Propofol ameliorates endotoxin-induced myocardial cell injury by inhibiting inflammation and apoptosis via the PPARγ/HMGB1/NLRP3 axis

HUI ZHAO, YING GU and HAI CHEN

Department of Anesthesiology, The Second Affiliated Hospital of Air Force Medical University, Xi'an, Shaanxi 710032, P.R. China

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Abstract. Endotoxin lipopolysaccharide (LPS) is one of the primary causes of myocardial injury. Propofol confers protective effects against LPS-induced myocardial damage; however, the biological functions and mechanisms underlying propofol are not completely understood. The present study aimed to investigate the effects of propofol on LPS-induced myocardial injury. Primary neonatal rat cardiomyocytes were treated with LPS to establish a myocardial injury model. LDH release in the culture media was measured using a LDH assay kit. The interactions between NLR family pyrin domain containing 3 (NLRP3), apoptosis-associated speck-like protein containing A CARD (ASC) and pro-caspase-1 were determined using a co-immunoprecipitation assay. Cell viability was measured using an MTT assay, and the levels of cell apoptosis were determined using flow cytometry, JC-1 staining (mitochondrial membrane potential) and caspase-3 activity assays. The mRNA expression levels of TNF-α, IL-6, IL-1β and IL-18, and the protein expression levels of NLRP3, ASC, pro-caspase-1, caspase-1 p10, pro-IL-1β, IL-1β, pro-IL-18, IL-18, high mobility group box-1 (HMGB1) and peroxisome proliferator-activated receptor γ (PPARγ) were analyzed using reverse transcription-quantitative PCR and western blotting analyses, respectively. ELISAs were performed to measure the production of inflammatory mediators, including TNF-α, IL-6, IL-1β and IL-18. The present results demonstrated that pretreatment with propofol significantly attenuated LPS-induced neonatal rat cardiomyocyte injury in a concentration- and time-dependent manner. Propofol pretreatment also significantly inhibited LPS-induced cardiomyocyte inflammation and apoptosis. The results suggested that propofol pretreatment inactivated HMGB1-dependent NLRP3 inflammasome signaling, which involved PPARγ activation. Therefore, the results indicated that propofol reduced endotoxin-induced cardiomyocyte injury by inhibiting inflammation and apoptosis via the PPARγ/HMGB1/NLRP3 axis, suggesting that propofol may serve as a potential therapeutic agent for septic myocardial damage.

Correspondence to: Professor Hai Chen, Department of Anesthesiology, The Second Affiliated Hospital of Air Force Medical University, 1 Xinsi Road, Xi'an, Shaanxi 710032, P.R. China
E-mail: haichenfmmu@yeah.net

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Introduction

Sepsis is a dysregulated host response that can lead to multiple organ dysfunction (1,2) and is the primary cause of morbidity and mortality among patients in intensive care units (ICUs) worldwide (3). The heart is one of the most susceptible organs to sepsis-induced damage and myocardial dysfunction is a primary cause of mortality in patients with sepsis (4). The bacterial endotoxin lipopolysaccharide (LPS) is the principal cause of septic heart failure due to stimulating excessive inflammatory mediator production, abnormal gene regulation and mitochondrial dysfunction (5-7). At present, no effective therapeutic strategies for sepsis-induced myocardial dysfunction have been used in the clinic. Thus, the precise mechanism underlying LPS-induced septic heart injury requires investigation to aid with the development of novel therapeutic agents.

LPS stimulates inflammatory responses by activating and releasing multiple cytokines, including IL-1β (8), which is an important contributor to myocardial dysfunction in sepsis (9). IL-1β expression, maturation and secretion processes are highly regulated by the NLR family pyrin domain containing 3 (NLRP3) inflammasome (10). The NLRP3 inflammasome consists of three essential components: NLRP3, an inflammasome sensor molecule; apoptosis-associated speck-like protein containing A CARD (ASC), an adaptor protein; and pro-caspase-11, an inflammatory protease (11). The activated NLRP3 inflammasome triggers caspase-1 activation, which promotes the cleavage and secretion of proinflammatory cytokines IL-1β and IL-18 (12). NLRP3 inflammasome activation-induced IL-1β production involves high mobility group box-1 protein (HMGB1) (13), which is a key cytokine in the promotion of cellular activation and inflammatory responses during LPS-induced myocardial

injury (14). Peroxisome proliferator-activated receptor γ (PPARγ) is implicated in the regulation of LPS-induced HMGB1 release in RAW 264.7 cells (15). PPARγ activation mediates LPS-induced inflammation and sepsis-related heart dysfunction in mice (16). Therefore, it was hypothesized that PPARγ activation to target HMGB1/NLRP3 signaling may alleviate LPS-induced myocardial cell injury.

Propofol (2,6-diisopropylphenol) is widely used to induce and maintain anesthetic effects, and sedate patients in ICUs (17). Alongside the anesthetic functions, propofol also displays pharmacological properties (18,19). A clinical study demonstrated that the antioxidant capacity of plasma is increased during propofol anesthesia (20), and propofol suppresses proinflammatory cytokine production in patients with sepsis (21). *In vitro* and *in vivo* experiments have suggested that propofol elicits cardioprotective effects (22,23). Intriguingly, propofol displays inhibitory effects on myocardial ischemia-reperfusion injury in various experimental models by reducing oxidative stress (24), protecting mitochondrial function (25) and suppressing apoptosis (26). However, whether propofol protects against LPS-induced myocardial cell injury is not completely understood.

The aim of the present study was to investigate the biological functions and the underlying mechanism of propofol in LPS-induced cardiomyocyte injury.

Materials and methods

Neonatal rat primary cardiomyocyte isolation and culture. The animal experimental protocol was approved by Air Force Medical University's Institutional Animal Care and Use Committee (approval no. 2017-01347). Healthy neonatal male Sprague-Dawley rats (male; age, 6-24 h; weight, 5-6 g) were obtained from the Institute of Zoology, Chinese Academy of Medical Sciences. The rats were maintained in specific pathogen-free conditions at 22±2˚C and 60% humidity, with a 12-h light/dark cycle and free access to food and water. The primary cardiomyocytes were prepared from ventricles of neonatal rats according to a previously described protocol with slight modifications (27,28). In brief, rats were euthanized with an intraperitoneal injection of sodium pentobarbital (200 mg/kg) under sterile conditions. Subsequently, hearts were collected from rats and cut into pieces in Ca^{2+}- and Mg^{2+}-free D-Hanks balanced salt solution (Beyotime Institute of Biotechnology) supplemented with 0.1% trypsin. Then, 0.1% type II collagenase was added to digest the cells four times. Cells were centrifuged at 4˚C and 120 x g for 5 min. The pellets were resuspended in DMEM (Gibco; Thermo Fisher, Scientific, Inc.) supplemented with 15% FBS (Gibco; Thermo Fisher, Scientific, Inc.), 100 U/ml penicillin and 100 µg/ml streptomycin. Cells were transferred into 60-mm primary culture dishes (Corning Inc.) precoated with 1% gelatin at 37˚C for 1.5 h to remove non-cardiomyocytes. Non-adherent cardiomyocytes were plated ($1x10^6$ cells/dish) into 60-mm gelatin-precoated primary culture dishes. After 24 h, cardiomyocytes were washed with PBS (Beyotime Institute of Biotechnology) thrice and cultured in serum-free maintenance medium [80% DMEM, 20% M199 (Gibco; Thermo Fisher, Scientific, Inc.), 100 U/ml penicillin and 100 µg/ml streptomycin]. To validate cardiomyocytes, striated muscle-specific sarcomeric α-actin monoclonal antibody was employed.

Cardiomyocyte transfection. Cardiomyocytes were cultured in serum-free maintenance medium at 37˚C with 5% CO_2. At 80% confluence, cells were transfected with 100 nM non-targeting small interfering RNA (si)-negative control (NC; siNC), siHMGB1 or siPPARγ (all purchased from Shanghai GenePharma Co., Ltd.) for 48 h at 37˚C using Lipofectamine RNAiMAX (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Following 48 h of transfection, the cells were used for subsequent experiments. The sequences of each siRNA were as follows: siNC, 5'-GCT CTGGAGCAGTTCCGATAT-3'; siHMGB1, 5'-CCATCACAG TGTTGTTAA-3'; and siPPARγ, 5'-TAACGAATGGGATTT GTCTG-3'.

Experimental design and cell treatments. Cardiomyocytes were divided into the following groups: i) Control, cardiomyocytes cultured in serum-free maintenance medium; ii) propofol, cardiomyocytes treated with propofol (Sigma-Aldrich; Merck KGaA; 12.5, 25, 50 or 100 µM) for 6, 12, 24 or 48 h; iii) LPS, cardiomyocytes stimulated with 1 µg/ml LPS (*Escherichia coli* 055:B5; Sigma-Aldrich; Merck KGaA) for 4 h; iv) propofol + LPS, cardiomyocytes treated with propofol (12.5, 25, 50 or 100 µM) for 6, 12, 24 or 48 h then stimulated with 1 µg/ml LPS for 4 h; v) propofol + LPS + siRNA, cardiomyocytes transfected with 100 nM siNC, siHBGB1 or siPPARγ for 24 h, treated with propofol (50 µM) for 24 h and then stimulated with 1 µg/ml LPS for 4 h; vi) propofol + LPS + inhibitor, cardiomyocytes pretreated with recombinant rat HMGB1 (rHMGB1; Chimerigen Laboratories; 100 ng/ml) or GW9662 (a PPARγ activation inhibitor; MedChemExpress; 10 µM) for 30 min followed by treatment with propofol (50 µM) for 24 h and then stimulation with 1 µg/ml LPS for 4 h. All the treatments were incubated at 37˚C in a humidified atmosphere containing 5% CO_2.

Cell viability assay. An MTT assay (Dojindo Molecular Technologies, Inc.) was performed to measure cell viability. Cardiomyocytes were seeded ($2x10^4$ cells/well) into a 96-well plate. Following culture for 24 h, cells were treated as aforementioned. Subsequently, 10 µl MTT (0.5 mg/ml) was added to each well and incubated at 37˚C for 4 h. Cell culture medium was removed and DMSO was added to dissolve the purple formazan crystals. The absorbance of each well was measured at a wavelength of 570 nm using a microplate reader (Bio-Rad Laboratories, Inc.). Cell viability is presented as a percentage of the control.

Measurement of lactate dehydrogenase (LDH) release. Following treatment, the culture media from each group was collected. LDH release in the culture media was measured using the LDH assay kit (Nanjing Jiancheng Bioengineering Institute) according to the manufacturer's protocol.

Measurement of cardiomyocyte apoptosis via flow cytometry. Cardiomyocyte apoptosis was determined using an Annexin V-FITC/PI apoptosis detection kit (BD Biosciences). Following treatment, cells were harvested, washed twice with ice-cold PBS (pH 7.4) and centrifuged at 300 x g at 4˚C for 5 min. Cells were resuspended in 1X binding buffer to a final concentration of $5x10^5$ cells/ml. Subsequently, 5 µl Annexin V-FITC

and 10 μl PI were added to the cells, gently vortexed and incubated in the dark at 37˚C for 15 min. Apoptotic cardiomyocytes were analyzed using a FACSCalibur flow cytometer (BD Biosciences) and Cell Quest software (version 3.3; BD Biosciences). Early (positive Annexin V-FITC staining) plus late apoptotic cells (positive PI staining) were counted to determine the levels of cardiomyocyte apoptosis.

Measurement of mitochondrial membrane potential (MMP). Cardiomyocyte MMP was measured using a 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolocarbocyanine iodide (JC-1) detection kit (Nanjing Jiancheng Bioengineering Institute). JC-1 is a dual-emission MMP sensing dye that selectively aggregates in polarized (healthy) mitochondria and aggregated JC-1 emits red fluorescence. Mitochondrial depolarization (loss of MMP) prevents JC-1 from entering the mitochondria, thus monomeric JC-1 remains in the cytosol and emits green fluorescence. Following treatment, cardiomyocytes were washed with PBS thrice, incubated with 200 μM JC-1 at 37˚C for 15 min and washed twice with PBS. The fluorescence of aggregated JC-1 (red) was visualized at an emission wavelength of 590 nm and the fluorescence of monomeric JC-1 (green) was visualized at a wavelength of 529 nm using a fluorescence microscope (magnification, x400; Carl Zeiss AG). The ratio of red to green fluorescence intensity was calculated to determine the MMP.

Caspase-3 activity assay. Caspase-3 activity was detected to evaluate cell apoptosis using a Caspase-3 activity assay kit (Beyotime Institute of Biotechnology) according to the manufacturer's protocol. Caspase-3 catalyzes the conversion of acetyl-Asp-Glu-Val-Asp *p*-nitroanilide (Ac-DEVD-pNA) into pNA, which displays a strong absorption peak at a wavelength of 405 nm. Following treatment, cardiomyocytes were lysed using RIPA lysis buffer (Beyotime Institute of Biotechnology) and centrifuged at 1,000 x g at 4˚C for 15 min. The supernatants were harvested to evaluate caspase-3 activity. The supernatant (50 μl) was added to 2X reaction buffer (50 μl) and Ac-DEVD-pNA (50 μl) and incubated at 37˚C for 4 h. Absorbance was measured at a wavelength of 405 nm using a microplate reader.

Reverse transcription-quantitative PCR (qPCR). Following treatment, total RNA was extracted from cardiomyocytes using TRIzol® (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. Total RNA was reverse transcribed into cDNA using PrimeScript RT master mix (Takara Bio, Inc.). The temperature protocol for the reverse transcription was 5 min at 25˚C, 60 min at 42˚C and 15 min at 70˚C. Subsequently, qPCR was performed using SYBR-Green Premix-Ex Tag kit (Takara Bio, Inc.) on an ABI 7500 Fast Real-time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The following thermocycling conditions were used for the qPCR: Initial denaturation for 10 min at 95˚C; followed by 40 cycles of 15 sec at 95˚C and 60 sec at 60˚C. The following primers were used for qPCR: TNF-α forward, 5'-TGGAACTGGCAGAAGAGGCACT-3' and reverse, 5'-GTTCAGTAGACAGAAGAGCGTGGTG-3'; IL-6 forward, 5'-AGAAAGAGTTGTGCAATGGCA-3' and reverse, 5'-GGCAAATTTCCTGGTTATATCC-3'; IL-1β forward, 5'-CACTACAGGCTCCGAGATGAACAAC-3' and reverse, 5'-TTGTCGTTGCTTGGTTCTCCTTGT-3'; IL-18 forward, 5'-GACTGGCTGTGACCCTATCTGTGA-3' and reverse, 5'-TTGTGTCCTGGCACACGTTTC-3'; and GAPDH forward, 5'-TCCATGACAACTTTGGTATCG-3' and reverse, 5'-TGTAGCCAAATTCGTTGTCA-3'. mRNA expression levels were quantified using the $2^{-\Delta\Delta Cq}$ method (29) and normalized to the internal reference gene GAPDH.

ELISA. Following treatment, cardiomyocyte culture media was harvested and centrifuged at 500 x g at room temperature for 5 min. The supernatants were pooled to measure TNF-α, IL-6, IL-1β and IL-18 levels using rat TNF-α (cat. no. 560479; BD Biosciences), IL-6 (cat. no. 550319; BD Biosciences), IL-1β (cat. no. ab255730; Abcam) and IL-18 (cat. no. ab213909; Abcam) ELISA kits, respectively, according to the manufacturer's protocols.

Western blotting. Following treatment, cardiomyocytes were harvested, lysed in RIPA lysis buffer (Beyotime Institute of Biotechnology) and centrifuged at 1,000 x g at 4˚C for 10 min. Protein concentrations in the supernatant were quantified using a BCA assay kit (Beyotime Institute of Biotechnology). Proteins (30 μg) were separated via 10% SDS-PAGE and transferred onto PVDF membranes (EMD Millipore). The membranes were blocked with 5% skimmed milk in TBS containing 0.1% Tween-20 (TBST) for 1 h at room temperature. Subsequently, the membranes were incubated at 4˚C overnight with primary antibodies targeted against: NLRP3 (Santa Cruz Biotechnology, Inc.; cat. no. sc-134306; 1:1,000), ASC (Santa Cruz Biotechnology, Inc.; cat. no. sc-514414; 1:1,000), pro-caspase-1 (Santa Cruz Biotechnology, Inc.; cat. no. sc-56036; 1:800), caspase-1 p10 (Santa Cruz Biotechnology, Inc.; cat. no. sc-514; 1:500), pro-IL-1β (Santa Cruz Biotechnology, Inc.; cat. no. sc-12742; 1:500), IL-1β (Santa Cruz Biotechnology, Inc.; cat. no. sc-515598; 1:1,000), pro-IL-18 (Abcam; cat. no. ab191860; 1:100), IL-18 (Abcam; cat. no. ab223293; 1:1,000), HMGB1 (Abcam; cat. no. ab79823; 1:10,000), PPARγ (Abcam; cat. no. ab272718; 1:1,000) and β-actin (Abcam; cat. no. ab6276; 1:5,000). Following primary antibody incubation, the membranes were washed three times with TBST and incubated with an HRP-conjugated goat anti-rabbit IgG (Abcam; cat. no. ab6721; 1:5,000), HRP-conjugated rabbit anti-mouse IgG (Abcam; cat. no. ab6728; 1:5,000) or HRP-conjugated goat anti-Armenian hamster IgG (Abnova; cat. no. PAB9133; 1:3,000) secondary antibodies for 1 h at room temperature. The membranes were washed three times with TBST. Protein bands were visualized using an ECL kit (Pierce; Thermo Fisher Scientific, Inc.) followed by exposure to X-ray films. β-actin was used as the loading control.

Co-immunoprecipitation (Co-IP). Following treatment, cardiomyocytes were harvested, lysed in RIPA lysis buffer (Beyotime Institute of Biotechnology) and centrifuged at 1,000 x g at 4˚C for 10 min. Protein concentrations in the supernatant were quantified using a BCA assay kit. Cell lysates (500 μg) were precleared with 20 μl of protein A/G-agarose beads (Santa Cruz Biotechnology, Inc.), according to the manufacturer's protocol, and incubated with anti-ASC antibody

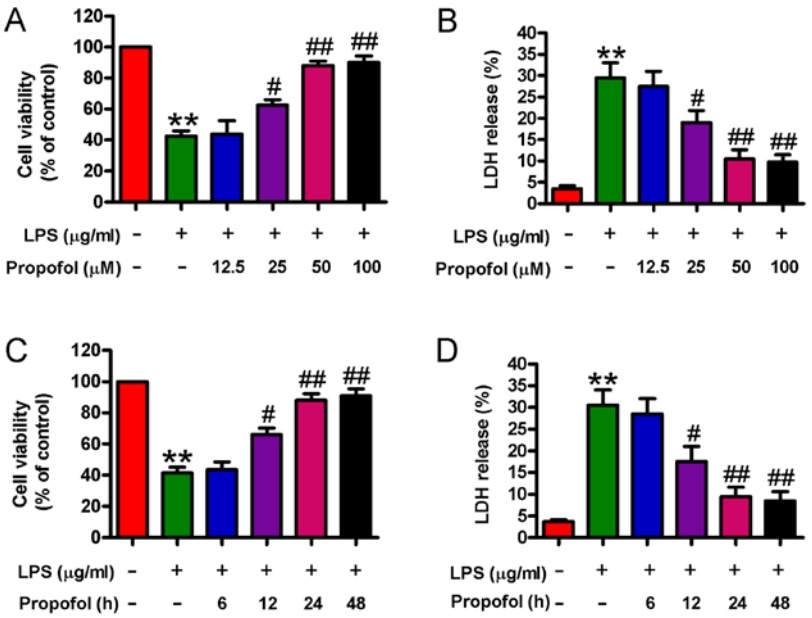

Figure 1. Propofol increases cell viability and inhibits LDH release in LPS-stimulated cardiomyocytes. Cardiomyocytes were pretreated with propofol (12.5, 25, 50 or 100 μM) for 24 h followed by stimulation with LPS (1 μg/ml) for 4 h. (A) Cell viability and (B) LDH release were determined by performing MTT and LDH release assays, respectively. Cardiomyocytes were pretreated with 50 μM propofol for different durations (6, 12, 24, and 48 h) followed by stimulation with LPS (1 μg/ml) for 4 h. (C) Cell viability and (D) LDH release were determined by performing MTT and LDH release assays, respectively. Data are from three independent experiments. **P<0.01 vs. control; $^{\#}$P<0.05 and $^{\#\#}$P<0.01 vs. LPS. LDH, lactate dehydrogenase; LPS, lipopolysaccharide.

(Santa Cruz Biotechnology, Inc. cat. no. sc-514414) or normal IgG at 4˚C overnight with gentle agitation. Subsequently, 20 μl protein A/G-agarose beads were added to the immune complexes and incubated for 6 h at 4˚C. The resultant mixtures were centrifuged at 250 x g at 4˚C for 5 min and the supernatants were removed. The pellets were washed three times with PBS and western blotting was performed according to the aforementioned protocol. Proteins were separated via SDS-PAGE and transferred onto PVDF membranes. The membranes were incubated with primary antibodies targeted against: NLRP3, ASC and caspase-1. Subsequently, the membranes were incubated with HRP-conjugated secondary antibodies. Protein bands were visualized using an ECL kit.

Statistical analysis. Data are presented as the mean ± SD from three independent experiments. Statistical analyses were performed using SPSS software (version 17.0; SPSS, Inc.). Comparisons among multiple groups were analyzed using one-way ANOVA followed by Tukey's post hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

Propofol protects cardiomyocytes against LPS-induced injury. Propofol has been reported to attenuate LPS-induced myocardial injury (22,23). The MTT and LDH release assays were performed to determine the effects of propofol on LPS-induced cardiomyocyte damage. Compared with the control group, LPS stimulation significantly reduced cardiomyocyte viability and enhanced LDH release, whereas propofol (25, 50, or 100 μM) pretreatment for 24 h significantly increased cell viability and decreased LDH release in LPS-stimulated cardiomyocytes in a concentration-dependent manner; however, there were no significant differences observed between the 50 and 100 μM propofol groups (Fig. 1A and B). Compared with the LPS group, LPS-stimulated cardiomyocytes that were pretreated with 50 μM propofol for different durations (12, 24, and 48 h) displayed significantly increased cell viability and decreased LDH release in a time-dependent manner; however, there was no significant difference observed between the 24 and 48 h groups (Fig. 1C and D). Therefore, 50 μM propofol treatment for 24 h was selected for subsequent experiments due to its protective effects against LPS-induced injury in cardiomyocytes. The results indicated that propofol ameliorated LPS-induced cardiomyocyte injury.

Propofol inhibits LPS-induced cardiomyocyte apoptosis and inflammation. The effect of propofol on LPS-induced cardiomyocyte apoptosis and inflammation was assessed. The flow cytometry results indicated that compared with the control group, LPS significantly increased cell apoptosis, which was significantly reduced by pretreatment with propofol (Fig. 2A and B). Propofol also significantly abolished LPS-induced loss of MMP (Fig. 2C). Moreover, LPS-induced increases in caspase-3 activity were significantly reduced by pretreatment with propofol (Fig. 2D). The mRNA expression levels of proinflammatory cytokines, including TNF-α and IL-6, were significantly increased by LPS treatment compared with the control group. However, pretreatment with propofol significantly decreased TNF-α and IL-6 expression levels in LPS-treated cardiomyocytes (Fig. 2E and F). Similarly, LPS-injured cardiomyocytes that were pretreated with propofol released significantly less TNF-α and IL-6 compared with the LPS group (Fig. 2G and H). The results demonstrated that propofol alleviated LPS-induced cardiomyocyte apoptosis and inflammation.

Propofol prevents LPS-induced NLRP3 inflammasome activation in cardiomyocytes. The NLRP3 inflammasome is

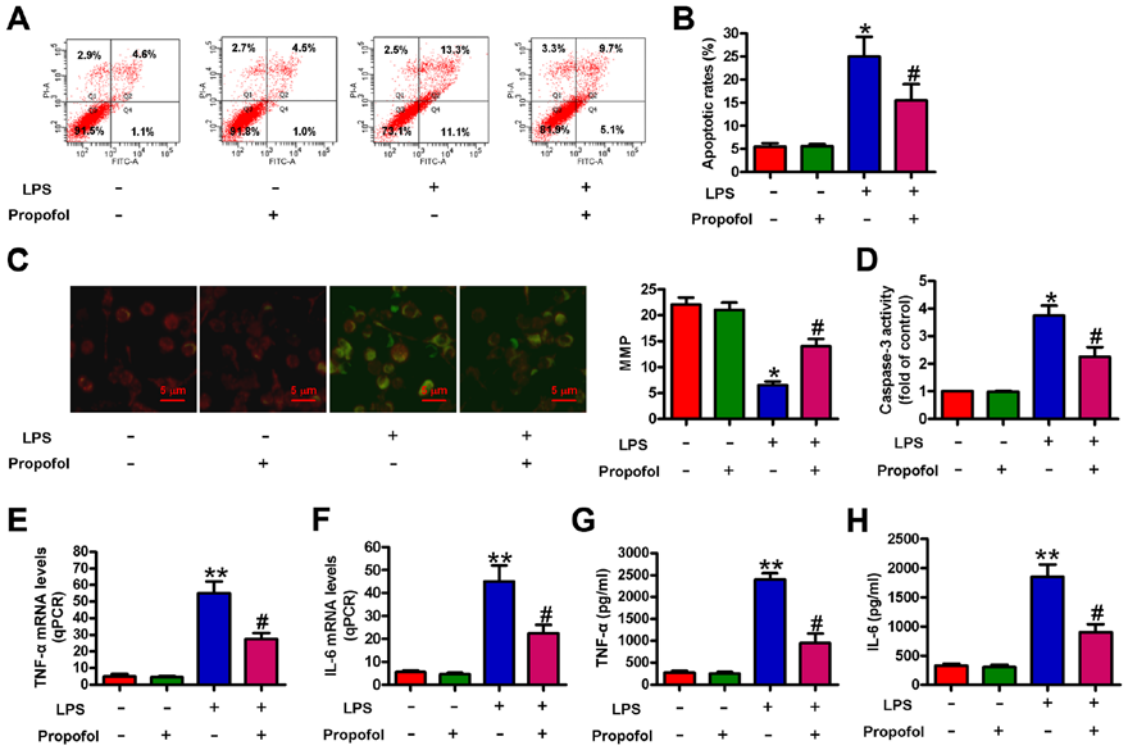

Figure 2. Propofol reduces LPS-induced cardiomyocyte apoptosis and inflammation. Cardiomyocytes were pretreated with 50 μM propofol for 24 h followed by stimulation with LPS (1 μg/ml) for 4 h. Cardiomyocyte apoptosis was (A) determined by flow cytometry and (B) quantified. (C) 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolocarbocyanine iodide staining was performed to assess the MMP. Scale bar, 5-μm. (D) Caspase-3 activity was measured to evaluate cell apoptosis. (E) TNF-α and (F) IL-6 mRNA expression levels were measured via reverse transcription-quantitative PCR. (G) TNF-α and (H) IL-6 levels in the supernatants were measured by performing ELISAs. Data are from three independent experiments. *P<0.05 and **P<0.01 vs. control; $^{\#}$P<0.05 vs. LPS. LPS, lipopolysaccharide; MMP, mitochondrial membrane potential; qPCR, quantitative PCR.

a prominent and early mediator of inflammatory responses in myocardial injury (30). NLRP3 inflammasome activation serves an important role in high glucose-induced H9c2 cell toxicity (31). Therefore, whether propofol prevented NLRP3 inflammasome activation in LPS-induced cardiomyocytes was investigated. Propofol markedly reduced LPS-induced increases in the protein expression levels of NLRP3, ASC, caspase-1 p10, IL-1β and IL-18 in cardiomyocytes (Fig. 3A). The Co-IP assay results demonstrated that the formation of the NLRP3-ASC-pro-caspase-1 complex was notably inhibited by pretreatment with propofol in LPS-injured cardiomyocytes (Fig. 3B). LPS-induced upregulation of IL-1β and IL-18 mRNA expression levels was significantly decreased by pretreatment with propofol (Fig. 3C and D). Propofol also significantly inhibited IL-1β and IL-18 release in LPS-injured cardiomyocytes (Fig. 3E and F). Collectively, the results suggested that propofol inhibited LPS-induced NLRP3 inflammasome activation in cardiomyocytes.

Propofol-mediated inactivation of the NLRP3 inflammasome is dependent on HMGB1 downregulation in LPS-injured cardiomyocytes. HMGB1 produced by cardiomyocytes contributes to LPS-induced myocardial dysfunction (14). To assess whether propofol reduced LPS-induced HMGB1 expression in cardiomyocytes, western blotting was performed. Compared with the control group, LPS notably increased HMGB1 expression levels, which were obviously reduced by pretreatment with propofol (Fig. 4A). HMGB1 activates the NLRP3 inflammasome in vascular smooth muscle cells (13). Subsequently, whether propofol-mediated inactivation of the NLRP3 inflammasome was HMGB1-dependent was investigated. siHMGB1 transfection markedly decreased HMGB1 protein expression levels in control and LPS-stimulated cardiomyocytes compared with siNC transfection (Fig. 4B). In LPS-stimulated cardiomyocytes, propofol-mediated downregulation of NLRP3, ASC, pro-caspase-1 and caspase-1 p10 protein expression levels were obviously counteracted by rHMGB1 pretreatment, but notably enhanced by siHMGB1 transfection (Fig. 4C). Similarly, rHMGB1 pretreatment significantly attenuated propofol-mediated inhibition of IL-1β and IL-18 release in LPS-stimulated cardiomyocytes, whereas HMGB1 knockdown enhanced the inhibitory effects of propofol on IL-1β and IL-18 release (Fig. 4D and E). The results suggested that propofol inhibited NLRP3 inflammasome activation by reducing HMGB1 expression in LPS-injured cardiomyocytes.

PPARγ contributes to propofol-mediated inactivation of the HMGB1-dependent NLRP3 inflammasome in LPS-injured cardiomyocytes. PPARγ inhibits LPS-induced HMGB1 release in RAW 264.7 cells (15). Previous studies have revealed that propofol represses inflammation by activating PPARγ in the renal tissues of sepsis model mice (32) and by upregulating PPARγ expression in THP-1 macrophage-derived foam cells (33). Compared with the control group, PPARγ expression was notably downregulated in LPS-injured cardiomyocytes, which was reversed by pretreatment with propofol (Fig. 5A). Subsequently, whether PPARγ was involved in propofol-mediated inactivation of the HMGB1-dependent

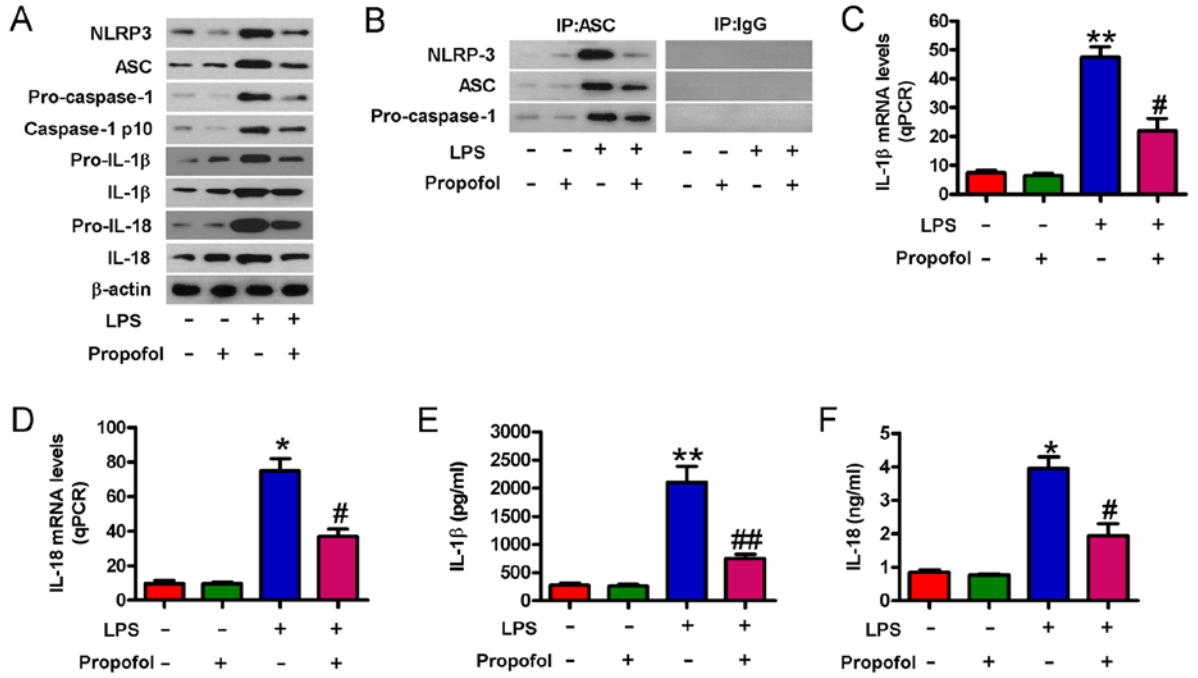

Figure 3. Propofol suppresses NLRP3 inflammasome activation in LPS-injured cardiomyocytes. Cardiomyocytes were pretreated with 50 μM propofol for 24 h followed by stimulation with LPS (1 μg/ml) for 4 h. (A) Western blotting was performed to measure the protein expression levels of NLRP3, ASC, pro-caspase-1, caspase-1 p10, pro-IL-1β, IL-1β, IL-18 and pro-IL-18. (B) The co-immunoprecipitation assay was performed to assess the relationship among NLPR3, ASC and pro-caspase-1. (C) IL-1β and (D) IL-18 mRNA expression levels were measured via reverse transcription-quantitative PCR. (E) IL-1β and (F) IL-18 levels in the supernatants were evaluated by performing ELISAs. Data are from three independent experiments. *P<0.05 and **P<0.01 vs. control; $^{\#}$P<0.05 and $^{\#\#}$P<0.01 vs. LPS. NLRP3, NLR family pyrin domain containing 3; LPS, lipopolysaccharide; ASC, apoptosis-associated speck-like protein containing A CARD; qPCR, quantitative PCR; IP, immunoprecipitation.

NLRP3 inflammasome in LPS-injured cardiomyocytes was assessed. In control and LPS-stimulated cardiomyocytes, PPARγ protein expression levels were obviously lower in siPPARγ-transfected cardiomyocytes compared with control or siNC-transfected cardiomyocytes (Fig. 5B). siPPARγ transfection or GW9662 treatment reversed propofol-mediated downregulation of HMGB1, NLRP3, ASC, pro-caspase-1 and caspase-1 p10 protein expression levels in LPS-injured cardiomyocytes (Fig. 5C). Moreover, propofol-mediated inhibition of IL-1β and IL-18 release was significantly reversed by siPPARγ transfection or GW9662 treatment in LPS-injured cardiomyocytes (Fig. 5D and E). The results indicated that PPARγ was involved in propofol-mediated inactivation of the HMGB1-dependent NLRP3 inflammasome in LPS-injured cardiomyocytes.

Discussion

In the present study, the effects and mechanisms underlying propofol in LPS-induced cardiomyocyte injury were investigated. Propofol pretreatment significantly enhanced cell viability and decreased LDH release in LPS-stimulated cardiomyocytes. Propofol also protected against LPS-induced cardiomyocyte apoptosis and inflammation. Moreover, LPS-induced NLRP3 inflammasome activation in cardiomyocytes was inhibited by pretreatment with propofol, which occurred via downregulation of HMGB1. In addition, PPARγ participated in propofol-mediated inactivation of the HMGB1-dependent NLRP3 inflammasome in LPS-injured cardiomyocytes. Overall, the results indicated that the cardioprotective role of propofol was partly reliant on its ability to inhibit activation of the HMGB1-dependent NLRP3 signaling pathway via activating PPARγ.

Myocardial injury is a distinctive characteristic of sepsis (4). LPS, the primary component of the outer membrane of Gram-negative bacteria, is one of the major causes of sepsis (34). LPS is commonly utilized to induce cardiomyocyte lesions (35-37). In the present study, cardiomyocyte viability was significantly decreased and LDH release was significantly increased in LPS-stimulated cardiomyocytes compared with control cells, suggesting that LPS induced cardiomyocyte damage. In LPS-induced myocardial injury, the excessive generation of inflammatory mediators is considered as one of the principal underlying mechanisms (38). Inflammatory mediators can result in loss of the MMP and caspase-3 activation, which ultimately leads to cardiomyocyte apoptosis (35). Cardiomyocyte apoptosis has been implicated in sepsis-induced myocardial dysfunction (39). In LPS-challenged mice, the myocardial levels of TNF-α and IL-1β are increased (40). In the present study, the levels of proinflammatory factors, such as TNF-α, IL-6, IL-1β and IL-18, were increased, and cell apoptosis, loss of the MMP and caspase-3 activation were enhanced in LPS-injured cardiomyocytes compared with control cells. Propofol is a widely used anesthetic agent that displays cardioprotective effects in various injury models (22-26). Propofol reduces the production of IL-1, IL-6 and TNF-α during LPS-induced myocardial injury *in vitro* and *in vivo* (22,23). Propofol attenuates H_2O_2-induced oxidative stress and apoptosis via the mitochondrial-mediated signaling pathway in neonatal rat

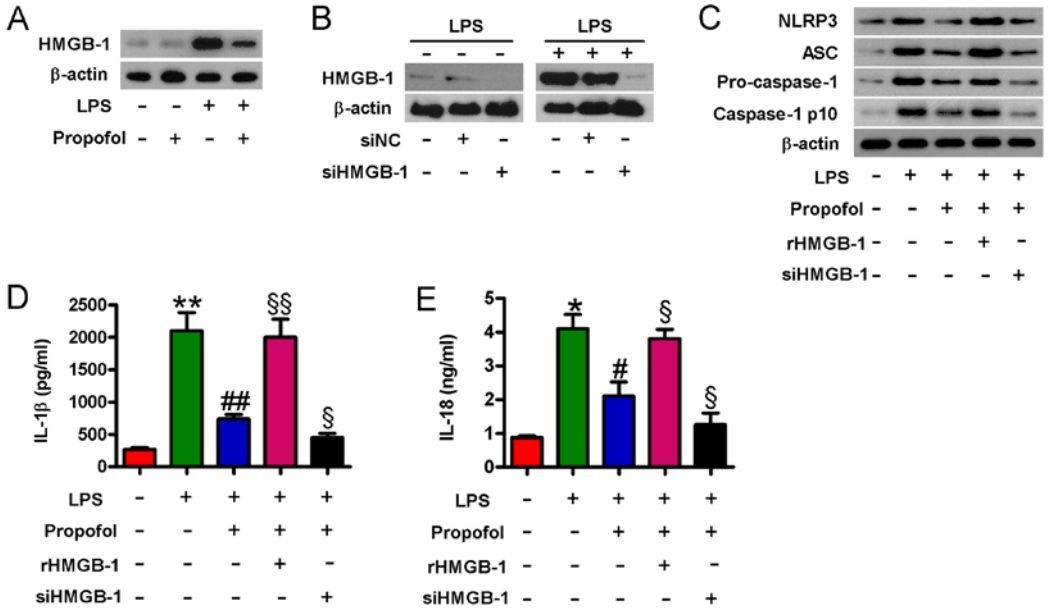

Figure 4. Propofol inactivates the NLRP3 inflammasome by downregulating HMGB1 in LPS-injured cardiomyocytes. (A) Cardiomyocytes were pretreated with 50 μM propofol for 24 h followed by stimulation with LPS (1 μg/ml) for 4 h. Western blotting was performed to measure HMGB1 protein expression levels. (B) Cardiomyocytes were transfected with 100 μM siHMGB1 or siNC for 48 h followed by stimulation with LPS (1 μg/ml) for 4 h. Western blotting was performed to measure HMGB1 protein expression levels. Cardiomyocytes were pretreated with rHMGB1 (100 ng/ml) for 30 min or transfected with 100 μM siHMGB1 or siNC for 24 h, followed by treatment with propofol (50 μM) for 24 h then stimulation with LPS (1 μg/ml) for 4 h. (C) NLRP3, ASC, pro-caspase-1 and caspase-1 p10 protein expression levels were measured via western blotting. (D) IL-1β and (E) IL-18 levels in the supernatants were evaluated by performing ELISAs. Data are from three independent experiments. *P<0.05 and **P<0.01 vs. control; $^{\#}$P<0.05 and $^{\#\#}$P<0.01 vs. LPS; §P<0.05 and §§P<0.01 vs. LPS + propofol. NLRP3, NLR family pyrin domain containing 3; HMGB1, high mobility group box-1; LPS, lipopolysaccharide; si, small interfering RNA; NC, negative control; ASC, apoptosis-associated speck-like protein containing A CARD; r, recombinant; ns, no significance.

cardiomyocytes (41). In the present study, the results indicated that propofol pretreatment protected cardiomyocytes against LPS-induced inflammation and apoptosis, as evidenced by decreased inflammatory cytokines, reduced apoptosis, alleviation of loss of the MMP and decreased caspase-3 activation in propofol-pretreated LPS-injured cardiomyocytes compared with LPS-injured cardiomyocytes. The results suggested that propofol may serve as a therapeutic agent for septic myocardial dysfunction due to its anti-inflammatory and anti-apoptotic properties.

NLRP3 inflammasome activation is a major contributor to myocardial injury (42). Activation of the NLRP3 inflammasome is a multi-step process that includes priming and activating (43). A priming stimulus increases the intracellular levels of pro-IL-1β and NLRP3, and the activation stimulus results in NLRP3 interacting with procaspase-1 via the adaptor protein ASC (44). The assembly of the NLRP3 inflammasome initiates caspase-1 activation via the cleavage of pro-caspase-1. The active form of caspase-1 causes enzymatic cleavage of pro-IL-1β and pro-IL-18, leading to IL-1β and IL-18 maturation and release (45). The present study indicated that propofol pretreatment markedly reduced LPS-induced increases in NLRP3, ASC, pro-caspase-1, caspase-1 p10, IL-1β and IL-18 expression levels, and IL-1β and IL-18 production in cardiomyocytes. Propofol pretreatment also prevented the association of NLRP3, ASC and pro-caspase-1 in LPS-injured cardiomyocytes. The results indicated that propofol pretreatment suppressed the priming and activation of NLRP3 inflammasome in LPS-injured cardiomyocytes.

HMGB1 can increase the expression of NLRP3 inflammasome components (NLRP3, ASC and caspase-1) (13). HMGB1 was originally described as a nuclear DNA-binding protein that is present in the nuclei and the cytoplasm, and is involved in maintaining the nucleosome structure and regulating gene transcription (46). HMGB1 is derived from stimulated macrophages or monocytes, and can be released from injured or necrotic cardiomyocytes (14,47). HMGB1 is an alarmin cytokine implicated in inflammation and cell injury during myocardial dysfunction (14). HMGB1 is an early mediator of inflammation and necrosis, and a late mediator of lethal sepsis (48,49). Chen *et al* (47) reported that LPS augments HMGB1 expression and facilitates its translocation from the nucleus to the cytoplasmic compartment in cardiomyocytes. HMGB1 antibody administration effectively decreases the mortality of LPS-induced sepsis model animals (50). In the present study, propofol pretreatment obviously decreased LPS-induced HMGB1 expression in cardiomyocytes. Propofol-induced reductions in the expression levels of NLRP3, ASC, pro-caspase-1 and caspase-1, and production of IL-1β and IL-18 were reversed by rHMGB1 pretreatment, but enhanced by siHMGB1 transfection in LPS-injured cardiomyocytes. The results suggested that propofol-mediated inactivation of the NLRP3 inflammasome was HMGB1-dependent in LPS-injured cardiomyocytes.

PPARγ, a ligand-inducible nuclear receptor belonging to the nuclear transcription factor PPAR superfamily, is expressed in a wide range of tissues and cells (51). PPARγ can protect against ischemia-reperfusion cardiomyocyte injury by regulating oxidative stress, inflammatory responses, glucose and lipid metabolism, and apoptosis (52). PPARγ activator inhibits LPS-induced TNF-α expression in neonatal rat cardiomyocytes (53). PPARγ agonist prevents LPS-mediated reductions

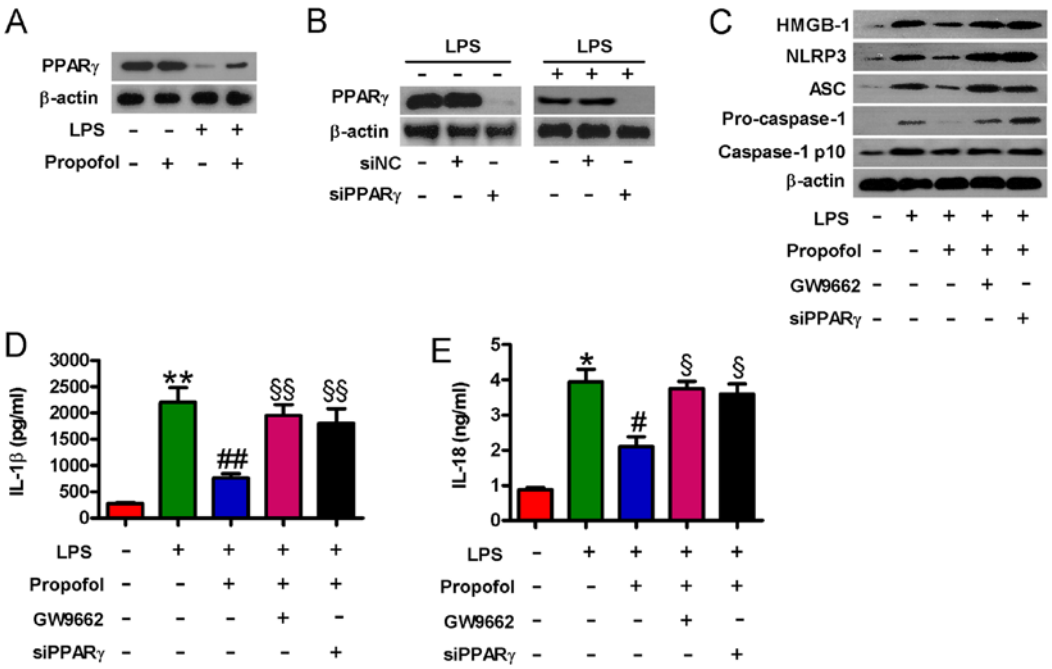

Figure 5. Propofol inhibits HMGB1-dependent NLRP3 inflammasome activation by activating PPARγ in LPS-injured cardiomyocytes. (A) Cardiomyocytes were pretreated with 50 μM propofol for 24 h followed by stimulation with LPS (1 μg/ml) for 4 h. Western blotting was performed to measure PPARγ protein expression levels. (B) Cardiomyocytes were transfected with 100 μM siPPARγ or siNC for 48 h followed by stimulation with LPS (1 μg/ml) for 4 h. Western blotting was performed to measure PPARγ protein expression levels. Cardiomyocytes were pretreated with GW9662 (10 μM) for 30 min or transfected with 100 μM siPPARγ or siNC for 24 h, followed by treatment with propofol (50 μM) for 24 h and then stimulation with LPS (1 μg/ml) for 4 h. (C) HMGB1, NLRP3, ASC, pro-caspase-1 and caspase-1 p10 protein expression levels were measured via western blotting. (D) IL-1β and (E) IL-18 levels in the supernatants were evaluated by performing ELISAs. Data are from three independent experiments. *P<0.05 and **P<0.01 vs. control; $^{\#}$P<0.05 and $^{\#\#}$P<0.01 vs. LPS; §P<0.05 and §§P<0.01 vs. LPS + propofol. HMGB1, high mobility group box-1; NLRP3, NLR family pyrin domain containing 3; PPARγ, peroxisome proliferator-activated receptor γ; LPS, lipopolysaccharide; si, small interfering RNA; NC, negative control; ASC, apoptosis-associated speck-like protein containing A CARD.

in the number of mitochondria and improves cardiac dysfunction in wild-type mice, and a high survival rate is observed in PPARγ-transgenic mice (16). Hwang *et al* (15) reported that PPARγ ligand rosiglitazone ablates LPS-stimulated HMGB1 release in RAW 264.7 cells, whereas siPPARγ transfection or GW9662 treatment abolishes the effect of rosiglitazone on HMGB1 release. Previous studies demonstrated the inhibitory effects of PPARγ on NLRP3 activation in monosodium urate-treated HK-2 cells and LPS/H_2O_2-challenged Kupffer cells (54,55). Propofol can upregulate PPARγ expression in THP-1 macrophage-derived foam cells (33). Consistently, the present study demonstrated that propofol enhanced PPARγ expression in LPS-injured cardiomyocytes, and GW9662 treatment or siPPARγ transfection alleviated propofol-mediated downregulation of LPS-induced increases in HMGB1, NLRP3, ASC, pro-caspase-1 and caspase-1 expression levels, and IL-1β and IL-18 production. Therefore, the results suggested that the potential beneficial effects of propofol against septic myocardial injury were dependent on PPARγ activation to suppress HMGB1-dependent NLRP3 inflammasome activation.

In summary, the present study suggested that the protective effects of propofol on LPS-induced cardiomyocyte injury were partially reliant on PPARγ activation, which inhibited activation of the NLRP3 inflammasome by downregulating HMGB1 expression during myocardial injury. Moreover, propofol reduced LPS-induced cardiomyocyte inflammation and apoptosis. Collectively, the present study suggested that propofol may serve as a potential therapeutic agent for septic myocardial dysfunction.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

HC conceived and designed the study. HZ performed the experiments and drafted the manuscript. YG analyzed the data. HZ and HC wrote the manuscript. All authors confirm the authenticity of all the raw data, and read and approved the final manuscript.

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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