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Atorvastatin attenuates myocardial remodeling induced by chronic intermittent hypoxia in rats: Partly involvement of TLR-4/MYD88 pathway

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ABSTRACT

Inflammatory processes and oxidative stress are known to play a key role in the development of cardiovascular complications such as cardiac hypertrophy induced by chronic intermittent hypoxia (CIH), the most characteristic pathophysiological change of obstructive sleep apnea syndrome (OSAS). Current evidence suggests that competitive inhibitors of 3-hydroxy-3-methylglutaryl-CoA coenzyme A reductase, such as atorvastatin, not only reduce blood lipids but also have anti-inflammatory and inhibit oxidative stress benefits. This study examined the protective role of atorvastatin in CIH-induced cardiac hypertrophy. Adult male wistar rats were subjected to 8 h of intermittent hypoxia/day, with/without atorvastatin for 6 weeks. Ventricular remodeling, toll-like receptor 4 (TLR-4), myeloid differentiation primary response protein 88 (MYD88), inflammatory agents and radical oxygen species were determined. As a result, we found that treatment with atorvastatin markedly inhibited the mRNA and protein expressions of TLR4, MYD88 and the downstream inflammatory agents and radical oxygen species. Administration of atorvastatin following CIH significantly ameliorated the myocardial injury, such as cardiac hypertrophy. In conclusion, Pre-CIH atorvastatin administration may attenuate TLR-4/MYD88 mediated inflammatory processes and oxidative stress in the injured rat myocardium, and this may be one mechanism by which atorvastatin ameliorated myocardial injury following CIH.

1. Introduction

Obstructive sleep apnea syndrome (OSAS) has emerged as a public health burden because of its serious clinical complications, especially cardiovascular diseases such as systemic hypertension [1], heart failure [2], stroke [3], pulmonary embolism [4], and other disorders. Untreated OSAS patients have higher cardiovascular morbidity and mortality compared patients treated with continuous positive airway pressure (CPAP) or healthy individuals [5,6]. The exact mechanisms underlying OSAS-induced cardiovascular disease are unclear. Inflammatory processes and oxidative stress are considered to play a key role in the development of cardiovascular complications such as cardiac hypertrophy induced by chronic intermittent hypoxia (CIH), the most characteristic pathophysiological change of OSAS [7–12]. The repetitive short cycles of desaturation followed by rapid reoxygenation experienced in OSAS patients is somewhat analogous to ischemia/reperfusion (I/R) injury [13]. Therefore, the pathology and mechanism of I/R injury may provide valuable insights into OSAS.

Toll-like receptor-4 (TLR-4), a type-I transmembrane receptor protein that plays a critical role in innate and adaptive immunity, has been reported to mediate myocardial I/R injury [14,15]. The role of TLR-4 in post-infarct maladaptive left ventricular (LV) remodeling has also been described, and is likely mediated via inflammatory cytokine production and extracellular matrix degradation [16,17]. Interestingly, monocytes from OSAS patients show a significant increase in TLR-4 surface expression [18]. Myeloid differentiation primary response protein 88 (MYD88), which is one of the most important adaptor molecules for TLR-4, and TLR-4-MYD88-dependent and independent pathways have been shown to participate in the pathophysiology of myocardial injury in a porcine model of I/R injury [19]. Therefore, we hypothesized that the TLR-4 signaling pathways might be involved in CIH/ reoxygenation induced myocardial injury.

Current evidence suggests that competitive inhibitors of 3-hydroxy-3-methylglutaryl-CoA coenzyme A reductase, such as atorvastatin, not only reduce blood lipids but also have inhibit oxidative stress and anti-inflammatory benefits. Meanwhile,

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Atorvastatin suppresses LPS-induced rapid upregulation of TLR-4 and its signaling pathway in endothelial cells and human $CD14^+$ monocytes [20,21], and protects hepatic and brain tissues against I/R injury by TLR-4 suppression in rodent models [22,23]. However, it is not known whether atorvastatin can reduce myocardial injury induced by CIH/reoxygenation. Here, we investigate the protective effect of atorvastatin in in rodent model of CIH/reoxygenation-induced myocardial injury.

2. Materials and methods

2.1. Reagents

Atorvastatin was purchased from Pfizer (Dalian, China), Antibodies against TLR-4 and MYD88 were acquired from Abgent (an Diego, USA), Enhanced chemiluminescence (ECL) Plus Western blot reagent was obtained from Bio-Rad (Hercules, USA), ABI 7500 real-time PCR system was obtained from Life Technologies (Carlsbad, CA, USA).

2.2. Animal model and experimental design

Twenty-eight male wistar rats (180–200 g) were purchased from the experimental animal center of Wuhan University (Wuhan, China). Animals were kept in a departmental animal house on a 12:12 h light–dark cycle under standard laboratory conditions (temperature 25 ± 2 °C, humidity 60 ± 5%). Rats were provided with standard rodent chow and and allowed free access to water. The experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the Tongji Medical College of Huazhong University of Science and Technology. Rats were randomly divided into four groups ($N = 7$): normoxia + vehicle, normoxia + atorvastatin, CIH + vehicle and CIH + atorvastatin. Atorvastatin (20 mg/kg) was dissolved in a mixture of dimethyl sulfoxide (DMOS 1%) and polyethylene glycol (PEG) 400 as previously described [24], and administered by oral gavage once a day for 6 weeks prior to exposure to intermittent hypoxia. The concentration of DMSO in the administered solution was <1%.

CIH was conducted as previously described [25], with some modification. Briefly, during the exposure periods, animals were induced in custom-built chambers (OxyCycler A84, Biospherix, Redfield, NY, USA) connected to a supply of O_2 and N_2 gas. Animals in the chambers were allowed free mobility, and water and food were provided ad libitum. The oxygen concentration in the chambers was controlled by a computer via introducing either N_2 or O_2 into the chambers. For the CIH group, intermittent hypoxia for 30 s of 21% O_2 and 30 s of 5% O_2 cyclically repeated for 8 h/day, 7 days/week during the daytime over 6 weeks. For the normoxic group, rats were placed in similar chambers under normoxic conditions. Twenty-four hours after the last exposure, rats were sacrificed with pentobarbital sodium (40 mg/kg administered by intraperitoneal injection). The hearts were excised and perfused with cold PBS and then preserved in the liquid nitrogen or 10% formalin for in vitro analyses.

2.3. Western blotting

Tissues from the left ventricle were used for Western blot analysis. Proteins were extracted from the free wall of the left ventricle using RIPA lysis buffer (Beyotime, jiangsu, China) containing a protease inhibitor cocktail to prevent protein degradation. Protein concentration was determined using a Bradford protein assay kit (Bio-Rad, Hercules, CA). All protein samples were boiled for 8 min with loading buffer to denature proteins. Samples containing 50 μg of protein were separated in 10% SDS–PAGE and then transferred to 0.45 μm nitrocellulose membranes (Bio-Rad). Membranes were blocked in 5% nonfat dry milk in TBST (10 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.05% Tween-20) for 1 h at room temperature. Membranes were incubated with rat monoclonal anti-TLR-4 and anti-MYD88 antibodies overnight at 4 °C. Both primary antibodies were diluted 1:1000 with 5% nonfat dry milk in TBST. Membranes were then incubated with the secondary antibody conjugated to horseradish peroxidase (diluted 1:3000) at room temperature for 2 h. Reactive proteins were analyzed with an ECL Western blotting detection system. All experiments were performed three or more times.

2.4. Real-time reverse transcription (RT-PCR)

Tissues from the left ventricle were used for RT-PCR. The total RNA was extracted using Trizol according to the manufacturer's instructions (Takara, Japan). The concentration of total RNA was measured by spectrophotometry and the OD_{260}/OD_{280} ratio was used to assess the RNA purity. RNA was reversed transcribed into cDNA using PrimeScript RT Master Mix (Takara, Ostu, Shiga, Japan) in a 20 μL reaction mixture according to the manufacturer's protocol. Real-time PCR assays were performed using an ABI 7500 Real-time PCR System (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's recommendations. The primes (Invitrogen, USA) for cDNA were used as follows: TLR-4 5′-TCA GTG TGC TTG

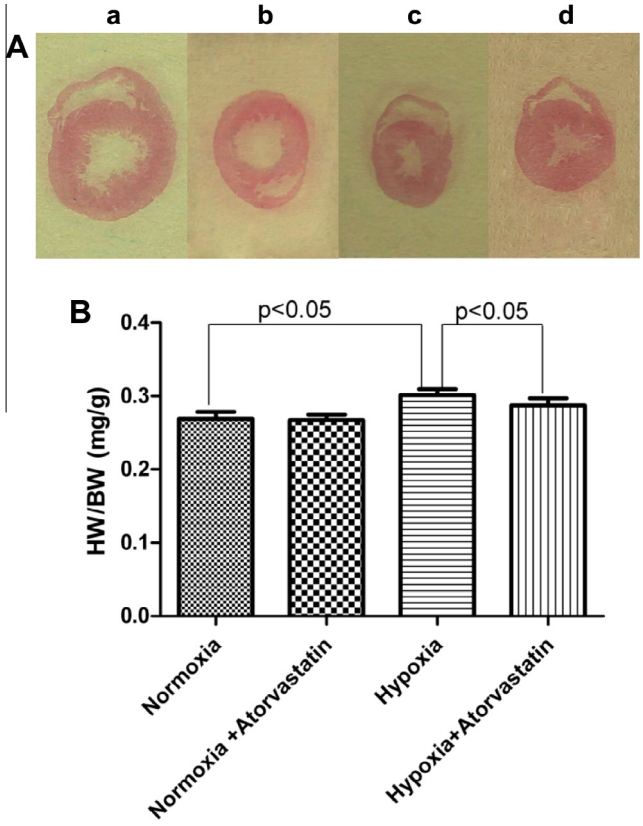

Fig. 1. Chronic intermittent hypoxia (CIH) causes myocardial hypertrophy. Male wistar rats were exposed to CIH for 6 weeks show clear evidence of myocardial hypertrophy compared to animals in normal oxygen concentrations. Pre-treatment with atorvastatin effectively inhibits CIH-induced myocardial hypertrophy. (A) Representative photomicrographs, (a) Normoxic, (b) Normoxic + Atorvastatin, (c) Hypoxia, (d) Hypoxia + Atorvastatin (B) Ratios of heart-to-body mass demonstrate myocardial hypertrophy ($N = 7$ rats per group) Results represent mean ± SD with means compared using one way ANOVA. A value of $P < 0.05$ is considered statistically significant.

TGG TAG CC-3′ and 5′-TCG TTT CTC ACC CAG TCC TC-3′; MYD88 5′-TAC GCA ACC AGC AGA AAC AG-3′ and 5′-ATT GGG GCA GTA GCA GAT GA-3′; IL-6 5′-TTG CCT TCT TGG GAC TGA TGT-3′ and 5′-TAC TGG TCT GTT GTG GGT GGT-3′; IL-1β 5′-ACT ATG GCA ACT GTC CCT GAAC-3′ and 5′-GTG CTT GGG TCC TCA TCC TG-3′; TNF-α 5′-CTT CTC ATT CCT GCT CGT GG-3′ and 5′-TCC GCT TGG TGG TTT GCT AC-3′; ICAM-1 5′-GGG ATG GTG AAG TCT GTC AA-3′ and 5′-GGC GGT AAT AGG TGT AAA TGG-3′. The cDNA$_S$ were detected in 96-well plates in duplicate using SYBR Premix Ex Taq^{TM} (Takara, Japan). The PCR conditions were 95 °C for 30 s, followed by 40 cycles at 95 °C for 5 s, 60 °C for 34 s and 95 °C for 15 s.

2.5. Lipid peroxidation assay

Lipid peroxidation was assessed using malondialdehyde (MDA) as an indicator of oxidative stress. The level of MDA production in myocardial tissues of rats was measured using a commercially available kit (Nanjing Jiancheng Institute of Biological Engineering,

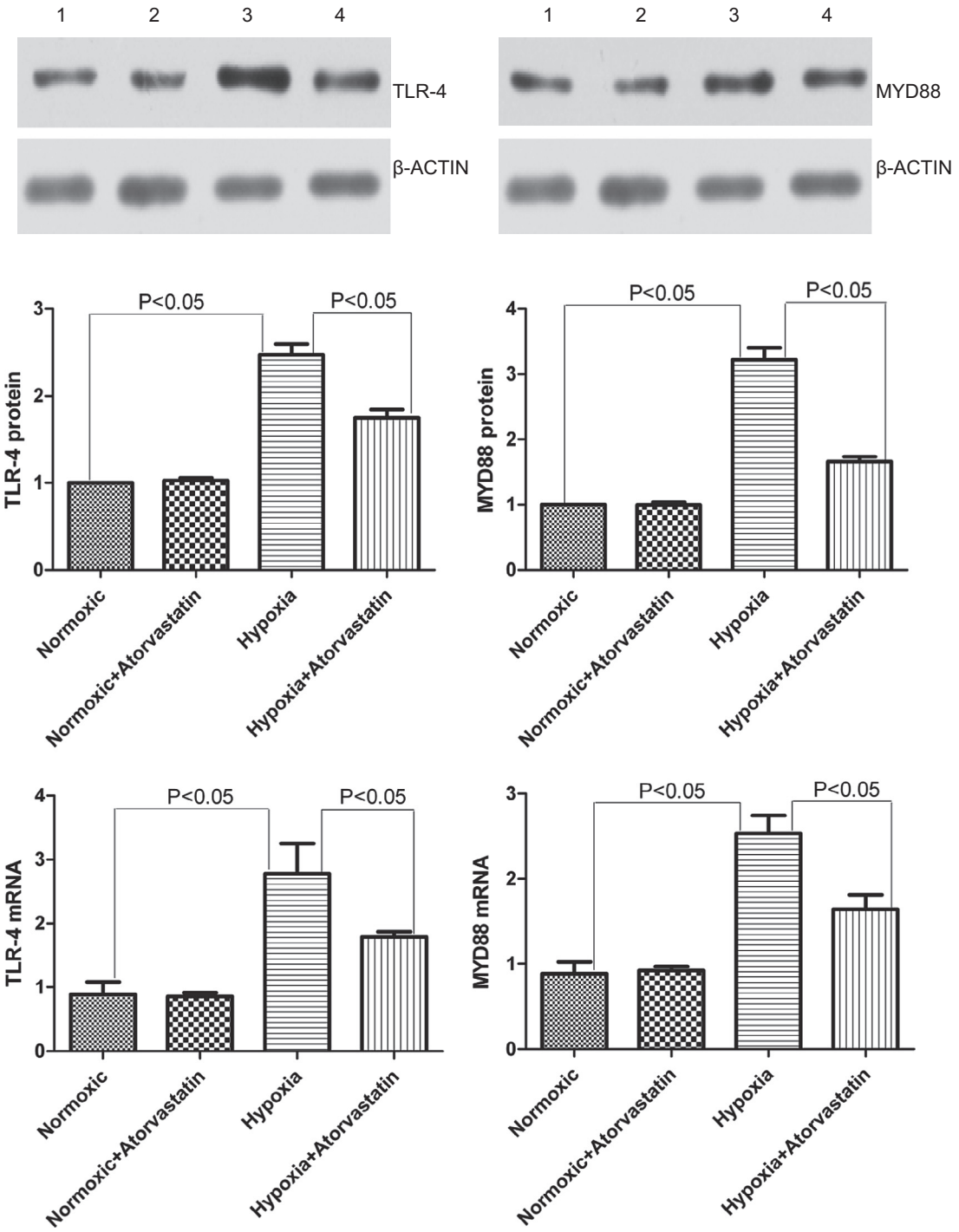

Fig. 2. mRNA and protein levels of TLR-4 and MYD88 are increased in the left ventricle during chronic intermittent hypoxia (CIH)-induced myocardial hypertrophy compared to exposure to normal oxygen concentration. Lane 1, Normoxic; Lane 2, Normoxic + Atorvastatin; Lane 3, Hypoxia; Lane 4, Hypoxia + Atorvastatin. Pre-treatment with atorvastatin significantly reduces TLR-4 and MYD88 mRNA and protein expression in the left ventricular myocardium. Results represent mean ± SD with means compared using one way ANOVA. A value of $P < 0.05$ is considered statistically significant.

Nanjing, China) according to the manufacturer' s instructions, as previously described [26].

2.6. Statistical analysis

All date were expressed as mean ± SD. Data were statistically analyzed using one way analysis of variance (ANOVA) for group comparisons. Student–Newman–Keuls post hoc tests were used when appropriate. A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. Inhibition of CIH-induced cardiac hypertrophy by atorvastatin

In this study, heart weight:body weight (HW:BW) ratio was used as an indicator of cardiac hypertrophy. Heart weight and body weight were measured on the day of sacrifice. We found that the HW:BW ratio was significantly increased in the CIH + vehicle group compared to the normoxic control group, and that these increases were markedly reduced by treatment with atorvastatin ($P < 0.05$; Fig. 1).

3.2. CIH exposure elevates TLR-4 and MYD88 mRNA and protein in the left ventricular

After 6 weeks of exposure to intermittent hypoxia, mRNA levels of TLR-4 and its signal mediator MYD88 were signficantly elevated in the left ventricle of the CIH + vehicle group compared to the normoxic group ($P < 0.05$; Fig. 2). Similarly, TLR-4 and MYD88 protein were significantly elevated in the left ventricle of the CIH + vehicle group compared to the control group, whereas these changes were significantly suppressed by treatment with atorvastatin ($P < 0.05$; Fig. 2).

3.3. Atorvastatin diminishes CIH-induced inflammatory response in the left ventricle

Inflammatory cytokines are important in the pathophysiological processes of myocardial injury induced by intermittent hypoxia. Therefore, we measured mRNA expression of inflammatory cytokines interleukin-6 (IL)-6, tumor necrosis factor (TNF)-α, IL-1β and intercellular adhesion molecule (ICAM)-1 in the left ventricle by RT-PCR. We found that 6 weeks of CIH stress significantly increased IL-6, TNF-α, IL-1β and ICAM-1 mRNA expression

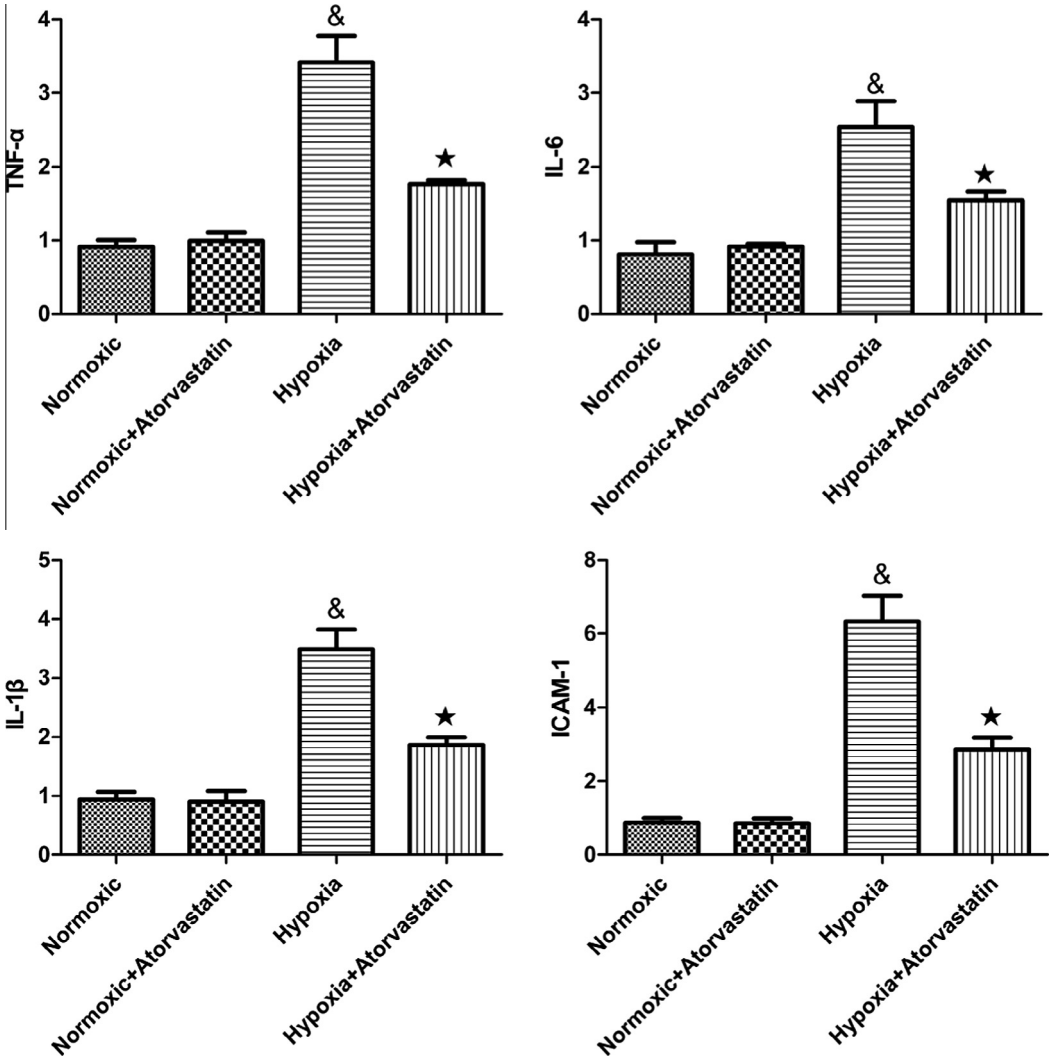

Fig. 3. Chronic intermittent hypoxia increases mRNA levels of inflammatory cytokines in the left ventricular (LV) myocardium. Intermittent hypoxia significantly increased expression of tumor necrosis factor (TNF)-α, interleukin (IL)-6, interleukin (IL)-1β and intercellular adhesion molecule (ICAM)-1 in the LV myocardium. Pre-treatment with atorvastatin significantly reduced expression of these cytokines in the LV myocardium. Results represent mean ± SD with means compared using one-way ANOVA. A value of $P < 0.05$ is considered statistically significant. $^{\&}$ $P < 0.05$ vs normoxia; ★ $P < 0.05$ vs hypoxia.

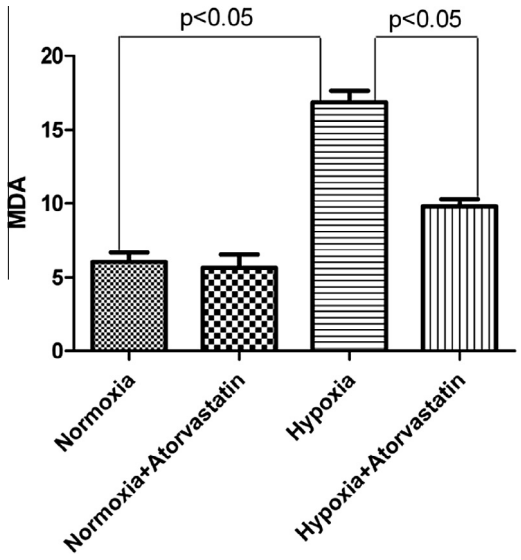

Fig. 4. MDA levels are increased in left ventricular (LV) myocardium by chronic intermittent hypoxia (CIH). Intermittent hypoxia significantly increases expression of MDA in the LV myocardium. Pre-treatment with atorvastatin significantly reduces MDA expression in the LV myocardium. Results represent mean ± SD with means compared using one-way ANOVA. A value of $P < 0.05$ is considered statistically significant.

in the LV myocardium. Expression of these cytokines was markedly reduced by atorvastatin treatment ($P < 0.05$; Fig. 3).

3.4. Inhibition of CIH-induced reactive oxygen species (ROS) in the left ventricle by atorvastatin

ROS are considered to play a key role in the pathophysiological processes of CIH-induced myocardial injury [12]. To assess the effect of CIH and atorvastatin treatment on myocardial injury in our model, we measured MDA production in the left ventricle. We found that MDA levels were significantly elevated in the LV myocardium of rats exposed to CIH compared to animals in the control group and that MDA production was suppressed by treatment with atorvastatin ($P < 0.05$; Fig. 4).

4. Discussion

The accurate pathophysiology of OSAS-induced cardiac dysfunction is still poorly understood. There is no doubt that the pathogenesis underlying OSAS-related myocardial damage is multifactorial; sympathetic activity, endothelial dysfunction, and metabolic anomalies may be involved. Current evidence suggests that inflammatory processes and oxidative stress may play a pivotal role in the pathogenesis of OSAS [7–12]. In the current study, we used a rodent model of CIH/reoxygenation to demonstrate local inflammatory processes and oxidative stress in myocardial tissue, and and non-bacterial-mediated TLR-4 signaling involved in these pathophysiological processes. Pathophysiology of injury and myocardial remodeling were attenuated with atorvastatin, a competitive inhibitor of 3-hydroxy-3-methylglutaryl-CoA coenzyme A reductase. These results may support the potential treatment of OSAS-induced cardiac dysfunction, as an alternative to continuous positive airway pressure (CPAP).

CIH/reoxygenation is the most characteristic pathophysiological feature of OSAS. Although there are no ideal animal models of sleep apnea, the murine model of CIH/reoxygenation is widely used in the field of CIH research. For our studies, we did not use a TLR-4 knockout mouse model, and we extended the time of hypoxia exposure to mimic the repeated apnea hypoxia in human OSAS as closely as possible.

Innate immunity is the first line of defense against foreign intruders in the body. The major targets of innate immune recognition are pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) [27]. Nonbacterial inflammatory response is one of the most important manifestation of DAMPs. Some damage signals of DAMPs such as tissue injury are endogenous and can initiate the innate immune response to maintain tissue homeostasis. However, inflammatory induced by innate immunity in the protection response is a double-edged sword, and sometimes the exaggerated inflammatory reaction counteracts the beneficial effects and contributes to maladaptive tissue damage such as myocardial I/R injury. Similarly, TLRs act as a bridge between the innate immune response and myocardial I/R injury [28].

TLRs are a class of cross-membrane protein receptors that recognize different pathogens or endogenous molecules released from damaged tissue to activate the immune system. To date, 11 TLR family members have been identified in mammals, many of which are involved in a variety of diseases. TLR-4 is expressed in mammalian cardiomyocytes. Growing evidence has demonstrated that TLR-4-induced innate immunity and inflammatory responses play a critical role in cardiovascular diseases [29]. Interestingly, endogenous molecules such as heat-shock protein 70, high- mobility group box-1, and myeloid-related protein 8/14 can activate TLR-4 were also found in OSAS patients [30–32]. Similarly, TLR-4 activity in patients with obstructive sleep apnea was recently investigated, demonstrating that OSAS is associated with enhanced expression and signaling events downstream of TLR-4 in circulating monocytes [18]. Furthermore, 8 weeks of CPAP treatment, downregulated TLR-4 expression and abrogated the release of inflammatory cytokines; however, the relationship between TLR-4 and inflammatory cytokines was not explored. In the current study, we found that rats exposed to intermittent hypoxia for 6 weeks showed induction of inflammatory genes such as IL-6, TNF-α, IL-1β, ICAM-1 in the left ventricle compared with normoxic control group. We also found that TLR-4 protein and mRNA levels were increased in the left ventricle compared with control group, and that pre-treatment with the non-specific TLR-4 inhibitor atorvastatin caused simultaneous reduction in the expression of TLR-4 and inflammatory genes. Therefore, we speculate that intermittent hypoxia upregulates the inflammatory response in the left ventricle partly through TLR-4, and that atorvastatin-mediated reduction of the inflammatory response in the left ventricle induced by intermittent hypoxia might involve TLR-4.

TLR-4 signaling is divided into MYD88-dependent and-independent pathways, the latter of which mediates myocardial injury induced by I/R injury [19]. After activation, the Toll/interleukin-1 (IL-1)-receptor (TIR)-domain-containing adaptor protein (also referred to as the MYD88-adapter like protein) mediates the TLR-4 signaling pathway. This in turn activates IL-1-receptor-associated kinases (IRAKs) and tumour–necrosis-factor-receptor-associated factor 6 (TRAF6), leading to the activation of the inhibitor of nuclear factor-κ B (IκB)-kinase (IKK) complex that induces expression of inflammatory cytokines such as IL-6, TNF-α, IL-1β, and ICAM-1 as well as ROS [33,34]. Similarly, in our present experiments we found that, compared to normal oxygen, intermittent hypoxia up-regulated TLR-4 and MYD88, and increased production of inflammatory genes and ROS expression in myocardial tissue. Importantly, we demonstrated that all of these effects were attenuated by atorvastatin.

Current evidence suggests that competitive inhibitors of 3-hydroxy-3-methylglutaryl-CoA coenzyme A reductase such as atorvastatin have a myriad of beneficial effects in addition to cholesterol lowering activity. Several experimental and clinical studies have shown the benefits of atorvastatin in various diseases.

The specific mechanisms that underlie the pleiotropic effects of atorvastatin are still under investigation. These effects include improved endothelial function, decreased vascular inflammation, inhibition of smooth muscle proliferation, and immunomodulation [35]. A negative regulatory role for atorvastatin in the TLR-4 signaling pathway has also been shown [20–23]. In the present study, we found that atorvastatin inhibited intermittent hypoxia-induced TLR-4 expression, reduced inflammatory response and oxidative stress, and improved myocardial tissue remodeling. Therefore, we speculate that atorvastatin attenuates CIH-induced myocardial injury in rats partly through the TLR-4 signaling pathway.

In summary, our study suggests that the underlying protective effects of atorvastatin on cardiomyocytes exposed to an intermittent hypoxic environment involve reduction of innate inflammation responses and oxidative stress mediated by TLR-4 and MYD88 in cardiomyocytes in vivo. However, the relationship between oxidative stress and inflammatory response is still unclear, and further investigation is needed to elucidate the potential role of other signaling pathways involved in the response of myocardial tissues to intermittent hypoxia.

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