintenance of mitochondrial integrity, which determines cell survival (23). Within the Bcl-2 family, BH3-only proteins, including Bcl-2-like 11, Bcl-2 binding component 3 or BH3 interacting domain death agonist (also termed Bim, Puma and Bid, respectively), neutralize antiapoptotic Bcl-2 proteins and directly activate rate-limiting cell death effectors Bax and Bcl-2 antagonist/killer 1 (also termed Bak). Upon activation, these cell death effectors form homodimers and assemble into higher order oligomers that allow the activation of caspase proteases lead to cell death (24). The present study demonstrated that DHM attenuated LPS-induced cardiomyocyte apoptosis by downregulating the expression of Bax, while upregulating Bcl-2 expression, in LPS-stimulated H9c2 cells. These results indicate a potential therapeutic role for DHM in heart disease.

TLR4 has critical roles in mediating inflammatory responses associated with heart diseases (7). TLR4 acts as a pattern recognition receptor, which are receptors that recognize molecular patterns associated with pathogens and damage (25). Once activated, TLR4 triggers an intracellular signaling response and causes the activation and translocation of NF-κB to the nucleus, which leads to an inflammatory response and cell apoptosis. The present study demonstrated that DHM treatment inhibited TLR4 expression and blocked the phosphorylation and nuclear translocation of NF-κB p65 in response to LPS. This indicates that the alleviating effect of DHM on LPS-induced inflammatory responses and apoptosis in cardiomyocytes may be mediated by inhibition of the TLR4/NF-κB pathway. In conclusion, the present study, to the best of our knowledge, is the first to demonstrate a protective effect of DHM on LPS-induced cardiomyocyte injury via inhibition of the TLR4/NF-κB signaling pathway. These results may be important for the development of strategies for the treatment of heart failure in septic shock. However, further studies are required before DHM may be considered for clinical usage in inflammatory disease.

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