Recombinant high-mobility group box 1 induces cardiomyocyte hypertrophy by regulating the 14-3-3η, PI3K and nuclear factor of activated T cells signaling pathways

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Abstract. High-mobility group box 1 (HMGB1) is released by necrotic cells and serves an important role in cardiovascular pathology. However, the effects of HMGB1 in cardiomyocyte hypertrophy remain unclear. Therefore, the aim of the present study was to investigate the potential role of HMGB1 in cardiomyocyte hypertrophy and the underlying mechanisms of its action. Neonatal mouse cardiomyocytes (NMCs) were co-cultured with recombinant HMGB1 (rHMGB1). Wortmannin was used to inhibit PI3K activity in cardiomyocytes. Subsequently, atrial natriuretic peptide (ANP), 14-3-3 and phosphorylated-Akt (p-Akt) protein levels were detected using western blot analysis. In addition, nuclear factor of activated T cells 3 (NFAT3) protein levels were measured by western blot analysis and observed in NMCs under a confocal microscope. The results revealed that rHMGB1 increased ANP and p-Akt, and decreased 14-3-3 protein levels. Furthermore, wortmannin abrogated the effects of rHMGB1 on ANP, 14-3-3 and p-Akt protein levels. In addition, rHMGB1 induced nuclear translocation of NFAT3, which was also inhibited by wortmannin pretreatment. The results of this study suggest that rHMGB1 induces cardiac hypertrophy by regulating the 14-3-3/PI3K/Akt/NFAT3 signaling pathway.

Introduction

High-mobility group box 1 (HMGB1) is released by activated cells, including cardiomyocytes and damaged or necrotic cells, and serves as an inflammatory cytokine. In addition, HMGB1 serves an important role in multiple organ pathologies, including cerebral, liver, lung and renal injury, and rheumatoid arthritis (1). It is likely that the specific properties of HMGB1 exhibit an effect on cardiomyocytes. Recently, it has been suggested that HMGB1 is involved in cardiovascular diseases (2), such as cardiac fibrosis, myocardial ischemia-reperfusion injury, heart transplantation, aortic valve calcification and sepsis-associated myocardial dysfunction (3-7). Although these previous reports have revealed that HMGB1 serves an essential role in the cardiovascular system, in which our previous study (8) also reported that HMGB1 induced cardiomyocyte hypertrophy, its physiological function in cardiomyocytes requires further investigation.

Cardiac hypertrophy is induced by several factors, including inflammatory factors and stresses, such as hypoxia (9). This process is initially considered beneficial, but it may progress to heart failure (10,11) under prolonged stress (12). It has been suggested that cardiac hypertrophy may be attenuated by controlling inflammation (13). HMGB1 acts as an inflammatory cytokine and serves an important role in cardiovascular pathology (14). Therefore, increased levels of circulating HMGB1 may be associated with human heart disease (15). For example, exogenous HMGB1 treatment in acute myocardial infarction induces cardiomyocyte survival by attenuating apoptosis and AMP-activated protein kinase-dependent autophagy (16). Our previous study demonstrated that HMGB1 may induce cardiomyocyte hypertrophy (8). In addition, HMGB1 may be partially derived from cardiomyocytes under pressure overload and may serve a crucial role in cardiac dysfunction (17). Notably, maintenance of stable nuclear HMGB1 levels prevents heart hypertrophy and failure by inhibiting DNA damage (18). However, to the best of our knowledge, the direct effects of exogenous HMGB1 treatment on cardiomyocytes remain elusive.

14-3-3 proteins are distributed ubiquitously in all eukaryotic organisms and serve a major role in stress response in several cells, including cardiomyocytes. The 14-3-3 protein family includes several highly conserved acid proteins, named according to their different isoforms (19). The 14-3-3 protein...
family includes several highly conserved acid proteins, named according to their different isoforms (β, ε, η, γ, τ, σ and ζ) detected in the cell cytoplasm and nucleus (20,21). 14-3-3η has been reported to serve an essential role in myocardial metabolism (22,23). Additionally, depletion of 14-3-3η increases cardiac hypertrophy, inflammation, fibrosis and apoptosis (24). It has previously been suggested that 14-3-3 proteins regulate the nuclear translocation of the nuclear factor of activated T cells (NFAT) (25,26), which in turn may induce pathological cardiac hypertrophy (27-29). In addition, a recent study has revealed that simvastatin upregulates 14-3-3 expression, which ultimately exerts beneficial effects through cardioprotection against pressure overload (30). In addition, 14-3-3 proteins interact with PI3K and NFAT3-mediated transcription in cardiomyocytes (19), which in turn phosphorylates its downstream target, Akt, resulting in physiological cardiac growth (31,32). The aforementioned results indicate that 14-3-3 proteins may be involved in the process of cardiac hypertrophy. However, whether HMGB1 interacts with 14-3-3 proteins to affect cardiac hypertrophy remains unclear, and, to the best of our knowledge, there are no studies on the association between HMGB1 and 14-3-3 proteins in cardiac hypertrophy.

Therefore, the present study hypothesized that HMGB1 may induce cardiomyocyte hypertrophy by regulating the 14-3-3 and PI3K/Akt signaling pathways. To test this hypothesis, neonatal mouse cardiomyocytes (NMCs) were isolated and treated with HMGB1. Subsequently, NMCs were treated with wortmannin, a specific PI3K inhibitor. Furthermore, to assess the nuclear translocation of NFAT in NMCs, the effects were observed by confocal microscopy.

Materials and methods

Preparation of NMCs and treatment. NMCs were isolated from 100 C57BL/6 mice (male; weight, 2±0.5 g; age, 1-3 days old), which were housed in a pathogen free facility with 50% humidity at 22˚C, with a 12-h light/dark cycle and free access to food and water. Mice were obtained from the Animal Center of the Fourth Military Medical University (Xi’an China). The mice were sacrificed by decapitation and heart cell isolation was performed using the Pierce Primary Cardiomyocyte Isolation kit (Thermo Fisher Scientific, Inc.), according to the manufacturer’s protocol. A monolayer of isolated NMCs was plated at a density of 3x10^5 cells/plate. NMCs were cultured in DMEM (Gibco; Thermo Fisher Scientific, Inc.) containing 1% penicillin-streptomycin (Thermo Fisher Scientific, Inc.) and 10% FBS (Thermo Fisher Scientific, Inc.) at 37˚C in a humidified atmosphere with 5% CO₂. The presence of fibroblasts was minimized by the addition of cardiomyocyte growth supplement (dilution, 1:1,000; Thermo Fisher Scientific, Inc.). Subsequently, NMCs were divided into the following four groups: The control (Ctrl) group; the wortmannin (Wort; kindly provided by Dr Jun L)‑treated group; the recombinant HMGB1‑treated (rHMGB1) group; and the rHMGB1 plus Wort (rHMGB1+Wort) group. In addition, rHMGB1 and wortmannin treatment of NMCs was performed according to our previous study (8). NMCs were treated with 200 ng/ml rHMGB1 (Sigma-Aldrich; Merck KGaA) or PBS for 24 h in the rHMGB1 or Ctrl groups, respectively, and with 100 nmol/l wortmannin for 60 min prior to exposure to PBS or rHMGB1 in the Wort or rHMGB1+Wort groups, respectively.

NMC protein synthesis measurement. Following treatment, NMCs were trypsinized, counted using a cell counting chamber (Beckman Coulter, Inc.) and lysed for further protein detection. Subsequently, protein concentration was determined using a Bradford protein assay (Bio-Rad Laboratories, Inc.). Finally, the protein synthesis per cell was calculated by dividing the total amount of protein by the number of NMCs.

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted from NMCs using the TRizol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.). RNA samples were quantified spectrophotometrically at 260 nm. Subsequently, RT-qPCR was performed as previously described (8). Briefly, RNA was exposed to RNase-free DNase I and 5 μg total RNA was reverse transcribed into cDNA using oligo(dT) and M-MuLV reverse transcriptase (Promega Corporation). The reverse transcription products served as templates for PCR using gene-specific primers (Table I) (33). qPCR was subsequently performed using a TaqMan™ Fast Advanced Master Mix (Applied Biosystems; Thermo Fisher Scientific, Inc.) using the 7500 Sequence Detector Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The thermocycling conditions were as follows: Initial step at 95˚C for 10 min, followed by 40 cycles at 95˚C for 15 sec and 60˚C for 1 min. Fluorescence signals of each gene were recorded during the elongation phase of each PCR cycle. The melting curve analysis was used to confirm the amplification specificity, and the RNA abundance was expressed as ΔΔCq. GAPDH expression served as the internal control, and each gene was quantified in duplicate. Finally, the RT-qPCR data were analyzed using the 2^ΔΔCq method (34).

Western blot analysis. NMCs were rinsed with PBS and lysed in buffer [Cell Lysis buffer (10X); cat. no. 9803; Cell Signaling Technology, Inc.] on ice. Subsequently, cell lysates were centrifuged (2,000 x g; 5-7 min; 4˚C), and the supernatant was collected. Following a Bradford assay to determine protein concentration, equal amounts of proteins (20 μg) were diluted in sample buffer (Thermo Fisher Scientific, Inc.), boiled and separated using 15% SDS-PAGE (Thermo Fisher Scientific, Inc.). The separated proteins were transferred to a nitrocellulose membrane (LI-COR Biosciences). Following blocking in 5 ml 1X Odyssey® blocking buffer (LI-COR Biosciences) for 1 h at room temperature, the nitrocellulose membranes were incubated with primary antibodies at 4˚C for 12 h. Subsequently, membranes were washed with 1X PBS-T (PBS with 0.1% Tween-20) and incubated with secondary antibodies for 1 h at room temperature. Finally, immunoreactive protein bands were analyzed by densitometry using the Odyssey® infrared imaging system (version 2.1; LI-COR Biosciences), and the specific protein bands were visualized using an Odyssey® scanner, then analyzed using Image Studio Lite version 5.0 (LI-COR Biosciences). The primary antibodies used in the present study were all diluted 1:1,000 and were: Anti-atrial natriuretic peptide (ANP; cat. no. ab126149; Abcam), anti-pan14-3-3
was detected using a TUNEL assay kit (Roche Diagnostics), according to the manufacturer's protocol. Briefly, following treatment NMCs (1x10^6 cells/well) were fixed with 4% paraformaldehyde at room temperature for 30 min and permeabilized with 0.2% Triton X-100 at room temperature for 30 min. The cells were subsequently incubated with TUNEL reagent for 1 h at room temperature. Thereafter, the cells were mounted with a mounting medium containing DAPI (1:1,000; Vector Laboratories, Inc.) at room temperature for 10 min. The TUNEL-positive and the total number of nuclei were counted in five independent experiments (3 randomly selected fields of view) under a Nikon Eclipse 80 fluorescent microscope (magnification, x10; Nikon Corporation).

**Confocal microscopy observation.** NFAT3 localization in NMCs was analyzed using confocal microscopy. Briefly, NMCs were isolated and cultured on laminin pre-coated Lab-Tek chamber slides (Thermo Fisher Scientific, Inc.). The secondary antibodies were goat anti-mouse IRDye® (1:10,000; cat. no. 926-68070; LI-COR Biosciences), goat anti-rabbit IRDye® (1:10,000; cat. no. 926-32211; LI-COR Biosciences) and goat anti-rat IRDye® (1:10,000; cat. no. 926-32219; LI-COR Biosciences). In addition, β-tubulin and histone protein levels served as a loading control for the total protein lysate, the cytosol and nuclear extracts, respectively.

**Statistical analysis.** All data were analyzed using GraphPad Prism 5 (GraphPad Software, Inc.) and presented as the mean ± SEM for continuous variables. One-way ANOVA was used to assess differences among groups followed by Tukey's post hoc test. P<0.05 was considered to indicate a statistically significant difference. The Ctrl group value was set as 1 to express the fold change.

**Results**

**rHMGB1 induces NMC hypertrophy.** Following exposure to rHMGB1 for 24 h, NMCs were harvested and their cellular protein content was analyzed. The results revealed that rHMGB1 treatment significantly increased the protein content per cell compared with that in the Ctrl group (Fig. 1A). Subsequently, the protein levels of ANP, a pathological cardiac hypertrophy marker, were detected using western blot analysis. Protein levels of ANP in the rHMGB1 group were significantly increased compared with those in the Ctrl group (Fig. 1B). In addition, the mRNA levels of ANP and brain natriuretic peptide (BNP), another pathological cardiac hypertrophy marker, were upregulated in the rHMGB1 group compared with those in the Ctrl group (Fig. 1C). Representative confocal images of NMCs are presented in Fig. 1D. Therefore, the present results suggest that rHMGB1 induces NMC hypertrophy.

**rHMGB1 decreases 14-3-3ζ protein levels, which can be partially reversed by wortmannin.** In the present study, western blot analysis was performed using anti-pan14-3-3 (an antibody against endogenous total 14-3-3 proteins) and anti-14-3-3ζ antibodies, to determine whether rHMGB1 could regulate their expression in NMCs. Following rHMGB1 treatment, 14-3-3ζ protein levels were decreased (Fig. 2A and B) and p-Akt levels were increased (Fig. 2A and F) in NMCs

| Gene    | Forward primer (5’→3’)                     | Reverse primer (5’→3’)     |
|---------|--------------------------------------------|-----------------------------|
| ANP     | AGGCAGTCGATTCTGCTTT                        | CGTGATAGATGAAGGCAGGAAG      |
| BNP     | TAGCCAGTCTCCAGAGCAATTTC                    | TTGGTCTTCCAGAGCTGTCTC       |
| GAPDH   | CCTCCTCCTTACCC                            | GCCCAAGATGCCCTTCAGT         |

ANP, atrial natriuretic peptide; BNP, brain natriuretic peptide.

**Table I. Primers used for reverse transcription-quantitative PCR.**
compared with those in the Ctrl group (Fig. 2). However, rHMGB1 treatment had no effect on the total endogenous 14-3-3 protein levels (Fig. 2A and D). Furthermore, wortmannin, a specific PI3K inhibitor, significantly inhibited the
rHMGB1-mediated effects on 14-3-3\(\eta\) protein levels and Akt phosphorylation (Fig. 2A, B and F). However, wortmannin alone had no significant effect on 14-3-3\(\eta\) levels compared with the Ctrl group (Fig. 2A and B). Additionally, no changes to pan14-3-3 protein levels were observed among the different groups (Fig. 2D). Finally, the effects of rHMGB1 treatment on the protein content per cell and ANP protein levels were inhibited by wortmannin (Fig. 2A, C and E). Overall, the present results suggest that rHMGB1 regulates 14-3-3\(\eta\) protein levels and hypertrophy in NMCs partially through the PI3K/Akt signaling pathway.

rHMGB1 induces NFAT3 nuclear translocation, which can be inhibited by wortmannin. In the present study, confocal microscopy was used to detect the subcellular location of NFAT3 and to determine whether NFAT3 is translocated to the nucleus following rHMGB1 treatment. In the rHMGB1 group, the levels of NFAT3 in the cytosol were decreased, while those in the nuclei were increased compared with the levels of NFAT3 in the Ctrl group (Fig. 3). Notably, wortmannin pretreatment inhibited the nuclear translocation of NFAT3 following rHMGB1 treatment. The results were verified using western blot analysis, demonstrating that rHMGB1 regulates 14-3-3\(\eta\) protein levels and hypertrophy in NMCs partially through the PI3K/Akt signaling pathway.

rHMGB1 has no effect on NMC apoptosis. Cardiac hypertrophy may lead to cardiomyocyte apoptosis during the heart failure process (35). In the present study, the apoptosis rate was measured using the TUNEL assay to detect TUNEL-positive nuclei and to determine whether rHMGB1 treatment induced NMC apoptosis. However, no significant differences in the number of TUNEL-positive nuclei were observed among the four groups (Fig. 5).

Discussion

The present study demonstrated that rHMGB1 induced NMC hypertrophy, decreased 14-3-3\(\eta\) protein levels and induced translocation of NFAT3 from the cytoplasm to the nucleus. The effects of rHMGB1 on NMC hypertrophy, 14-3-3\(\eta\) levels and NFAT3 translocation may be partially blocked by wortmannin, a specific PI3K inhibitor. However, rHMGB1 alone did not affect NMC apoptosis. Overall, the present results support the hypothesis that exogenous HMGB1 induces cardiomyocyte hypertrophy via the 14-3-3/PI3K/Akt/NFAT signaling pathway.

HMGB1 is a ubiquitous nuclear protein that is released from cell nuclei following tissue damage (36). Endogenous HMGB1 is located in the nucleus (37) and is actively secreted by innate immune cells (38) or other cells under stress conditions (6). Therefore, HMGB1 is found in cells and in the systemic
ciliation. It has been reported that endogenous HMGB1 may serve an important role in myocardium pathology (39). In addition, patients with myocarditis display increased systemic HMGB1 levels, suggesting its involvement in the pathogenesis of inflammatory cardiomyopathy (2). However, the effects of secreted HMGB1 on the cardiovascular system remain unclear, and the effect of exogenous HMGB1 treatment on cardiomyocytes requires further investigation. The results of the present study revealed that rHMGB1 treatment increased ANP protein levels, BNP and ANP mRNA synthesis, and protein content per cell, highlighting its role in the induction of NMC hypertrophy. In the present study, HMGB1 increased the intracellular levels of ANP and BNP in cardiomyocytes. However, whether HMGB1 is able to enhance the secretion of ANP or BNP was not the main purpose of the present study; this should be analyzed in vivo in future studies. The aforementioned results suggest that both endogenous and exogenous HMGB1 serve a role in myocardial modifications, which is consistent with a previous study demonstrating that exogenous HMGB1 treatment induced cardiomyocyte survival in a murine myocardial infarction model (16).

In the present study, HMGB1 activated NFAT3 by promoting its translocation to the nucleus, thereby upregulating its nuclear expression. Additionally, it has been documented that NFAT3 is regulated by 14-3-3 proteins (25,26), exhibits inflammatory effects and induces pathological hypertrophy in cardiac myocytes (27-29).

14-3-3 proteins interact with several proteins, including PI3K, Akt and NFAT3 in diabetic cardiomyopathy (40). 14-3-3 proteins are dimeric phosphoserine-binding molecules separated into several isoforms, including the β, γ, ε, ζ, η, θ and σ isoforms (19). These proteins bind to their target proteins and modify their function by altering their intracellular localization and phosphorylation status (20). Several studies have concluded that 14-3-3 proteins, particularly the 14-3-3η isoform, are involved in diabetic cardiomyopathy (40,41). However, whether 14-3-3η is involved in rHMGB1-induced hypertrophy, an independent predictor of cardiovascular morbidity and mortality, requires further investigation. The results of the present study revealed that the levels of total 14-3-3 proteins in NMCs did not change in the presence of rHMGB1. However, the protein levels of 14-3-3η were significantly decreased in rHMGB1-treated NMCs, resulting in significant induction of NMC hypertrophy. Therefore, the present results suggest that 14-3-3η and not total 14-3-3, may serve a major role in rHMGB1-induced NMC hypertrophy.

NMCs were treated with a specific PI3K inhibitor prior to exposure to rHMGB1. Notably, the levels of 14-3-3η were partially preserved in rHMGB1-treated NMCs following wortmannin pretreatment. Additionally, wortmannin partially
inhibited NFAT3 nuclear translocation. The present results suggest that PI3K, Akt and NFAT3 may interact with 14-3-3ζ and may influence rHMGB1-induced hypertrophy. The proposed model of rHMGB1-mediated regulation of NMC hypertrophy is illustrated in Fig. 6.

Furthermore, it has been reported that cardiomyocyte hypertrophy may eventually lead to apoptosis (35). Therefore, a TUNEL assay was performed to determine the rHMGB1-induced apoptosis rate in NMCs. However, no statistically significant differences were observed in the NMC apoptosis rate between the Ctrl and the rHMGB1-treated groups. The present finding is consistent with a previous study demonstrating that HMGB1 does not induce cardiomyocyte apoptosis under normal conditions (42). However, it has been reported that endogenous HMGB1 contributes to ischemia-reperfusion-induced myocardial apoptosis (39). A potential explanation for these contrasting findings may reside in the different environments or distinct receptors on different cells. Therefore, further investigation is required to investigate the role of exogenous HMGB1 in different cell types.

In conclusion, the present study demonstrated that extracellular HMGB1 treatment induced NMC hypertrophy potentially through the 14-3-3ζ/PI3K/Akt/NFAT signaling pathway. Therefore, 14-3-3 may be an important factor that links HMGB1, PI3K/Akt and NFAT3 in cardiomyocytes.

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Availability of data and materials
The datasets used and/or during the present study are available from the corresponding author on reasonable request.

Authors’ contributions
FS conceived the study, FS, MS and JZ performed the experiments. YL and JT analyzed the experimental data, assessed the raw data and confirm the authenticity of all the raw data. FS prepared the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate
The present experimental procedures were performed according to the ethical guidelines of the 1964 Declaration of Helsinki. All animal experiments were approved by the Animal Care and Welfare Ethics Committee of the Fourth Military Medical University (Xi’an, China).

Patient consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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