# Original Article Atorvastatin attenuates inflammation and oxidative stress induced by ischemia/reperfusion in rat heart via the Nrf2 transcription factor

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**Abstract:** The role of atorvastatin in inflammation and oxidative stress induced by ischemia/reperfusion is currently not well understood. The aim of this study was toinvestigate whether atorvastatin modulates neutrophil accumulation, TNF- $\alpha$  induction and oxidative stress and to examine the possible role of the nuclear factor erythroid 2-related factor 2 (Nrf2)/antioxidant response element (ARE) pathway in an ischemia/reperfusion injured rat heart model. Rats were randomly assigned into tosham operation group, myocardial ischemia/reperfusion (MI/R) group, MI/R + atorvastatin group. Myocardial infarct area, myeloperoxidase (MPO), serum creatinine kinase (CK) and lactate dehydrogenase (LDH) levels were monitored. The results indicate that compared to MI/R, atorvastatin reduced myocardial infarction area, MPO level, serum CK and LDH levels, and both serum and myocardial TNF- $\alpha$ production. In addition, atorvastatin increased SOD and GSH-PX activity and decreased MDA content. Atorvastatin also enhanced levels of Nrf2 and heme oxygenase-1. In summary, our data suggests that atorvastatin exerts significant cardioprotective effects following myocardial ischemia, possibly through the activation of the Nrf2/ARE signaling pathway.

Keywords: Atorvastatin, myocardial ischemia, Nrf2, HO-1

#### Introduction

The inflammatory reaction induced by ischemia/reperfusion is one of the most important elements leading tomyocardial ischemia-reperfusion (MI/R) injury [1]. During the process of inflammation, various cytokines are released, including tumor necrosis factora (TNF-a), interleukin 6 (IL-6) and IL-8 [2]. Of these, TNF- $\alpha$  is known to trigger the inflammatory reaction caused by MI/R. In addition, vascular endothelial cell injuries, and infiltration by inflammatory cells such as neutrophils, which are activated by cytokines and adhesion molecules, are also involved in the inflammatory process. As such, TNF- $\alpha$  activity and the amount of neutrophil infiltration are often considered as positive indicators of an inflammatory reaction. In addition to inflammation, oxidative stress also plays a pivotal role in MI/R-induced injury [3].

Nuclear factor erythroid 2-related factor 2 (Nrf2), a key transcription factor, plays an indis-

pensable role in the induction of endogenous antioxidant enzymes against oxidative stress. Under physiological conditions, Nrf2 is localized within the cytoplasm by binding to its negative regulator, Kelch-like ECH associating protein 1 (Keap1), which promotes Nrf2 ubiguitination by the Cul3-Rbk1 complex and subsequent degradation by the proteasome [4]. However, upon exposure to reactive oxygen species (ROS), Nrf2 is liberated from the regulatory Keap1-Nrf2 complex and translocates from the cytoplasm to the nucleus. Within the nucleus, Nrf2 binds to the antioxidant response element (ARE), a regulatory enhancer region within gene promoters. This binding triggers the production of many phase II detoxifying and antioxidant enzyme genes such as hemeoxygenase 1 (HO-1) and NAD(P)H: quinoneoxidoreductase 1 (NQ01), both of which protect cells against oxidative stress and against a wide range of other toxins [5, 6]. In agreement, many recent reports have demonstrated that Nrf2 plays a critical



**Figure 1.** The comparison of myocardial infarct area (INF/AAR%) in each group. TTC-Evans blue double staining suggested that compared with the MI/R group, atorvastatin significantly reduced the infarct area. \*P < 0.05 vs. sham group; \*P < 0.05 vs. MI/R group.

role in the induction of endogenous antioxidant enzymes that protect against oxidative damage in a variety of diseases. For example, Nrf2 is reportedly to protect cardiac fibroblasts and cardiomyocytes against oxidative stress by increasing detoxification pathways and antioxidant potentials [7, 8].

Atorvastatin is a member of the statin class of inhibitors. Through the inhibition of 3-hydroxy-3-methylglutaryl-coenzymeA (HMG-CoA) reductase, statins have revolutionized the treatment of hypercholesterolemia. The beneficial effects of HMGCoA reductase inhibitors are usually attributed to their ability to reduce endogenous cholesterol synthesis. However, statins have a wide spectrum of other effects as well. For instance, in addition to decreasing the ability of blood to form clots, statins are responsible for a variety of biochemical alterations, including a reduced accumulation of esterified cholesterol in macrophages, an increase in endothelial nitric oxide (NO) synthetase, a reduction of the inflammatory process, and an increased stabil-

ity of atherosclerotic plagues [9]. Studies have shown that treatment with statins leads to a significant reduction in the levels of proinflammatory cytokines, such as TNF, IL-1 and IL-6 [10]. In a separated study, atorvastatin was found to significantly decreased bone resorption markers, including serum levels of IL-6 [11]. In agreement, atorvastatin has been used to inhibit metalloproteinases [12, 13], osteoclastogenesis and bone destruction, as well as the expression of the receptor activator of nuclear factor-kappaB ligand (RANKL) [14]. However, the role of atorvastatin in inflammation and oxidative stress induced by myocardial ischemia/reperfusion is not well understood. Therefore, the present study aims to investigate the role of the Nrf2/ARE signaling pathway in the cardioprotective effects of atorvastatin in a rat model of MI/R.

### Materials and methods

#### Reagents

Atorvastatin was purchased from Sigma Chemical Co. (St. Louis, MO, USA). MPO assay kit, CK test kit and LDH assaykit were purchased from Jiancheng Bioengineering Institute (Nanjing, China). TNF- $\alpha$  ELISA kit was purchased from R&D Corporation, (USA). BCA protein quantification kit was purchased from Bio-Rad (USA). GSH-PX and SOD activity assay kit and MDA content assay kit were purchased from Jiancheng Bioengineering Institute (Nanjing, China).

### Animals

Thirty adult male Sprague-Dawley rats (250-300 g) were purchased from the Center of Experimental Animal in Jilin University, China. All animals used in this study were cared for in accordance with the Guide for the Care and Use of Laboratory Animals published by the United States National Institute of Health (NIH



Figure 2. The comparison of MPO activity in each group. Compared with MI/R group, the MPO activity in MI/R + Atorvastatin group reduced significantly. \*P < 0.05 vs. sham group; #P < 0.05 vs. MI/R group.



Figure 3. The comparison of serum CK activity in each group. Compared with MI/R group, atorvastatin reduced the serum CK activity significantly. \*P < 0.05 vs. sham group; \*P < 0.05 vs. MI/R group.

publication no. 85-23, revised 1996), and all procedures were approved by the Committee of Experimental Animals of Jilin University.

# Myocardial ischemia-reperfusion model and experimental protocol

Male Sprague-Dawley rats (250-300 g) were anesthetized i.p. with sodium pentobarbital (Sigma, St. Louis, USA, 40 mg/kg). Myocardial ischemia was produced by exteriorizing the heart with a left thoracic incision followed by a slipknot (5-0 silk) around the left anterior descending coronary artery (LAD). After 30 min



Figure 4. The comparison of serum LDH activity in each group. Compared with MI/R group, atorvastatin reduced the serum LDH activity significantly. \*P < 0.05 vs. sham group; #P < 0.05 vs. MI/R group.

of ischemia, the slipknot was released and the animal received 120 min of reperfusion.

Rats were randomly assigned to three experimental groups. There were 10 rats in each group: (1) sham group: silk was drilled underneath the LAD but the LAD was not ligated; (2) MI/R group: LAD was ligated for 30 min and then allowed 120 min reperfusion with receiving vehicle (0.9% NaCl i.v.); (3) MI/R + Atorvastatin group: Atorvastatin (10 mg/kg, i.v.) was administered 5 min prior to reperfusion.

### Assay of myocardial infarct area

After reperfusion, myocardial infarct size was determined by means of a double-staining technique and a digital imaging system (infarct area/area at risk × 100%) [15].After reperfusion, coronary blood flow was again blocked and Evans blue (2%, 4 ml) was injected by the rapid distribution of the right ventricle into the body. The heart was guickly removed to a -20°C refrigerator for cryopreservation. The heart was cut into 1 mm slices, placed in 1% 2,3,5-triphenyltetrazolium chloride (TTC) solution, incubated for 15 min, and then placed in 4% formaldehyde solution overnight. Evans blue stained area (blue staining, non-ischemic area), TTC stained area (red staining, ischemic area) and non-TTC stained area (white, infarct area) were analyzed with a digital imaging system by computer. Myocardial infarct area (infarct area/ area at risk%, INF/AAR%) were calculated.



**Figure 5.** The comparison of levels of TNF- $\alpha$  in myocardium and serum in each group. Compared with the MI/R group, atorvastatin reduced TNF- $\alpha$ level significantly. \**P* < 0.05 vs. sham group; \**P* < 0.05 vs. MI/R group.

### Determination of myeloperoxidase (MPO) level

After reperfusion, the myocardial tissue was placed at -70°C for preservation. MPO test kit was used to detect level of MPO in the myocardial tissue according to manufacturer's instruction.

# Detection of creatine kinase (CK) activity

Following reperfusion, blood was obtained from the carotid artery and was maintained at room temperature for 30 min. Next, the serum was collected by centrifugation at 3,000 × g for 20 min at 4°C and placed at -70°C for preservation. According to the manufacturer's instructions, the CK test kit was utilized to detect the serum CK activity.

# Determination of lactate dehydrogenase (LDH) level

Following reperfusion, blood was obtained from the carotid artery and was placed at room temperature for 30 min. Next, the serum was collected by centrifugation at  $3,000 \times g$  for 20 min at 4°C and placed at -70°C for preservation. According to the manufacturer's instructions, the CK test kit was utilized to detect the serum LDH levels.

# Detection of TNF-α level

After reperfusion, the levels of TNF- $\alpha$  in myocardial tissue homogenate and serum were detected in strict accordance with manufacturer's instructions.

# Detection of SOD, GSH-PX activity and MDA content

Superoxide dismutase (SOD), glutathione peroxidase (GSH-PX), and malondialdehyde (MDA) were measured by using assay kits according to the manufacturer's instructions.

# Western blot analysis

Protein extracts were prepared from ischemic myocardium or heart in sham group by homogenization in a RIPA buffer. Protein concentration was assayed using the BCA Protein Assay reagent kit (Novagen, Madison, WI), and equal amounts (50 µg) of protein per sample were separated by electrophoresis on a 10% polyacrylamide gel and electrotransferred to a nitrocellulose membrane. The nonspecific binding of antibodies was blocked using TBS with 5% nonfat milk and with 0.1% Tween-20 for 2 h at room temperature. Membranes were then incubated overnight (4°C) with the primary antibody at the appropriate dilution (rabbit anti-Nrf2, 1:200, Santa Cruz Biotechnology; rabbit anti-HO-1, 1:2000, Santa Cruz Biotechnology). Protein loading was normalized by Western blotting to lamin B1 (lamin B1, 1:500, Santa Cruz Biotechnology) or anti-β-actin (1:2000; Santa Cruz Biotechnology) in blocking buffer overnight at 4°C. Subsequently, the mem-





**Figure 6.** Atorvastatin ameliorates oxidative stress after myocardial ischemia/reperfusion injury. A. Atorvastatin enhanced glutathione peroxidase (GSH-PX) activity. B. Atorvastatin enhanced superoxide dismutase (SOD) activity. C. Atorvastatin significantly decreases the content of malondialdehyde (MDA). \*P < 0.05 vs. sham group; #P < 0.05 vs. MI/R group.

branes were incubated for 1 h at room temperature with the corresponding horseradish peroxidase-conjugated secondary antibodies (1: 3000, goat anti-rabbit; 1:5000, goat antimouse, both from Santa Cruz Biotechnology). Finally, immunoreactive bands were detected by chemiluminescence using ECL Plus Western Blotting Detection kit (Millipore, USA), according to the manufacturer's instructions. Nrf2 expression levels were normalized to lamin B1, while HO-1 expression level was normalized to  $\beta$ -actin.

### Statistical analysis

Data is presented as means  $\pm$  SD. Statistical significance was evaluated by a Student's t-test for unpaired data or Dunnett's t-test, preceded by one-way analysis of variance (ANOVA). Values of P < 0.05 were considered statistically significant.

#### Results

Atorvastatin reduced the myocardial infarction area

MI/R induced a significant infarction area as shown in the MI/R group. Compared with the MI/R group, atorvastatin significantly reduced myocardial infarction area (P < 0.05) (**Figure 1**).

#### Atorvastatin inhibited neutrophil infiltration

Neutrophils contain a certain amount of myeloperoxidase (MPO), which reliably accounts for 5% of their total dry cell weight. Therefore, the activity of MPO in the myocardium can be considered as an indication of the overall level of neutrophil infiltration. In the absence of MI/R, the overall level of MPO activity in the sham group was low, whereas MPO activity was significantly increased in the MI/R group. Im-



**Figure 7.** Western blot analysis for Nrf2 and HO-1 expression. A, B. Representative photographs of the Western blot show Nrf2 and HO-1protein levels in sham, MI/R and MI/R + Atorvastatin respectively. C, D. The graphs show that Nrf2 and HO-1, and protein levels were increased after MI/R and more significantly induced by treatment with atorvastatin. Data were expressed as mean  $\pm$  SD (n = 10 in each group). \**P* < 0.05 versus the sham group, #*P* < 0.05 versus the MI/R group.

portantly, atorvastatin treatment significantly decreased myocardial MPO activity in comparison to the MI/R group (**Figure 2**).

# Atorvastatin reduced the activity of serum CK in MI/R rats

The activity of CK increased significantly in the MI/R group compared with that in the sham group. However, atorvastatin treatment significantly decreased CK activity compared with that in the MI/R group (**Figure 3**).

### Atorvastatin reduced LDH level in MI/R rats

The activity of LDH increased significantly in the MI/R group compared with that in the sham group. Atorvastatin treatment dramatically decreased CK activity compared with that in the MI/R group (**Figure 4**).

# Atorvastatin reduced TNF- $\alpha$ levels in serum and MI/R tissue

The MI/R injury results in production of a large amount of TNF- $\alpha$ . Therefore, myocardial and serum TNF- $\alpha$  levels were examined to detect the degree of injury. Compared with the MI/R group, atorvastatin significantly decreased the

levels of TNF- $\alpha$  in both myocardium and serum (Figure 5).

Atorvastatin elevated antioxidant enzymes activities and decreased MDA content

To investigate whether atorvastatin affects oxidative stress damage, we evaluated the activities of antioxidant enzyme and the content of MDA. The activities of GSH-PX and SOD were decreased significantly in the MI/R group compared with those in the sham group. However, atorvastatin treatment significantly elevated GSH-PX and SOD activities compared with those in the MI/R group. The content of MDA is an index of lipid peroxidation, which increased significantly after myocardial I/R injury. Atorvastatin treatment significantly decreased the MDA content compared with that in the MI/R group (**Figure 6**).

# Effects of atorvastatin on Nrf2 and HO-1 expressions

Western blot analysis showed weak signals for Nrf2 and HO-1 in the sham group. In contrast, significant increases in protein expression for Nrf2 and HO-1 were found in the MI/R group (P < 0.05), and these increases were further

intensified by treatment with atorvastatin (Figure 7).

# Discussion

The major findings in the present study are: (1) Atorvastatin attenuates MI/R injury by inhibiting neutrophil infiltration and TNF- $\alpha$  production. (2) Atorvastatin attenuates MI/R injury by elevating SOD and GSH-PX activity and decreasing MDA content. (3) The mechanism of atorvastatin activity is potentially associated with the activation of Nrf2 expression.

Inflammatory reaction plays a critical role in myocardial ischemia/reperfusion injury [1]. The release of inflammatory cytokines and the aggregation and infiltration of inflammatory cells are the key steps in inflammation [16].

TNF- $\alpha$  is primarily produced by macrophages, and it acts to promote inflammatory cascade by increasing the release of other proinflammatory cytokines and by influencing neutrophil recruitment [17]. TNF- $\alpha$ , as an important cytokine in inflammation, plays a pivotal role in the inflammation induced by MI/R [18]. Indeed, TNF-a can induce the release of other inflammatory mediators, increase the expression of cell adhesion factors, and promote the adhesion of neutrophils to endothelial cells. In addition, TNF- $\alpha$  has a negative inotropic effect, resulting in inhibited myocardial contractility and reduced blood pressure. TNF-α can also induce cardiomyocyte apoptosis and participate in ventricular remodeling [19]. Previous studies suggest that the level of TNF- $\alpha$  increases significantly after MI/R [20], while the administration of a neutralizing TNF-α monoclonal antibody attenuated edema, and was conducive to the recovery of cardiac function [21].

MI/R injury seems to be triggered in part by neutrophil activation. The underlying mechanisms include: (1) Cell damage caused by the release of oxygen free radicals, proteolytic enzymes and cytotoxic substances. (2) The released inflammatory mediators cause vascular endothelial cell damage, vascular permeability increasing, and edema formation. (3) Further activation of inflammatory cells increase further the inflammatory response [22]. (4) Neutrophils adhesion to vascular endothelium and small blood vessels occlusion result in no-reflow phenomenon. Previous studies have demonstrated the link between neutrophil infiltration and ischemia/ reperfusion injury. For instance, removal of neutrophils or the use of drugs to inhibit of neutrophil activity, have both been shown to reduce ischemia/reperfusion injury [23, 24]. In agreement, in the present study, we observed that neutrophil accumulation and TNF- $\alpha$  production increased significantly following MI/R. Importantly, we also found that atorvastatin reduced the accumulation of neutrophils and TNF- $\alpha$  production, strongly indicating that atorvastatin attenuates neutrophil-mediated I/R injury.

Under physiological conditions ROS are generated at low levels and play important roles in signaling and metabolic pathways [25], however, under pathologic conditions such as MI/R, their overproduction leads to oxidative stress. This can lead to cell damage to nervous tissue, including DNA oxidation, promoting chain reactions of membrane lipid peroxidation, and alterations in membrane fluidity [26, 27]. As ROS produces malondialdehyde (MDA), an end product of lipid peroxidation, the levels of MDA were utilized to estimate the extent of ROS following MI/R. Our results show that MI/R induced elevation in MDA levels was markedly decreased by treatment with atorvastatin, indicating that the cardioprotection conferred by atorvastatin may be in part due to its attenuation of lipid peroxidation following MI/R. following the overproduction of harmful ROS, endogenous antioxidants are able to detoxify them, however, this results in a depletion of their cellular stores. [28]. Superoxide dismutase (SOD) and glutathione peroxidase (GSH-PX) are thought to be two dominant enzymes acting as free radical scavengers that act to prevent ROS accumulation [29]. SOD scavenges the superoxide anion radical  $(0^{-})$  by catalyzing its dismutation to  $H_2O_2$ , which is further converted to water by GSH-PX, at the expense of glutathione [30]. In the present study, atorvastatin appeared to be effective in stimulating the activities of SOD and GSH-PX, and therefore, our data suggests that atorvastatin also protects against MI/R injury through the amelioration of oxidative stress.

Ischemia-reperfusion enhances Nrf2 dissociation from Keap1, thus facilitating Nrf2 translocation to the nucleus, binding to the ARE, and activation of phase II detoxifying and antioxidant genes [31]. The Nrf2/ARE pathway affects cell survival through a variety of mediators, including apoptotic proteins such as Bcl-2 and Bax [32] and phase II enzymes such as HO-1 [33]. HO-1, is considered a stress protein, and is regarded as a sensitive and reliable indicator of cellular oxidative stress [34]. Our results suggest that atorvastatin attenuates inflammation and oxidative stress induced by MI/R injury by activating the expression of Nrf-2, which in turn upregulates HO-1 expression.

In conclusion, the present study demonstrates that atorvastatin attenuates myocardial ischemia/reperfusion injury. This protective effect of atorvastatin is mediated through the inhibition of neutrophil infiltration and TNF- $\alpha$  production, and an increase in SOD and GSH-PX activity. Furthermore, atorvastatin likely significantly decreases lipid peroxidation, as MDA production is strongly reduced. Finally, we show for the first time that atorvastatin cardioprotection is also closely associated with Nrf2/ARE pathway.

# Disclosure of conflict of interest

None.

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