Original Paper

Atorvastatin Protects Myocardium Against Ischemia-Reperfusion Injury Through Inhibiting miR-199a-5p

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Key Words

Atorvastatin • Ischemia-reperfusion injury • Glycogen synthase kinase-3 β (GSK-3 β) • microRNA • miR-199a-5p

Abstract

Objective: This study aimed to evaluate the protective effects of atorvastatin against myocardial ischemia/reperfusion (I/R) injury in cardiomyocytes and its possible underlying mechanism.

Method: Direct cytotoxic effect of OGD/R on cardiomyocytes with and without atorvastatin pretreatment was evaluated. Effects of atorvastatin on expression of GSK-3 β and miR-199a-5p were determined using RT-PCR and Western blot. In addition, GSK-3 β expression with miR-199a-5p upregulation and downregulation was detected using RT-PCR, Western blot, and immunohistochemistry. **Results:** Pretreatment with atorvastatin significantly improved the recovery of cells viability from OGD/R ($p < 0.05$). In addition, the atorvastatin pretreatment significantly increased GSK-3 β expression both in mRNA level and protein level and decreased miR-199a-5p expression in mRNA level ($p < 0.05$). Upregulation and downregulation of miR-199a-5p respectively decreased and increased GSK-3 β expression both in mRNA level and protein level. **Conclusion:** These results suggested that atorvastatin provides the cardioprotective effects against I/R injury via increasing GSK-3 β through inhibition of miR-199a-5p.

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Introduction

Percutaneous coronary intervention (PCI) is a critical part of the intervention of acute myocardial infarction (AMI) patients [1]. However, myocardial tissue suffers from myocardial ischemia/reperfusion (I/R) injury during revascularization, which results in poor clinical outcomes [2]. Apoptosis of cardiomyocytes is known to be an important mechanism of

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I/R injury [3-5]. Accumulating evidence has shown that apoptosis of cardiomyocytes is suppressed by glycogen synthase kinase-3 β (GSK-3 β) [6-8]. Therefore manipulation of GSK-3 β is a promising strategy for myocardial protection in I/R injury. The GSK-3 family of serine/threonine kinases plays an important role in various pathologic processes of heart, including pressure overload and ischemic injury. It is comprised of two isoforms (α and β) which are encoded by distinct genes and are ubiquitously expressed. Numerous studies have shown that GSK-3 β , but not GSK-3 α is cardioprotective [9-10].

MicroRNAs regulate gene expression at the posttranscriptional level by degrading or blocking translation of target messenger RNA (mRNA) [11, 12]. Various studies have shown that increased miR-199a-5p expression is responsible for pathophysiological alterations contributing to and promoting the development of heart diseases including atrial fibrillation and heart failure [13, 14]. Therefore, therapeutic interventions targeting miR-199a-5p represent promising strategies for the treatment of I/R injury. Statins were found to prevent heart failure, acute coronary syndrome (ACS), and cardiovascular risk [15-17]. Numerous studies have demonstrated the cardioprotection against myocardial I/R injury of statins in animal experiments and clinical studies [18, 19]. However, the major molecular mechanisms of the cardioprotective effect of statins have not been completely elucidated. Therefore, we hypothesized that cardioprotection by statins may be associated with inhibition of apoptosis in myocardial I/R injury through miR-199a-5p /GSK-3 β pathway.

This work examined the effects of atorvastatin on H9c2 cardiomyoblast cells and neonatal rat cardiac ventricular cardiomyocytes by detecting changes in the mRNA and protein expression levels of GSK-3 β . Moreover, we firstly observed the influence of atorvastatin on miR-199a-5p expression and its influence on GSK-3 β expression. We further analyzed the mechanism involved in the effects of atorvastatin on myocardial I/R injury to provide a theoretical basis and new intervention targets for the prevention and treatment of myocardial I/R injury in the clinic.

Materials and Methods

Drugs and reagents

Atorvastatin and LiCl were purchased from Sigma-Aldrich Corp. Antibodies for GSK-3 β , Caspase 9 and Bcl-2 were provided by BD Biosciences. Antibody for Cytochrome C was from Abcam. Antibodies for α -actin, β -actin and GAPDH were provided by Sigma. LY294002 was purchased from Cayman Chemical. Syn-rno-miR-199a-5p miScript miRNA Mimic, anti-rno-miR-199a-5p miScript miRNA Inhibitor, AllStars Negative Control siRNA, AllStars Mm/Rn Cell Death Control siRNA, and HiPerFect Transfection Reagent was purchased from QIAGEN. 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium (MTS) was purchased from Promega.

Cell culture and treatment

Neonatal rat cardiac ventricular cardiomyocytes were prepared from 1 to 3 day-old neonatal SD rat and cultured as previously described [20, 21]. The cells were maintained in a humidified incubator (37°C with 5% CO₂). Brdu (0.1mM) was added to the medium for the first 2 days to inhibit fibroblast growth. Neonatal rat myocytes were cultured to 70% confluence and then serum starved in basal medium (DMEM) for 24h before the drug treatment. H9c2 rat myocardial cells (ATCC CRL 1446) were cultured in DMEM supplemented with 10% FBS (Hyclone), penicillin G (100 U·mL⁻¹), streptomycin (100 μ g·mL⁻¹) and glutamine (2 mM). The neonatal rat cardiac ventricular cardiomyocytes and H9c2 cells were treated with oxygen-glucose deprivation/recovery (OGD/R) with or without pretreatment with as described previously with minor modification [22]. The pretreatment included incubation with 1 μ M atorvastatin for 3 hours or 10 μ M GSK-3 β inhibitor LiCl for 1 hour. Then cells were rinsed twice, incubated in glucose-free DMEM and subsequently placed in an anaerobic chamber containing a mixture of 95% N₂ and 5% CO₂ at 37°C for 6 h. Following OGD, glucose was added to normal levels (final concentration: 4.5 mg·mL⁻¹), and cells were incubated under normal growth conditions (95% air and 5% CO₂) for an additional 18 h, as OGD/R, unless otherwise specified.

Neonatal rat cardiac ventricular cardiomyocytes identification

After culture for different time periods, the samples were rinsed with PBS, fixed with 4% paraformaldehyde for 20 min, and then permeabilized with glycine/triton solution (PBS/0.1% Triton/10 mM glycine). The samples were incubated with mouse monoclonal anti- α -actin (1:300 dilution) (cat# A7811; Sigma) at 4°C overnight. After rinses, the samples were then incubated with anti-mouse IgG-FITC (1:300 dilution) (cat# F2012; Sigma) for 60 min at 37°C. Nuclei were counter-stained with 100 nM DAPI (Invitrogen) for 30 min followed by rinses with PBS. Fluorescent images were taken with a spectral confocal microscope imaging system (Leica TCS SP2).

Cell viability assays

Cell viability was assessed by the CellTiter 96 Aqueous One Cell Proliferation assays (promega). Cells were seeded at 5×10^3 per well in 96-well plates overnight. After the treatment cells were incubated with 20 μ l /100 μ l MTS for 1 h. Cell viability was determined by measuring the absorbance at 490 nm using a microplate reader (M200PRO, TECAN, Austria).

Measurement of LDH activity

A supernatant was obtained from the culture medium by centrifugation for 1 min (1000 r/min). The activity of LDH in the supernatant was detected using a commercial kit (Jiancheng, China).

Analysis of GSK-3 β mRNA expression

Total RNA was extracted with TriZol Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The cDNA was synthesized from total RNA (0.5 μ g) using the Reverse Transcription System (Promega) according to the manufacturer's instructions. Subsequently, the cDNA was subject to real-time polymerase chain reaction (PCR) using GoTaq qPCR Master Mix (Promega). Reactions were performed on a 7500 Real-Time PCR System (Applied Biosystems). The primers used for real-time PCR were as follows: GSK-3 β forward 5'-TCCATTCCTTTGGGATCTGCC -3' and reverse 5'-ATCAGCTCTGGTGCCCTGTAGTAC -3'; β -actin forward 5'-CAGGGTGTGATGGTGGG -3' and reverse 5'-GGAAGAGGATGCGGCAG-3'. The fold change in expression of each gene was calculated using the $\Delta\Delta$ Ct method [23].

Analysis of miR-199a-5p expression

Total RNA was extracted with TriZol Reagent (Invitrogen) according to the manufacturer's instructions. qRT-PCR was performed using All-in-One miRNA qRT-PCR Detection Kit (GeneCopoeia). Reactions were performed on a 7500 Real-Time PCR System (Applied Biosystems). The primers used for real-time PCR were as follows: rno-miR-199a-5p and U6 (GeneCopoeia). The fold change in expression each gene was calculated using $\Delta\Delta$ Ct method.

Transfection of H9c2

H9c2 cells were seeded in 6-well plates for 12 hours. Before transfection, the medium was replaced with DMEM without serum and antibiotics. The 10nM, 20nM, and 50nM AllStars Mm/Rn Cell Death Control siRNA (QIAGEN) were used to incubate for 24 hours, 48 hours, and 72 hours to select the best condition. Transfection efficiency was observed and evaluated under the microscope. Ultimately transfection with 20nM and was selected due to the highest efficiency which was over 70%. HiPerFect Transfection Reagent (QIAGEN) was used as transfection reagents. 20 nM Syn-rno-miR-199a-5p miScript miRNA Mimic and anti-rno-miR-199a-5p miScript miRNA Inhibitor were used to respectively overexpress and inhibit miR-199a-5p expression. AllStars Negative Control siRNA was used as negative control.

Western blot analysis

The proteins were separated by 10% SDS-PAGE, then transferred onto PVDF membranes and blotted with specific antibodies against GSK-3 β (1:2000), caspase 9 (1:2000), BCL-2 (1:2000), cytochrome C (1:5000), GAPDH (1:1000), and β -actin (1:1000) at 4°C overnight, and then incubated with the horseradish peroxidase-conjugated secondary antibody (1:5000) at 37°C for 1 h. They were then processed for chemiluminescent detection according to the manufacturer's instructions (Santa Cruz Biotechnology). All bands were evaluated by densitometry with Quantity One V4.6.2 software (Bio-Rad, Hercules, CA, USA). Bands of interest were normalized against GAPDH and β -actin. Data are presented as relative density ratios.

Immunofluorescence assay

The cells were fixed with 4% paraformaldehyde for 20 min, and then permeated with glycine/triton solution (PBS/0.1% Triton/10 mM glycine). The samples were incubated with mouse monoclonal anti-GSK-3 β (1:100 dilution) (BD Biosciences) at 4°C overnight. After rinses, the samples were then incubated with anti-mouse IgG-FITC (1:300 dilution) (BD Biosciences) for 40 min at 37°C. Nuclei were counter-stained with 100 nM DAPI (Invitrogen) for 30 min followed by rinses with PBS. Fluorescent images were taken with a spectral confocal microscope imaging system (Leica TCS SP2).

Statistical analysis

Statistical analysis was performed using SPSS 13.0. The data were expressed as the mean $\pm$ standard deviation (SD). Differences were analyzed by one-way analysis of variance (ANOVA) followed by the Newman-Keuls test; values of $P < 0.05$ were considered statistically significant. The 2- $\Delta\Delta C_t$ method was used for the RT-PCR analysis.

Results

Atorvastatin protects H9c2 myocardial cells from OGD/R toxicity by increasing cell viability and decreasing LDH release and expression of Caspase 9, Bcl-2, and Cyto C

To evaluate whether atorvastatin protects H9c2 myocardial cells from OGD/R, we examined the direct cytotoxic effect of OGD/R on H9c2 cells with and without atorvastatin pretreatment. The data shown in Fig. 1A indicates that atorvastatin protects against OGD/R. The cell viability was significantly decreased after OGD/R compared to the control group ($p < 0.05$). As shown in Fig. 1A, B, and C, pretreatment with atorvastatin increased cell viability and reduced OGD/R-induced LDH release and cell damage-related proteins including Caspase 9, Bcl-2, and Cyto C.

Atorvastatin increases GSK-3 β and miR-199a-5p expression of H9c2 myocardial cells

We further examined the effect of OGD/R on GSK-3 β and miR-199a-5p expressions of H9c2 cells with and without atorvastatin pretreatment. As shown in Fig. 2A and C, the GSK-3 β mRNA and protein expression levels were significantly decreased after OGD/R compared to the control group and normoxia group ($p < 0.05$). Pretreatment with atorvastatin increased both mRNA and protein expression levels of GSK-3 β . As shown in Fig. 2B, miR-199a-5p

Fig. 1. Effects of atorvastatin on H9c2 myocardial cell viability, LDH release, and expression of Caspase 9, Bcl-2, and Cyto C. A. Graph showing cell viability in the three groups. B. Graph showing LDH release in the three groups. C. The level of Caspase 9, Bcl-2, and Cyto C proteins in the three groups. * Compared with the control group, $p < 0.05$; # Compared with the OGD/R group, $p < 0.05$.

Fig. 2. Effects of atorvastatin on expression of GSK-3β and miR-199a-5p of H9c2 myocardial cells. A. Graph showing GSK-3β mRNA levels in the three groups. B. Graph showing miR-199a-5p mRNA levels in the three groups. C. The level of GSK-3β proteins in the three groups. * Compared with the control group, $p < 0.05$; # Compared with the OGD/R group, $p < 0.05$.

Fig. 3. The protective effects of atorvastatin on H9c2 myocardial cells through GSK-3β. A. Graph showing cell viability in the five groups. B. Graph showing LDH release in the five groups. C. The level of Caspase 9, Bcl-2, and Cyto C proteins in the five groups. * Compared with the control group, $p < 0.05$; # Compared with the OGD/R group, $p < 0.05$.

mRNA level was significantly increased after OGD/R ($p < 0.05$), whereas pretreatment with atorvastatin significantly reserved it ($p < 0.05$).

Atorvastatin exerts its protective effects against OGD/R through increasing GSK-3β expression

To analyze the exact mechanism of atorvastatin protecting H9c2 cardiomyoblast cells from OGD/R, we examined the effect of GSK-3β inhibitor LiCl on H9c2 cells with and without atorvastatin pretreatment. As shown in Fig. 3A, the cell viability was significantly decreased

Fig. 4. Effects of miR-199a-5p on GSK-3 β expression. A. Graph showing GSK-3 β mRNA level in the four groups. B. Graph showing miR-199a-5p mRNA level after transfection. C. The level of GSK-3 β proteins in the three groups. ** Compared with the control group, $p < 0.01$.

Fig. 5. Effects of miR-199a-5p inhibitor on H9c2 myocardial cells viability, LDH release, and expression of Caspase 9, Bcl-2, and Cyto C. A. Graph showing cell viability in the four groups. B. Graph showing LDH release in the four groups. C. The level of Caspase 9, Bcl-2, Cyto C, and GSK-3 β proteins in the four groups. D. Immunohistochemical images showing GSK-3 β expression in the four groups. * Compared with the control group, $p < 0.05$. # Compared with the OGD/R group, $p < 0.05$.

in LiCl + atorvastatin + OGD/R group compared to the control group ($p < 0.05$). As shown in Fig. 3B and C, pretreatment with LiCl significantly increased atorvastatin-inhibited LDH release and cell damage-related proteins including Caspase 9, Bcl-2, and Cyto C compared to the control group ($p < 0.05$).

Fig. 6. Effects of miR-199a-5p inhibitor on H9c2 myocardial cells viability, LDH release, and expression of Caspase 9, Bcl-2, and Cyto C. A. Graph showing cell viability in the four groups. B. Graph showing LDH release in the four groups. C. The level of Caspase 9, Bcl-2, Cyto C, and GSK-3β proteins in the four groups. D. Immunohistochemical images showing GSK-3β expression in the four groups. * Compared with the control group, $p < 0.05$. # Compared with the OGD/R group, $p < 0.05$.

Upregulation and downregulation of miR-199a-5p respectively decreased and increased GSK-3β expression

To evaluate the effect of miR-199a-5p on GSK-3β expression, we examined the effects of miR-199a-5p mimic and inhibitor transfections on GSK-3β expression. As shown in Fig. 4A and C, the miR-199a-5p mimic transfection significantly decreased GSK-3β expression both in mRNA and protein levels, while the miR-199a-5p inhibitor transfection increased them ($p < 0.01$).

Downregulation of miR-199a-5p protects H9c2 myocardial cells from OGD/R by increasing cells viability and decreasing LDH release and expression of Caspase 9, Bcl-2, and Cyto C

To evaluate whether miR-199a-5p inhibitor protects H9c2 cardiomyoblast cells from OGD/R, we examined the direct cytotoxic effect of OGD/R on H9c2 cells with and without miR-199a-5p inhibitor pretreatment. As shown in Fig. 5A, B, and C, pretreatment with miR-199a-5p inhibitor increased cell viability and reduced OGD/R-induced LDH release and cell damage-related proteins including Caspase 9, Bcl-2, and Cyto C.

Atorvastatin protects neonatal rat cardiac ventricular cardiomyocytes from OGD/R by increasing cell viability and decreasing LDH release and expression of Caspase 9, Bcl-2, and Cyto C via increasing GSK-3β

To evaluate whether atorvastatin protects neonatal rat cardiac ventricular cardiomyocytes from OGD/R as it does for H9c2 myocardial cells, we first verified neonatal

rat cardiac ventricular cardiomyocytes (Fig. 6A). Then we examined the direct cytotoxic effect of OGD/R on neonatal rat cardiac ventricular cardiomyocytes with and without atorvastatin pretreatment. The data shown in Fig. 6B indicates that atorvastatin protects against OGD/R. The cell viability was significantly decreased after OGD/R compared to the control group ($p < 0.05$). As shown in Fig. 6B and C, pretreatment with atorvastatin increased cell viability and reduced OGD/R-induced cell damage-related proteins including Caspase 9, Bcl-2, and Cyto C. As shown in H9c2 myocardial cells, the protective effects of atorvastatin were through miR-199a-5p /GSK-3 β pathway as well (Fig. 6D, E, F, and G).

Discussion

Apoptosis is a vital pathophysiological mechanism associated with myocardial I/R injury. Prevention and treatment of myocardial apoptosis are an important area of research in myocardial infarction. It was previously reported that atorvastatin could protect vascular smooth muscle cells from TGF- β 1-stimulated calcification by inducing autophagy via suppression of the β -catenin pathway [24]. We found that atorvastatin protected cardiomyocytes from apoptosis caused by OGD/R. In our study, cardiomyocytes showed high level of apoptosis after OGD/R which was significantly attenuated by atorvastatin pretreatment, suggesting that attenuation of apoptosis may be the mechanism that enables atorvastatin to protect against myocardial I/R injury.

Furthermore, this study suggests that GSK-3 β may be involved in the cardioprotective mechanism of atorvastatin on myocardial I/R injury. GSK-3 β , a serine/threonine kinase, participates in various cell activities through phosphorylation of the substrate protein [25]. GSK-3 β has received increasing attention because of its involvement in some serious diseases including neurological disease, cancer, and I/R injury. In the cardiovascular system, GSK-3 β has major roles in glucose metabolism, cardiomyocyte hypertrophy [26], and cell death [27]. Our data shows that the mRNA and protein levels of GSK-3 β were decreased by OGD/R, whereas atorvastatin pretreatment reversed these effects. This finding is consistent with previous reports demonstrating downregulation of GSK-3 β in response to I/R injury.

MicroRNAs (miRNAs) are small (~22 nucleotides) non-coding RNAs that regulate gene expression and play an important role in numerous cardiovascular diseases [28]. They function as endogenous intracellular regulators of mRNA translation. Although the significance of miR-199a-5p in myocardial I/R injury has not been studied, it is recognized as a hypoxia-sensitive miRNA [29]. Therefore we speculate that the anti-apoptotic mechanism of atorvastatin may be responsible for the downregulation of miR-199a-5p. We found that GSK-3 β was decreased in the miR-199a-5p mimic transfection group, whereas it was increased in the miR-199a-5p inhibitor transfection group. Therefore, we believe that downregulation of miR-199a-5p contributes to the cardioprotective effect of atorvastatin against myocardial I/R injury.

To our knowledge, this is the first study showing that atorvastatin pretreatment alters GSK-3 β via miR-199a-5p after OGD/R, which raises the possibility that atorvastatin protects myocardium from I/R injury through modulating miR-199a-5p. Wang H et al. [30] showed that inhibition of miR-199a-5p reduces the replication of HCV via regulating the pro-survival pathway. Sun L et al. [31] showed that inhibition of miR-199a-5p reduced cell proliferation in autosomal dominant polycystic kidney disease through targeting CDKN1C. All those previous studies suggest that miR-199a-5p might be a novel target for treatment of various diseases. Our study, for the first time, showed that miR-199a-5p mRNA level was significantly increased after OGD/R ($p < 0.05$), whereas pretreatment with atorvastatin significantly reserved it ($p < 0.05$). In addition, we further showed that downregulation and upregulation of miR-199a-5p mRNA by transfection respectively increased and decreased GSK-3 β both in mRNA and protein levels suggesting that miR-199a-5p mRNA exert its effects via GSK-3 β . It is noteworthy to mention that other microRNAs, such as miR-31[32] and miR-17[33], have also been found to be involved in cardiac ischemia/reperfusion injury.

In conclusion, our study suggests a potential mechanism of this helpful effect in which atorvastatin attenuates OGD/R-induced apoptosis in cardiomyocytes by upregulating specific survival proteins such as GSK-3 β via downregulation of miR-199a-5p. For the first time, our data provide new insight into the mechanisms underlying the effectiveness of atorvastatin in reducing apoptosis in myocardial I/R injury. Statins is a standard ingredient of the therapeutic regimen in hypercholesterolemia. Recently, it was found to prevent cardiovascular diseases, although the exact mechanism remains unclear. The results presented here demonstrated that atorvastatin increases the cardioprotective effects of GSK-3 β by inhibiting miR-199a-5p.

Disclosure Statement

The authors declared that there is no conflict of interests.

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Erratum

In the article by Zuo et al., entitled “Atorvastatin Protects Myocardium against Ischemia-Reperfusion Injury through Inhibiting miR-199a-5p” [Cell Physiol Biochem 2016;39:1021-1030 (DOI: 10.1159/00044780)] there are a few mistakes:

- In page 1022, the second to the last line “...for an additional 18h,...” should be changed to “...for an additional 1h,...”

- The legend of Figure 6 is wrong and should be changed into:

Fig. 6. Effects of Atorvastatin on neonatal rat cardiac ventricular cardiomyocytes viability, LDH release, and expression of Caspase 9, Bcl-2, and Cyto C. A. Images showing the verification of neonatal rat cardiac ventricular cardiomyocytes; B. Graph showing cell viability in the three groups; C. The level of Caspase 9, Bcl-2, Cyto C, and GSK-3 β proteins in the three groups; D. Graph showing GSK-3 β mRNA level in the three groups. E. Graph showing miR- 199a-5p mRNA level in the three groups; F. Graph showing LDH release in the five groups; G. The level of Caspase 9, Bcl-2, and Cyto C proteins in the five groups. *compared with the control group, $p<0.05$. # compared with the OGD/R group, $p<0.05$.

The authors sincerely apologize for the errors.