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Original Article

ATF3/EGR1 regulates myocardial ischemia/reperfusion injury induced autophagy and inflammation in cardiomyocytes



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Article Info

Abstract



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Myocardial ischemia/reperfusion injury (MIRI) is an irreversible adverse event during the management of coronary heart disease that lacks effective controls. The underlying mechanism of MIRI still requires further investigation. Recent studies have suggested that overexpression of ATF3 protects against MIRI by regulating inflammatory responses, ferroptosis, and autophagy. The downstream target of ATF3, EGR1, also showed cardioprotective properties against MIRI by promoting autophagy. Therefore, further investigating the effect of ATF3/EGR1 pathway on MIRI-induced inflammation and autophagy is needed. Cardiomyocyte MIRI model was established by challenging H9C2 cells with hypoxia/reoxygenation (H/R). The ATF3 overexpression-H/R cell model by transfecting ATF3 plasmid into the H9C2 cell line. The transcription levels of ATF3 and EGR1 were determined using RT-qPCR, the levels of TNF- α and IL-6 were determined using ELISA kits, the protein expression of LC3 I, LC3 II, and P62 was determined via WB, and microstructure of H9C2 cell was observed by transmission electron microscopy (TEM). Overexpression of ATF3 significantly downregulated Egr1 levels, indicating that EGR1 might be the target of ATF3. By upregulating ATF3 levels, the extracellular levels of the inflammatory cytokines TNF- α and IL-6 significantly decreased, and the protein expression of the autophagy markers LC3 I, LC3 II, and P62 significantly increased. TEM results revealed that the cell line in the H/R-ATF3 group exhibited a higher abundance of autophagosome enclosures of mitochondria. The results indicated that ATF3/EGR1 may alleviate inflammation and improve autophagy in an H/R-induced MIRI model of cardiomyocytes.

Keywords: Autophagy, ATF3/EGR1, Ischemia/reperfusion injury, Cardiomyocyte, Inflammation.

1. Introduction

At present, the most effective clinical treatment of coronary heart disease is to restore blood flow at the early stage to reduce oxidative damage, apoptosis and cardiomyocyte death to the greatest extent [1]. However, during the clinical management of MI, myocardial ischemia/reperfusion injury (MIRI) commonly occurs. MIRI refers to the irreversible event that arises when oxygenated blood flow is restored in ischemic tissues [2]. The underlying mechanism of MIRI has not been fully clarified. As reported, multiple factors, such as oxidative stress, calcium overload, mitochondrial dysfunction, cell death-related pathways, inflammation, and others, are all related to the pathogenesis of MIRI [3]. Although the efficacy of myocardial ischemia and reperfusion treatment has been continuously improving due to advances in percutaneous coronary intervention techniques and the use of more effective antiplatelet and anticoagulant drugs, there is still a lack of promising and targeted medicine or methods for attenuating MIRI [4]. Hence, to better understand the pathological mechanism of MIRI and to provide potential targets for future MIRI clinical management, investigating the novel mechanism of MIRI is needed.

Accumulative studies suggest that activating transcrip-

tion factor (ATF3), a stress-response factor, plays a critical role in the cardioprotective property against MIRI [5,6]. Liu et al. reported that overexpression of ATF3 protects against cardiac microvascular ischemia/reperfusion injury by inhibiting the TLR4/NF- κ B pathway and alleviating oxidative stress [7]. Recent research indicated that ATF3 overexpression inhibits erastin-induced cardiomyocyte ferroptosis by upregulating FANCD2 promoter activity, which revealed a novel mechanism by which ATF3 mitigates MIRI through ferroptosis suppression [8]. Moreover, it has been reported that the efficacy of ATF3 in regulating autophagy as well as alleviating inflammation responses contributes to its heart protection against pressure-overload heart failure, maladaptive cardiac remodeling, cardiac hypertrophy and other cardiac injuries [9-11], indicating that autophagy adjustment and inflammation amelioration of ATF3 might be a novel mechanism in MIRI treatment as well. Since the principal target of ATF3 is early growth response 1 (EGR1) in cardiomyocytes [12-15], which promotes autophagy and suppresses apoptosis to protect against MIRI in cardiomyocytes [16], we hypothesized that the effect of ATF3 against MIRI might be realized through interacting with EGR1 to regulate inflammation and autophagy.

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In the present study, we investigated the specific mechanism of ATF3/EGR1 in regulating MIRI-induced autophagy and inflammation in the H/R model of a myocardial cell line. We found that overexpression of ATF3 in cardiomyocytes pronouncedly downregulated the levels of EGR1 and inflammatory cytokines and promoted autophagy after H/R challenge. These findings suggest that the ATF3/EGR1 signaling pathway plays a crucial role in MIRI amelioration via autophagy and inflammation regulation and that interfering with the ATF3/EGR1 pathway might be a potential MIRI treatment in clinical practice.

2. Materials and Methods

2.1. Reagents

Fetal calf serum (10270-106) (GIBCO, Invitrogen Corporation, NY, USA); penicillin–streptomycin (V900929) (Sigma, St. Louis, MO, USA); DMEM culture medium (GIBCO, NY, USA); trypsin (KGY0012) (KeyGEN, Nanjin, China); StarSignal Western Protein Marker(10-200 kDa) (M227-01) (GenStar, Beijing, China); Tween 20, acrylamide, sodium dodecyl sulfate, PMSF (Solon, OH, USA); RIPA tissue lysate, 1.5 mol/L Tris HCl (pH 6.8), 1.5 mol/L Tris HCl (pH 8.8) (Beyotime, Shanghai, China); electrochemiluminescence solution (Thermo Fisher Scientific, Pittsburgh, PA, USA); Anti-SQSTM1/p62 antibody (ab109012), Anti-LC3A/B antibody (ab128025) (Abcam, Cambridge, UK); TNF-alpha ELISA kit (E-EL-R2856c); IL-6 ELISA kit (E-EL-R0015c) (Elabscience, Wuhan, China); TRIzol (9108) (TaKaRa, Tokyo, Japan); iScript cDNA synthesis kit (1708891) (Bio-Rad, Hercules, CA, USA); TB Green Premix Ex Taq™ (RR420) (TaKaRa, Tokyo, Japan); DEPC-treated Water (R0603) (Sangon Biotech, Shanghai, China).

2.2. Cell culture, transfection, and H/R model establishment

The H9C2 cell line (rat myocardial cells) was purchased from the American Tissue Culture Collection (ATCC, Rockville, MD, USA). Cells were cultured with DMEM supplemented with penicillin (100 U/mL), streptomycin (100 µg/mL), and 10% FBS. The H9C2 cell line was divided into the following 4 groups: NC group, H/R group, H/R+ATF3-NC group, and H/R+ATF3 group. The H9C2 cell line at the logarithmic growth stage was digested and counted, and the cell suspension was inoculated into 6-well plates (1×10^6 cells/well) according to the groups.

H/R treatment was performed according to the groups after transfection for 24 h. The H9C2 cell line in the H/R+ATF3 group was transfected with the ATF3 overexpression plasmid, while the H/R+ATF3-NC group was transfected with vector as a control using Lipofectamine 3000 according to the manufacturer's instructions. Twenty-four hours after transfection, apart from the NC group, the H9C2 cell line was incubated in a 37°C incubator with 95% N₂ and 5% CO₂ for 3 hours. Then, the culture medium was replaced, and the cells were incubated with 95% air and 5% CO₂ (37°C) for 3 hours and 21 hours to establish the H/R cellular model.

2.3. Enzyme-linked immunosorbent assay (ELISA)

The treated cell culture medium of each group was absorbed and centrifuged at $1000 \times g$ for 20 min, and the supernatant was taken for detection. The extracellular le-

vels of TNF- α and IL-6 were detected using ELISA kits according to the manufacturer's protocol.

2.4. Western blot assay

Pancreatic enzyme-collecting cells were subjected to centrifugation, followed by discarding the supernatant. The cell samples were then washed twice with prechilled PBS. Subsequently, 1 mL of RIPA with added PMSF was added to each 100 µL compressed volume of cell sample to achieve complete lysis. The samples were then centrifuged at 4°C and $12000 \times g$ for 5 minutes to extract supernatant that contained the total proteins in the samples. Quantification of the total proteins was conducted using BCA kits. Finally, the samples were incorporated with $5 \times$ loading buffer and heated in a boiling water bath for 10 min. The prepared protein samples were then separated in a 10% SDS-PAGE system and transferred to a PVDF membrane (0.45 µm) (Millipore, Schwalbach, Germany). The membrane was incubated with primary antibodies against LC3 I, LC3 II, and P62 at 4 °C overnight and then incubated with the corresponding HRP-labeled secondary antibody. The expression levels of proteins were detected with a JP-K6000 chemiluminescence imager and analyzed with ImageJ software.

2.5. Quantitative Real-time PCR (RT-qPCR)

Total RNA was extracted using TRIzol reagent and purified following the trichloromethane-isopropanol-ethanol process. The total RNA concentration was detected using a Nanodrop 2000, and RNA samples were reversed transcribed using an iScript cDNA synthesis kit after dilution. Quantitative analysis was performed using TB Green Premix Ex Taq™ according to the manufacturer's protocol and analyzed by the $2^{-\Delta\Delta Ct}$ method.

2.6. Transmission Electron Microscopy (TEM)

Cells were fixed with 2.5% glutaraldehyde fixative, stained after sectioning, and observed and photographed using TEM.

2.7. Statistical analysis

All data are shown as the mean \pm SD. GraphPad Prism 9 (Version 9.5.0, La Jolla, CA, USA) software was used to analyze the statistical significance between groups by t-test. Data were considered statistically significant when $P < 0.05$.

3. Results

3.1. Overexpression of ATF3 significantly inhibited *Egr1* transcription in the H/R model of cardiomyocytes

First, we determined the transcription of ATF3 in each group of cardiomyocytes using RT-qPCR. Compared with the control, the mRNA level of ATF3 was significantly downregulated in the H/R model group, as well as in the ATF3 negative control group transfected with the ATF3 vector plasmid (Figure 1A, $P < 0.0001$). After transfection with the ATF3 overexpression plasmid, the mRNA level of ATF3 was significantly upregulated (Figure 1A, $P < 0.0001$), indicating that the H9C2 cell line was successfully transfected.

To investigate whether ATF3 interacts with EGR1 in the H/R model of cardiomyocytes, we determined the transcription level of *Egr1* after overexpressing ATF3. The *Egr1* level in the H/R model or ATF3-NC cells was

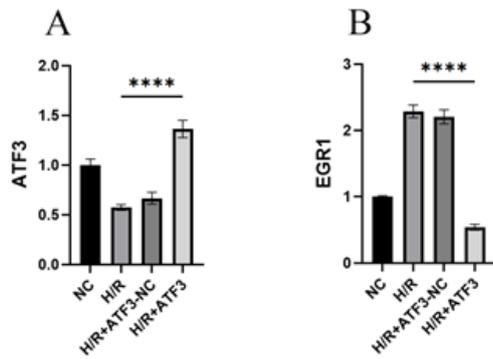


Fig. 1. Overexpression of ATF3 significantly downregulated *Egr1* levels in the H/R model of cardiomyocytes. (A) H/R challenge significantly downregulated ATF3 mRNA levels, while ATF3 overexpression significantly upregulated ATF3 mRNA levels. (B) ATF3 overexpression downregulates *Egr1* levels after H/R challenge. All data are shown as the mean \pm SD. NC: control; H/R: model; H/R+ATF3-NC: H/R model cells transfected with ATF3-NC plasmid; H/R+ATF3: H/R model cells transfected with ATF3 overexpression plasmid. (** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$)

markedly upregulated compared with that in the control (Figure 1B, $P < 0.0001$), while the *Egr1* level was significantly downregulated in ATF3-overexpressing cells (Figure 1B, $P < 0.0001$).

3.2. Overexpression of ATF3 significantly ameliorated H/R-induced cardiomyocyte inflammation

To evaluate the inflammatory responses in the H9C2 cell line at 6 hours and 24 hours after H/R treatment, we determined the levels of TNF- α and IL-6 via ELISA kits. As shown in Figure 2, compared with the NC group, H/R challenge significantly increased the expression of inflammatory cytokines, including TNF- α and IL-6, at 6 and 24 hours post H/R modeling ($P < 0.0001$). Meanwhile, TNF- α and IL-6 levels between the H/R and H/R+ATF3-NC groups were not significantly different ($P > 0.05$). Compared with the model and ATF3-NC groups, ATF3 overexpression significantly downregulated the levels of TNF- α and IL-6 at 6 and 24 hours after H/R modeling ($P < 0.0001$). Therefore, ATF3 attenuates H/R-induced inflammation in cardiomyocytes.

3.3. Overexpression of ATF3 significantly upregulated autophagy in the H/R model of cardiomyocytes

To investigate whether ATF3 regulates autophagy in the H/R model of cardiomyocytes, we determined the expression levels of the autophagy marker proteins LC3 I, LC3 II, and P62 at 6- and 24-hours post-H/R modeling. Compared with the control, the protein expression of LC3 I and LC3 II in the model group significantly increased at both time points measured (Figure 3A-B, $P < 0.01$). The expression of P62 in the model group was also markedly upregulated at 6 hours (Figure 3C, $P < 0.001$) and 24 hours (Figure 3C, $P < 0.01$) after modeling. The autophagy level in the ATF3-negative control group showed no significant difference from that in the control. After ATF3 expression was upregulated in the H/R-induced H9C2 cell line, the protein expression of LC3 I, LC3 II, and P62 was pronouncedly upregulated at both time points (Figure 3A-D, $P < 0.01$). The TEM results revealed that the cell line in the H/R-ATF3 group exhibited a higher abundance of au-

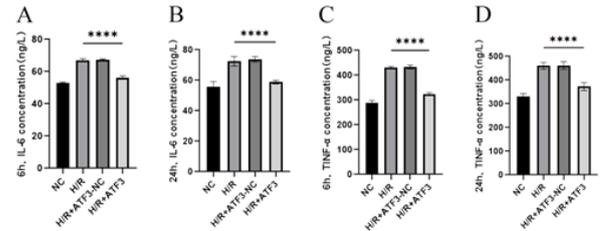


Fig. 2. Overexpression of ATF3 significantly inhibited H/R-induced inflammation in cardiomyocytes. (A-B) ATF3 overexpression downregulated TNF- α levels at 6 and 24 hours after H/R challenge. (C-D) ATF3 overexpression downregulated IL-6 levels at 6 and 24 hours after H/R challenge. All data are shown as the mean \pm SD. NC: control; H/R: model; H/R+ATF3-NC: H/R model cells transfected with ATF3-NC plasmid; H/R+ATF3: H/R model cells transfected with ATF3 overexpression plasmid. (** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$)

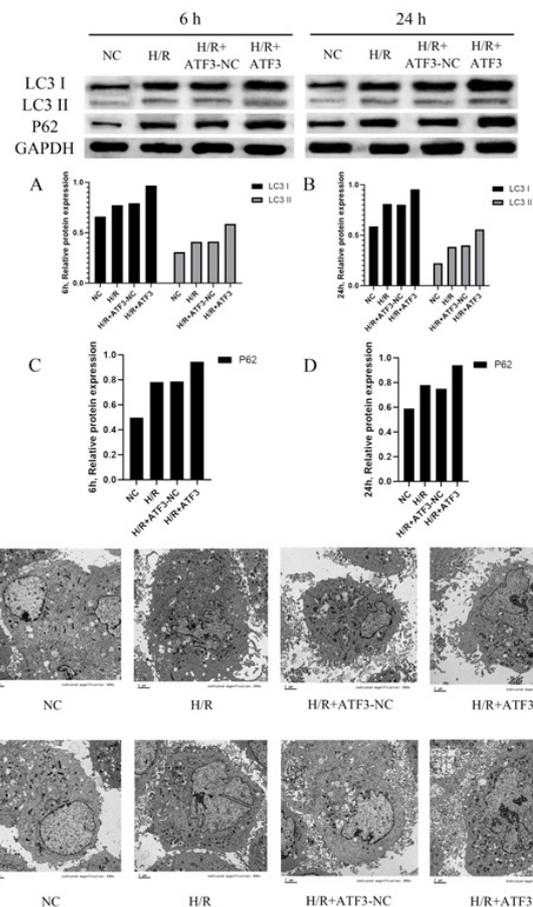


Fig. 3. Overexpression of ATF3 significantly upregulated autophagy in the H/R model of cardiomyocytes. (A-B) ATF3 overexpression increased LC3 I and LC3 II levels at 6 and 24 hours after H/R challenge. (C-D) Overexpressing ATF3 increased P62 levels at 6 and 24 hours after H/R challenge. (E-F) Improved cellular autophagy was observed via TEM in ATF3-overexpressing H9C2 cells at 6 and 24 hours after H/R challenge. All data are shown as the mean \pm SD. NC: control; H/R: model; H/R+ATF3-NC: H/R model cells transfected with ATF3-NC plasmid; H/R+ATF3: H/R model cells transfected with ATF3 overexpression plasmid. (** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$)

tophosome enclosures of mitochondria at 6- (Figure 3E) and 24-hours (Figure 3F) after H/R challenge, indicating enhanced autophagy by overexpressing ATF3.

4. Discussion

As the probability of risk factors such as hypertension, smoking, hyperlipidemia, and diabetes increases, the incidence of cardiovascular diseases (CVD) has been rising year by year, accounting for 45.5% and 43.16% of the main mortality rate in rural and urban populations, respectively [17]. Among CVDs, coronary heart disease-induced myocardial infarction (MI) is one of the major concerns that contribute to sudden cardiac death [18]. Primary percutaneous coronary intervention therapy is a highly effective method for alleviating MI symptoms, which can rapidly improve cardiac ischemia and minimize injuries to myocardial tissues [19]. However, restoring blood flow in ischemic sections may lead to secondary injuries to myocardial tissues, such as MIRI [20]. Despite developments in MIRI clinical management, there is still a lack of promising and targeted ways to prevent or mitigate MIRI. Thus, exploring novel targets in the pathogenesis of MIRI is conducive to improving clinical MIRI management. In this study, we aimed to investigate the effect of ATF3/EGR1 on autophagy and inflammation regulation against MIRI in the H/R model of H9C2 cardiomyocytes.

First, we constructed an ATF3 plasmid and transfected it into the H9C2 model cell line to establish ATF3-overexpressing cardiomyocytes. We determined the expression of *Atf3* using RT-qPCR to examine the transfection efficacy. The results showed that *Atf3* levels were significantly suppressed after H/R modeling, while plasmid transfection significantly upregulated *Atf3* levels, suggesting the successful transfection of the ATF3 plasmid. Importantly, we observed a significant downregulation of *Egr1* transcription in ATF3 overexpression-H/R model cells, which indicated that the increased level of ATF3 inhibited the expression of EGR1. EGR1, also known as NGFI-A, zif268, krox-24, and TIS85, is a transcription factor that is poorly expressed under normal conditions but is activated under vascular injury [21]. The latest study reports that EGR1 is evaluated following H/R treatment, consistent with our results and that impairing EGR1 expression promotes autophagy and suppresses apoptosis to protect against MIRI in cardiomyocytes [16, 22]. Fan et al. found that knockdown of EGR1 suppressed the expression of inflammatory cytokines and the NF- κ B pathway in an acute myocardial infarction model in mice [23]. The studies above indicate that EGR1 might be a negative regulator of vascular inflammatory responses and a positive mediator of autophagy to exert cardioprotective effects against MIRI. Our results indicated that EGR1 is the downstream target of ATF3; therefore, we aimed to further investigate the effect of ATF3/EGR1 on inflammatory responses and autophagy in the MIRI cardiomyocyte model.

By using ELISA kits, we determined the extracellular levels of inflammatory cytokines, including TNF- α and IL-6, in the ATF3 overexpression-MIRI model of the H9C2 cell line. We found that ATF3 overexpression significantly reduced the levels of TNF- α and IL-6, indicating that ATF3 could inhibit H/R-induced inflammatory responses. By using WB assays, we determined the protein expression of key autophagy proteins. We found that LC3 I, LC3II, and P62 expression was upregulated after ATF3 overexpression, indicating that ATF3 could promote autophagy in an H/R model of cardiomyocytes. It is suggested that the expression of LC3 II and P62 is not affected in the ischemia phase of MIRI, but in the reperfusion stage, the

expression of LC3 II and P62 is increased [24]. Eventually, an increase in the LC3-II/LC3-I ratio and an increase in the levels of LC3-II and P62 are observed, indicating autophagy activation [25, 26]. Our results showed increased levels of these key markers in autophagy with increasing ATF3 levels, indicating that ATF3 overexpression might promote autophagy to protect against H/R injuries in the H9C2 cell line.

Overall, our study revealed the potential effect of ATF3 in modulating EGR1 expression as well as cellular inflammation and autophagy in the H/R-induced MIRI model of H9C2 cardiomyocytes.

5. Conclusion

ATF3 overexpression significantly upregulated *Egr1* transcription, improved autophagy, and alleviated the inflammatory response in an H/R-induced MIRI model of cardiomyocytes.

Conflict of interests

The author has no conflicts with any step of the article preparation.

Consent for publications

The author read and approved the final manuscript for publication.

Ethics approval and consent to participate

No human or animals were used in the present research.

Informed consent

The authors declare not used any patients in this research.

Availability of data and material

The data that support the findings of this study are available from the corresponding author upon reasonable request

Authors' contributions

Li Li and Gang Fu designed the study and performed the experiments, Gang Fu and Caiyun Liu collected the data, Caiyun Liu and Yanling Liu analyzed the data, and Li Li prepared the manuscript. All authors read and approved the final manuscript.

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