HMGA1 Attenuates Doxorubicin-Induced Cardiomyocyte Pyroptosis By Inhibiting SOX9

Yaxiu Liu (✉ 15200872966@163.com)

Central South University Xiangya School of Pharmaceutical Sciences <https://orcid.org/0000-0001-6492-7495>

Yao Tang

Central South University Xiangya School of Pharmaceutical Sciences

Hui Fu

Fenyang College Shanxi Medical University

Shuang Fu

Central South University Xiangya School of Pharmaceutical Sciences

Xinbin Zheng

Central South University Xiangya School of Pharmaceutical Sciences

Yeshuo Ma

Central South University Xiangya School of Pharmaceutical Sciences

Deling Yin

Zhejiang University School of Medicine Second Affiliated Hospital <https://orcid.org/0000-0001-8887-6178>

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Abstract

Doxorubicin (DOX) is widely used as an anti-tumor drug with severe cardiotoxicity, encephalotoxicity, nephrotoxicity and so on, especially cardiotoxicity, which severely limit its application. Researchers have extensively studied the mechanisms of DOX-induced cardiotoxicity. However, the underlying mechanism of DOX-induced cardiotoxicity needs to be further evaluated. Studies reveal that High-mobility group AT-hook1 (HMGA1) and Sex-determining-region-Y (SRY)-related HMG box-containing protein 9 (SOX9) contribute to caspase-3-mediated apoptosis, but whether HMGA1 and SOX9 participate in caspase-3/gasdermin E (GSDME)-mediated pyroptosis remains unknown. This study was performed to investigate whether HMGA1 and SOX9 participate in DOX-induced cardiomyocyte pyroptosis induced by DOX in vitro, and to reveal the molecular mechanisms of HMGA1 and SOX9 in regulating DOX-induced cardiomyocyte pyroptosis via caspase/GSDME pathway. Results showed that the expression of HMGA1 is significantly up-regulated while SOX9 is down-regulated in HL-1 cells after DOX treatment. We found that both inhibition of HMGA1 by small interfering RNA (siRNA) and overexpression of SOX9 by transfection of SOX9 plasmid significantly promote cardiomyocyte pyroptosis induced by DOX. In addition, HMGA1 interacts with SOX9. Finally, our results show that silencing SOX9 reverses cardiomyocyte pyroptosis induced by silencing HMGA1 after DOX treatment.

Introduction

Doxorubicin (DOX) is a kind of anthracycline anti-tumor drug with extensive use. However, DOX is toxic to many healthy organs, especially the heart, which severely limit its application[1]. Recently, researchers have extensively studied and addressed the mechanisms of DOX-induced cardiotoxicity, including DNA damage, excessive reactive oxygen species (ROS) generation, mitochondrial dysfunction, endoplasmic reticulum (ER)-mediated apoptosis, and disturbances to calcium homeostasis[2-6]. It has been reported that doxorubicin induces cardiomyocyte pyroptosis[7], but the mechanisms remain to be further evaluated.

Pyroptosis, a new form of programmed cell death, is characterized by featuring cell swelling, large bubbles blowing from the plasma membrane and cell lysis[8]. Pyroptosis is regulated via caspase-1-dependent or caspase-4/5/11-dependent mechanism[9]. Recent studies identify that the family of pore-forming proteins known as gasdermin mediate pyroptosis and most characterized of which is gasdermin D (GSDMD)[10-13], which is a substrate of both caspase-1 and caspase-4/5/11[8, 9]. Caspase-1 and caspase-4/5/11 cleave GSDMD to form the membrane pore-forming GSDMD-N domain, and then the N-terminal of GSDMD binds to the plasma membrane to form pores, resulting in pyroptosis[14-16]. GSDME is specifically cleaved by caspase-3, generating a GSDME-N fragment that performs membranes for inducing pyroptosis, which highly resembles the effect of the GSDMD N-terminus[17]. Recently, we reported that DOX induces pyroptosis through the Bnip3/caspase-3/GSDME pathway in HL-1 cardiomyocytes[18]. However, the mechanisms underlying DOX-induced cardiomyocyte pyroptosis through caspase-3/GSDME pathway needs to be further investigated.

High-mobility group AT-hook1 (HMGA1) is an architectural transcription factor that participates in several fundamental processes[19]. Studies have shown that HMGA1 protein can not be detected in normal tissues, but it is elevated in human cancer cells[20]. Subsequent evidences have shown that both overexpression and knockdown of HMGA1 can aggravate apoptosis through different signaling pathways[21], which revealed that HMGA1 can be both anti-apoptotic and pro-apoptotic. HMGA1 silencing in BxPC3 and MiaPaCa2 cells increases in gemcitabine-induced apoptosis and caspase-3 activation[22]. Apoptosis-related protein caspase-3 mediates pyroptosis by cleaving GSDME, which suggests that HMGA1 may play a role in pyroptosis. As report goes that DOX interacts with a promoter region of HMGA1 gene in MCF7 cell line[23]. However, the role of HMGA1 in cardiomyocyte pyroptosis induced by DOX is not known yet.

SOX9 is a member of Sex-determining-region-Y (SRY)-related HMG box-containing (SOX) proteins that participates in cell differentiation, chondrogenesis, neural development, apoptosis and so on[24, 25]. Evidences show that in myocardial tissues of myocardial ischemia-reperfusion injury (MI/RI) rats, SOX9 silencing reduces cardiac function damage and suppressed oxidative stress, inflammation, cardiomyocyte apoptosis and myocardial enzymes[26]. Recent study indicates that HMGA1 and SOX9 are correlated in human intestine [27], the effect of this interaction on cardiomyocyte pyroptosis induced by DOX remains unknown.

In this study, we investigate the role of HMGA1 and SOX9 in cardiomyocyte pyroptosis after DOX treatment. Our results reveal a novel mechanism by which HMGA1 attenuates cardiomyocyte pyroptosis induced by doxorubicin via inhibiting SOX9.

Materials And Methods

Cell culture

Atrial-derived HL-1 cardiomyocytes were purchased from the American Type Culture Collection (ATCC) and cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco) containing 10% fetal bovine serum (FBS) and incubated in a humidified atmosphere containing 5% CO₂ at 37°C. The cells were treated with DOX (Selleck, Shanghai, China) at indicated concentrations (5 µM) for 9 h.

LDH release assay

LDH release in cells or serum was assessed using LDH Assay Kit (Beyotime Biotechnology, China) according to the manufacturer's instructions[18].

Cell viability assay

HL1 cells were seeded into 96-well plates at a concentration of 5,000 cells/well. The next day, the cells were treated with DOX for 9 h. Cell viability was detected by the Cell Counting Kit-8 assay (CCK8, Bimake)

according to the manufacturer's instructions. We measured the Optical density (OD) values at 450 nm by an Infinite™ M200 Microplate reader (Tecan, Mannedorf, Switzerland).

Microscope imaging

To observe the morphology of pyroptotic cells, the cells were first incubated into a 6-well plate and then treated with DOX. Still bright-field images were taken with Nikon TE2000 microscope.

Western blot analysis

Protein from HL-1 cells were purified with RIPA Lysis Buffer System. In short, equal amounts (30 µg) of proteins were separated by 12% SDS-polyacrylamide gel electrophoresis and blotted to Immobilon® PVDF Membranes (Merck KGaA, Darmstadt, Germany). Membranes were blocked in 5% non-fat milk for 1.5 h at room temperature and then incubated with the primary antibodies at 4°C overnight. After incubated with HRP-conjugated secondary antibodies (Goat anti-rabbit IgG, Proteintech, China) for 2 h at room temperature, the immune-complexes were visualized using the enhanced chemiluminescence (ECL) substrate (Cwbio, Beijing, China). The intensity of visualized protein bands was captured and analyzed by Image Lab™ software with tubulin as control for normalization (The antibodies manufacturers are shown in Table 1).

Cell transfection

The siRNA duplexes corresponding to HMGA1, SOX9 and negative control siRNA (Si-Ctrl) were purchased from RiboBio (Guangzhou, China). SOX9 expression vector, pcDNA3.1-SOX9 was constructed by cloning full-length wild-type SOX9 coding sequence into pcDNA3.1. HL-1 cardiomyocytes were transfected with siRNA or plasmids for 48 h with Lipofectamine 2000 reagent kit (Invitrogen, Carlsbad, CA). After 48 h, we treat the transfected cells with DOX.

Co-immunoprecipitation

HL-1 cells lysate was purified with ice-cold IP lysis buffer (Thermo Fisher Scientific, USA). The lysate was transferred into a microcentrifuge tube and centrifuged at 2500 rpm for 10 minutes. Then, we transferred the supernatant into a new microcentrifuge tube to determine the protein concentration and perform further analysis. Briefly, the protein A/G PLUS-Agarose (Santacruz Biotechnology, CA, USA), the target antibody and the lysate were mixed together and then incubated at 4°C overnight. Next day, we centrifuged the mixture at 2500 rpm for 10 minutes and washed the precipitated complex with phosphate buffer saline. Repeat this step at least five times. We used HMGA1 antibody as a bait antibody to capture SOX9 protein, and normal rabbit IgG (Cell Signaling Technology, USA) as a negative control. The control was processed in the same way as the Co-IP sample. Lysates from both control and DOX treated cells without immunoprecipitation were used as the positive control (input). After co-immunoprecipitation, the proteins pulled down by HMGA1 antibody were analyzed by Western blot.

Statistical analysis

The data were presented as means $\pm$ SD. Statistical analysis was performed by Graphpad Prism 6. The data were analyzed using one-way analysis of variance (ANOVA) and Student's t-test. A value of $P < 0.05$ was considered to be statistically significant.

Results

HMGA1 is up-regulated in DOX-induced pyroptosis in HL-1 cardiomyocytes.

To determine whether HMGA1 contributes to cardiomyocyte pyroptosis induced by DOX, we first analyzed the expression of HMGA1 in HL-1 cardiomyocytes following DOX treatment. As shown in Fig. 1a, the protein expression of HMGA1, GSDME-N, Cl-caspase 3 showed to be remarkably increased after exposure to DOX. We also found pyroptotic morphology with swollen cells and vesicle-like pyroptotic bodies (Fig. 1b), as well as increased LDH release (Fig. 1c) and decreased cell viability (Fig. 1d). These results indicate that HMGA1 may play a role in DOX-induced cardiomyocyte pyroptosis.

HMGA1 is required for cardiomyocyte pyroptosis induced by DOX.

To investigate the role of HMGA1 in the pyroptosis, small interfering RNA of HMGA1 (Si-HMGA1) was utilized in HL-1 cardiomyocytes. Once transfected, cells were stimulated by DOX for inducing pyroptosis. Western blot revealed that the expression of GSDME-N and Cl-caspase 3 characteristically increased by silencing HMGA1 (Fig. 2a). We also found that knockdown of HMGA1 significantly increased the number of pyroptotic cells (Fig. 2b), enhanced LDH release (Fig. 2c) and decreased cell viability (Fig. 2d) in DOX-treated cells. In a word, these data reveal that HMGA1 plays a role in cardiomyocyte pyroptosis induced by DOX.

HMGA1 interacts with SOX9 in DOX-induced cardiomyocyte pyroptosis.

It has been reported that there is an interaction between HMGA1 and SOX9 in human colorectal cancer[27]. Thus, we then investigated whether HMGA1 interacted with SOX9 following DOX treatment. As shown in Fig. 3a, inhibition of HMGA1 by Si-HMGA1 dramatically enhanced the expression of SOX9 compared with Si-Ctrl group. Moreover, co-immunoprecipitation showed that HMGA1 bound to SOX9 and SOX9 expression was significantly decreased following DOX treatment (Fig. 3b). These results suggest that HMGA1 interacts with SOX9 in cardiomyocytes after DOX treatment.

SOX9 participates in the regulation of cardiomyocyte pyroptosis induced by DOX

We then investigate whether SOX9 engaged in DOX-induced cardiomyocyte pyroptosis. Results showed that overexpression of SOX9 markedly increased DOX-promoted GSDME-N and Cl-caspase 3 in HL-1 cardiomyocytes (Fig. 4a). Our results also showed that overexpression of SOX9 significantly upregulated the number of pyroptotic cells (Fig. 4b), elevated LDH release (Fig. 4c) and decreased cell viability (Fig. 4d). Therefore, these results reveal that SOX9 is of great importance in cardiomyocyte pyroptosis after DOX treatment.

SOX9 involves in DOX/HMGA1-induced cardiomyocyte pyroptosis

As shown in Fig. 3a, HMGA1 affected the expression of SOX9. What puzzles us is if there exists a regulatory relationship between HMGA1 and SOX9. To explore the function of SOX9 in DOX/HMGA1-induced HL-1 cardiomyocyte pyroptosis, small interfering RNA of HMGA1 (Si-HMGA1) and SOX9 (Si-SOX9) were respectively utilized in HL-1 cardiomyocytes to knock down the expression of HMGA1 and SOX9, followed by DOX treatment. Results showed that the expression of GSDME-N and Cl-caspase 3 (Fig. 5a), the number of pyroptotic cells (Fig. 5b), LDH release (Fig. 5c) and cell viability (Fig. 5d) in the Si-HMGA1/SOX9+DOX group have no difference compared to the Si-Ctrl group. What's interesting is that knockdown of HMGA1 increased expression of SOX9, but inhibition of SOX9 could not alter the expression of HMGA1 (Fig. 5a), suggesting that HMGA1 inhibits SOX9. Generally speaking, HMGA1 attenuates pyroptosis by inhibiting the expression of SOX9.

Discussion

There is growing evidence that the use of doxorubicin is greatly limited due to its cardiotoxicity[28], which is an unsolved and troublesome problem. There are various ways of doxorubicin induced cardiomyocyte death, one of which is pyroptosis. The previous study showed that Bnip3 could regulate the focal death of myocardial cells induced by doxorubicin through caspase-3/GSDME pathway[18].

Studies have found that HMG protein family plays an important role in cardiovascular diseases, including myocardial hypertrophy[29], myocardial infarction[30], pulmonary hypertension[31], diabetic cardiomyopathy[19] and so on. For example, HMGB1 triggers the HMGB1/RAGE/cathepsin B signaling pathway to activate canonical pyroptosis in endothelial cells, which suggests that endothelial cell pyroptosis may play a significant role in coronary endothelial damage in Kawasaki disease[32]. Also, as a member of the HMGs, various studies have demonstrated that HMGA1 participates in tumor transformation[33] and apoptosis [34]. But its role in pyroptosis is still unknown yet. In our study, the data displayed that inhibition of HMGA1 exacerbates the cleavage of caspase-3 and DOX-induced pyroptosis while HMGA1 is obviously elevated in cardiomyocytes.

SOX9 is involved in many physiological processes, such as cell differentiation, apoptosis and so on. Interestingly, HMGA1 and SOX9 are positively correlated in human intestine[27]. Which occurs to my mind is that whether HMGA1 interact with SOX9 in HL-1 cardiomyocytes. It's worth noting that our studies reveal that SOX9 is decreased and it promotes DOX-induced cardiomyocyte pyroptosis. Western blot and Co-IP analysis reveal that HMGA1 binds to SOX9 directly or indirectly, which needs to be further addressed in the future studies. As far as we know, our study is the first to reveal that HMGA1 inhibits cardiomyocyte pyroptosis by regulating SOX9 negatively after DOX treatment.

Conclusion

In summary, the findings of the present study demonstrate that HMGA1 regulates SOX9 negatively, inhibits cardiomyocyte pyroptosis induced by DOX, and provides a potential target for therapeutic intervention.

Declarations

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Conflicts of interest

The authors declare that they have no competing interests.

Availability of data and materials

All relevant data are within this published paper.

Code availability

Not applicable.

Authors' contributions

XYL and DY conceived and designed the experiments in the manuscript. XYL performed the experiments. XYL analyzed data, plotted the graphs for figures. XYL wrote the manuscript. DY made manuscript revisions. All authors read and approved the final manuscript.

Ethics approval

Not applicable.

Consent to participate

Not applicable.

Consent for publication

Not applicable.

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Tables

Table 1. Antibodies used for Western blot

Name	Description	Manufacturer
Anti-Tubulin	Rabbit monoclonal, 55 kDa	Proteintech (11224-1-AP)
Anti-GSDME	Rabbit monoclonal, 55; 34 kDa Rabbit monoclonal, 35 kDa	Abcam (ab215191)
Anti-caspase-3		CST (#9662S)
Anti-CI-caspase 3	Rabbit monoclonal, 17 kDa	CST (#9664S)
Anti-HMGA1	Rabbit monoclonal, 17 kDa	Abcam (ab129153)
Anti-SOX9	Rabbit monoclonal, 56 kDa	Abcam (ab185966)

Figures

Figure 1

HMGA1 is up-regulated in DOX-induced pyroptosis in HL-1 cardiomyocytes. HL-1 cells were treated with doxorubicin (5 μ M) for 9 h. a The expression of HMGA1, GSDME-N and CI-caspase 3 were analyzed by Western blot (N = 3). b Representative microscopic images of HL-1 cells. White arrowheads indicate pyroptotic cells. c Relative levels of LDH release (N = 3). d Cell viability (N = 3). All the data are presented as the mean $\pm$ SD. **P< 0.01, ***P< 0.001 compared with indicated groups.

Figure 2

HMGA1 is required for cardiomyocyte pyroptosis induced by DOX. HL-1 cells were pretreated with HMGA1 siRNA (Si-HMGA1) or Si-Ctrl (negative control) for 48 h, followed by DOX (5 μ M) treatment for 9 h. **a** The expression of HMGA1, GSDME-N and Cl-caspase 3 were analyzed by western blot (N = 3). **b** Representative microscopic images of HL-1 cells. White arrowheads indicate pyroptotic cells. **c** Relative levels of LDH release (N = 3). **d** Cell viability (N = 3). All the data are presented as the mean $\pm$ SD. *P < 0.05, **P < 0.01 compared with indicated groups.

Figure 3

HMGA1 interacts with SOX9 in DOX-induced cardiomyocyte pyroptosis. HL-1 cells were pretreated with HMGA1 siRNA (Si-HMGA1) or Si-Ctrl (negative control) for 48 h, followed by DOX (5 μ M) treatment for 9 h. a The expression of HMGA1 and SOX9 were analyzed by western blot (N = 3). b The expression of HMGA1 and SOX9 were examined by Co-immunoprecipitation (N = 3). All the data were shown as mean $\pm$ SD. **P < 0.01 compared with indicated groups.

Figure 4

SOX9 participates in the regulation of cardiomyocyte pyroptosis induced by DOX. HL-1 cells were pretreated with SOX9 plasmid (SOX9) or empty vector for 48 h, followed by DOX (5 μ M) treatment for 9 h. a The expression of SOX9, GSDME-N and Cleaved-caspase-3 were analyzed by Western blot (N = 3). b Representative microscopic images of HL-1 cells. White arrowheads indicate pyroptotic cells. c Relative levels of LDH release (N = 3). d Cell viability (N = 3). All the data are presented as the mean $\pm$ SD. *P < 0.05, **P < 0.01, ***P < 0.001 compared with indicated groups.

Figure 5

SOX9 involves in DOX/HMGA1-induced cardiomyocyte pyroptosis. HL-1 cells were transfected with Si-HMGA1, Si-SOX9 (SOX9 siRNA) and Si-Ctrl for 48 h, followed DOX (5 μ M) treatment for 9 h to examine: a The expression of SOX9, GSDME-N and Cl-caspase-3 were analyzed by Western blot (N = 3). b Representative microscopic images of HL-1 cells. White arrowheads indicate pyroptotic cells. c Relative levels of LDH release (N = 3). d Cell viability (N = 3). All the data are presented as the mean $\pm$ SD. *P < 0.05, **P < 0.01, ***P < 0.001, ns > 0.05 compared with indicated groups.