Original Article

Atorvastatin Attenuates TNF-alpha Production via Heme Oxygenase-1 Pathway in LPS-stimulated RAW264.7 Macrophages*

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Abstract

Objective To assess the effect of atorvastatin on lipopolysaccharide (LPS)-induced TNF-α production in RAW264.7 macrophages.

Methods RAW264.7 macrophages were treated in different LPS concentrations or at different time points with or without atorvastatin. TNF-α level in supernatant was measured. Expressions of TNF-α mRNA and protein and heme oxygenase-1 (HO-1) were detected by ELISA, PCR, and Western blot, respectively. HO activity was assayed.

Results LPS significantly increased the TNF-α expression and secretion in a dose- and time-dependent manner. The HO-1 activity and HO-1 expression level were significantly higher after atorvastatin treatment than before atorvastatin treatment and attenuated by SB203580 and PD98059 but not by SP600125, suggesting that the ERK and p38 mitogen-activated protein kinase (MAPK) pathways participate in regulating the above-mentioned effects of atorvastatin. Moreover, the HO-1 activity suppressed by SnPP or the HO-1 expression inhibited by siRNA significantly attenuated the effect of atorvastatin on TNF-α expression and production in LPS-stimulated macrophages.

Conclusion Atorvastatin can attenuate LPS-induced TNF-α expression and production by activating HO-1 via the ERK and p38 MAPK pathways, suggesting that atorvastatin can be used in treatment of inflammatory diseases such as sepsis, especially in those with atherosclerotic diseases.

Key words: Lipopolysaccharide; Tumor necrosis factor-α; Heme oxygenase-1; Atorvastatin

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INTRODUCTION

Sepsis, an overwhelming systemic response to infection characterized by systemic inflammation and widespread tissue injury, is increasingly common. Approximately 2.3 cases of severe sepsis per 100 hospital discharges in the USA in 1995, can result in an annual burden of approximately 751,000 cases, 68% of which need treatment in intensive care unit (ICU)[1]. Moreover, the incidence and mortality of sepsis seem to increase in many countries[2-4].

Septic shock is a systemic inflammatory response syndrome (SIRS) and generally accepted that it is not only due to the infection itself, but also related to the host response to infection. Pathogenic microorganisms and their products trigger inflammatory response and activate expression of inflammatory genes, leading to release of a series of inflammatory mediators[5]. Sepsis and septic shock remain the leading cause of death for patients in a noncardiac ICU, although many efforts have been made and significant advances have been achieved in their treatment[2,5]. Major factors contributing to the high morbidity and mortality of septic shock include lack of effective treatment and other diseases occurring as sepsis such as coronary artery disease (CAD)[6]. In fact, many patients in cardiac care unit (CCU) also died of septic shock occurring as cardiovascular diseases, especially severe heart failure and/or CAD. Therapeutic strategy has been developed so that the molecular mechanisms of sepsis can be understood and new pathways can be sought for the treatment of sepsis.

Statins, a class of lipid-lowing drugs, can inhibit enzyme 3-hydroxy-3methylglutary-coenzyme A (HMG-CoA) reductase. It has been shown that statins can decrease blood lipids levels especially cholesterol and the incidence of cardiovascular events. It is no doubt that the cholesterol-lowering property plays an important role in prevention of cardiovascular events. Recently, more attentions have been paid to its non-lipid-lowing effects, especially its anti-inflammatory effects[7-10]. Accordingly, whether statins are valuable for classic inflammatory diseases such as sepsis has been extensively studied. It was recently reported that application of statins in the clinical setting can prevent lipopolysaccharide (LPS) or bacterial infection-related sepsis[11-16]. So far, it is still controversial and undetermined[17-21]. Statins, as the therapeutic drug combined with other pivotal anti-infection drugs, are still deemed to be promising for sepsis. However, their potential mechanisms are complex. As we know, inflammatory cells (e.g. macrophages) and inflammatory factors such as TNF-α play an important in the pathogensis of sepsis. Whether atorvastatin can attenuate TNF-α production in lipopolysaccharide (LPS)-stimulated macrophages was studied in the present study, hoping to ascertain the potential value of atorvastatin for sepsis.

MATERIALS AND METHODS

Cell Culture

Murine RAW264.7 macrophages were obtained from American Type Culture Collection (ATCC, USA) and maintained in RPMI 1640 medium supplemented with penicillin (100 U/mL), streptomycin (100 μg/mL) and 10% fetal bovine serum (FBS) at 37 °C in humidified aircontaining 5% CO_2.

Reagents

RPMI 1640 medium, FBS, peniciline, and streptomycin were obtained from Hyclone, and LPS was purchased from Sigma. Atorvastin was a gift by Pfizer Pharmaceutical Co (China). PD98059, SP600125, and SB203580 (specific inhibitor of ERK, JNK, and p38 MAPK inhibitor) were obtained from Calbiochem. Antibodies to HO-1, ERK, p38 MAPK, and secondary antibody conjugated with horseradish peroxidase (HRP) were purchased from Cell Signaling Technology (CST).

Cell Viability Assay

Cell viability was assayed by MTT assay. Briefly, cells (2×10^4 cells/well) in 96-well plates were treated with different concentrations of atorvastatin (0, 5, 20, 50, 100 μmol/L) or LPS (0, 2, 10, 50, 100 ng/mL) at 37 °C in an atmosphere containing 5% CO_2 for 18 h. Then, 50 μL of RPMI 1640 medium containing MTT (5 mg/mL) was added to each well, followed by incubation for 3 h at 37 °C. A stock solution of MTT was prepared in phosphate-buffered saline (PBS), diluted in RPMI 1640 medium and added to cell-containing wells at a concentration of 5 mg/mL, after the culture medium was removed. The plates were incubated for 4 h at 37 °C in an atmosphere containing 5% CO_2. At the end of incubation, the medium was aspirated and discarded. The formazan crystals in viable cells were dissolved in 100 μL DMSO for 10 min. The optical density (OD) of each

well was measured on a multi-scan reader with a 570 nm wavelength filter. All experiments were performed at least three times.

ELISA

After treatment of RAW264.7 macrophages, culture supernatants were collected and TNF-α concentration in the supernatants was measured using a commercial ELISA kit (R&D System Co., USA) according to its manufacturer's instructions. Three independent experiments were performed.

RNA Preparation and RT-PCR

After treatment of RAW264.7, total RNA was isolated from the cells using Trizol according to its manufacturer's instructions (Invitrogen, USA). RNA was transcribed using the ImProm-IITM Reverse Transcription System (Promega, USA). TNF-α mRNA expression was detected by RT-PCR. SYBR green PCR master mix was obtained from Promega (USA). Housekeeping β-actin mRNA was amplified as an internal control. The primers used were TNF-α (forward 5'-ACC CCC TGA GTC TGC TCA AT-3' and reverse 5'-CCT GGT GGG ACT TGG TTG TA-3'), HO-1 (forward 5'-TCC GAT GGG TCC TTA CAC TC-3' and reverse 5'-ATT GCC TGG ATG TGC TTT TC-3'), β-actin (forward 5'-CTG GTC GTA CCA CAG GCA TT-3' and reverse 5'-CTC TTT GAT GTC ACG CAC GA-3'). The primers were tested by 40 cycles of PCR at 95 °C for 20 s and at 60 °C for 1 min, before separated in ethidium bromide containing agarose gels. The PCR efficiency was examined by serially diluting the template cDNA. The melting curve data were collected for assessing the PCR specificity, and proper negative controls were set in each assay. The mRNA level in each gene was normalized to β-actin mRNA. All samples were tested by 3 duplications, and the data were obtained from three independent experiments.

Western Blotting Assay

Total protein was extracted from cells and protein concentration was measured with protein extraction kit and BCA protein assay kit, respectively (Beyotime Institute of Biotechnology, Beijing, China). β-mercaptoethanol was added to a final concentration of 1%, after which each sample was denatured by boiling for 5 min, followed by heating and then subjected to 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) gel. Electrophoresis productions were transferred onto polyvinylidene difluoride (PVDF) membrane (Millipore), blocked with 3% dry milk/0.1% Tween-20, incubated with primary antibodies and HRP-conjugated secondary antibodies (1:1000), respectively. Finally, proteins were visualized using the ECL plus system (Beyotime Biotechnology, China) according to its manufacturer's instructions. Immunoblotting signals were quantitated using an ImageMaster DVS. The data were obtained from three independent experiments.

Cells Transfection with siRNA

HO-1 siRNA and negative control siRNA were purchased from Invitrogen. The sequence of HO-1 siRNA (5'-end to 3'-end) is UUACAUGGCAUAAAUUC CCACUGCC. The RAW264.7 cells were placed on 6-well plates and incubated overnight with a transfection medium without antibiotics. After that, siRNA of 100 nmol/L was introduced into the cells using Lipofectamine® 2000 (Life Technologies, Invitrogen) according to its manufacturer's instructions. The silencer control siRNA was used as a negative control and introduced into the cells using the same protocol.

HO Activit yAssay

HO enzyme activity was assayed by bilirubin generation as previously described[22]. Briefly, after incubation, RAW264.7 macrophages were washed with PBS. Harvested cells were sonicated and centrifuged (18,000×g, 10 min, 4 °C), and the obtained microsomal pellet was re-suspended in ice-cold potassium phosphate buffer (100 mmol/L). Supernatant proteins (1 mg) were then incubated in the dark with 200 μL reaction mixture containing mouse liver cytosol (1 mg protein), 50 μmol/L hemin, 1 mmol/L NADPH, 2 mmol/L glucose-6-phosphate, and 0.2 unit glucose-6-phosphate dehydrogenase in 100 mmol/L potassium phosphate buffer, pH 7.4, at 37 °C for 1 h. Bilirubin was then extracted with 1 mL chloroform and quantitated with a double-beam spectrophotometer. HO-1 activity was expressed as nanomoles of bilirubin formed per mg protein per hour. Three independent experiments were performed.

Statistical Analysis

All data were expressed as mean±SD. Numeric values were analyzed for the presence of normal distribution. For comparison between multiple groups, statistical significance was tested by ANOVA

with SPSS 13.0 statistical software. $P<0.05$ was considered statistically significant.

RESULTS

Effect of Atorvastatin and LPS on Cell Viability

No significant difference was found in cell death rate among groups ($P>0.05$) (data not shown), suggesting that different atorvastatin or LPS concentrations can not significantly alter the cell viability.

Effects of Atorvastatin on Supernatant TNF-α Level in LPS-treated RAW264.7 Macrophages

After treatment with 100 μmol/L atorvastatin for 1 h, RAW264.7 macrophages were cultured with medium in the presence of 0, 2, 10, 50, 100 ng/mL LPS for 6 h or in the presence of 100 ng/mL LPS for 0, 2, 6, 12, 24 h. LPS increased the TNF-αlevel in a dose- and time-dependent manner (Figure 1A, 1B), which could be significantly attenuated by 100 μmol/L atorvastatin.

Effects of Atorvastatin on TNF-α mRNA Expression in LPS-treated RAW264.7 Macrophages

The cells were cultured and treated as above. As shown in Figure 1, LPS increased the TNF-α mRNA expression in a dose-dependent and time-dependent manner (Figure 1C, 1D) , which could be also significantly reduced by 100 μmol/L atorvastatin.

Effects of Atorvastatin on HO Activity and HO-1 Expression

To determine whether atorvastatin can attenuate inflammatory response by inducing HO-1 expression and secretion, RAW264.7 macrophages were treated with atorvastatin at the doses of 0, 5, 20, 50, 100 μmol/L for 6 h or with 100 μmol/L atorvastatin at 0, 1, 3, 6, 12 h. The HO activity was assayed as above, and the HO-1 mRNA and protein

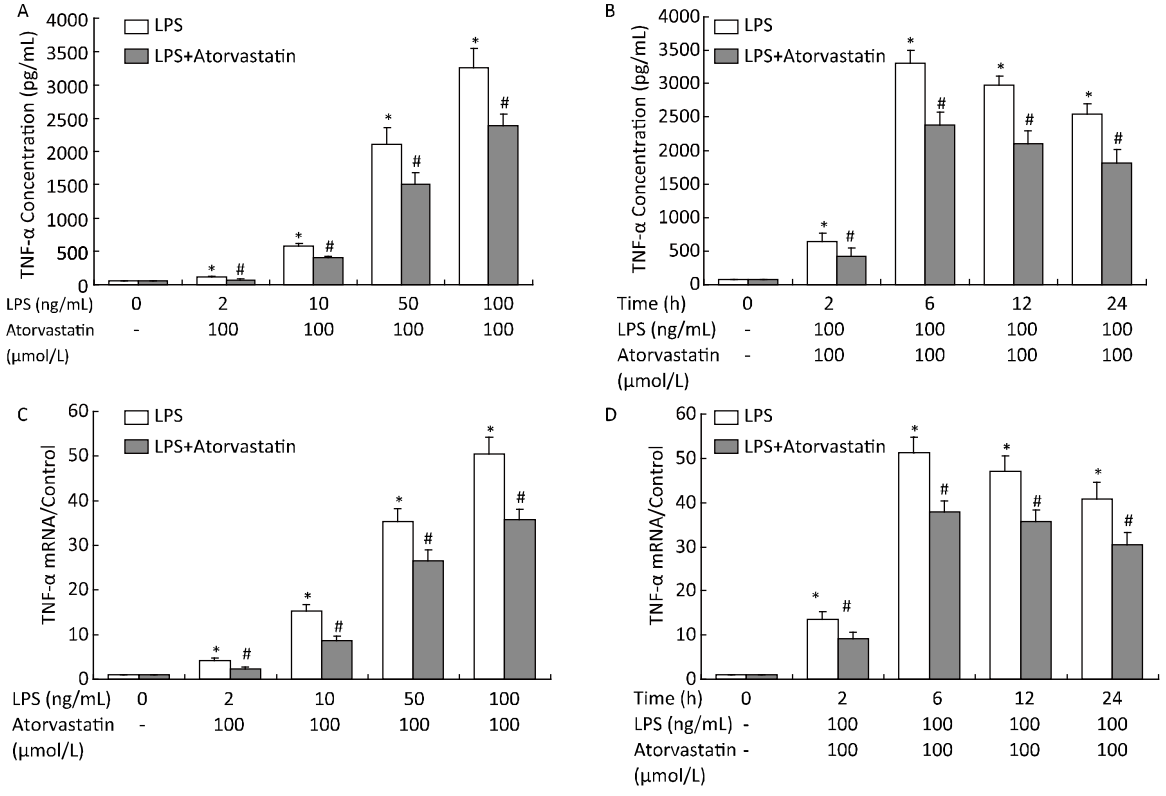

Figure 1. Effects of atorvastatin on TNF-α levels in LPS-treated RAW264.7 macrophages. Cells were treated with atorvastatin (100 μmol/L) or vehicle for 30 min, followed by with different LPS doses (0, 2, 10, 50, 100 ng/mL) or with 100 ng/mL LPS at different time points (0, 2, 6, 12, 24 h). Dose-dependent effects (A, C) and time-dependent effects (B, D) of LPS on supernatant TNF-α level and mRNA expression in RAW264.7 macrophages and the regulation effects of atorvastatin were respectively detected by ELISA and PCR, respectively. $^{*}P<0.01$ *vs.* control group, $^{\#}P<0.01$ *vs.* LPS group.

expression levels were measured by RT-PCR and Western blot, respectively. Atorvastatin increased the HO-1 mRNA and protein expression levels in a dose-and time-dependent manner (Figure 2A, 2B, 2D), up-regulated the HO activity and down-regulated the HO-1 activity in a dose-dependent manner (Figure 2C).

Role of MAPK Pathway in Atorvastatin- activated-HO-1 Expression

As an important regulation pathway of signal transduction, MAPK pathway plays a pivotal role in regulating genes expression. To evaluate the mechanism of atorvastatin-mediated HO-1 activation, RAW264.7 macrophages were treated with 50 μmol/L MAPK pathway inhibitors (PD98059, SB203580, SP600125) for 2 h before 6-h treatment with 100 μmol/L atorvastatin.

Atorvastatin activated the ERK and p38 MAPK pathways in a dose-dependent manner (Figure 3B). The ERK inhibitor (PD98059) and p38 MAPK inhibitor (SB203580) significantly reduced the atorvastatin-induced HO-1 expression. However, the JNK inhibitor (SP600125) did not significantly change the HO-1 mRNA and protein expression (Figure 3A, 3C).

Role of HO-1 in LPS-induced TNF-αExpression and Secretion in RAW264.7 Macrophages

To evaluate the role of HO-1 in LPS-induced TNF-α expression and secretion regulated by atorvastatin, RAW264.7 macrophages were cultured in medium with or without transfection siRNA before treatment with 100 μmol/L atorvastatin and 100 ng/mL LPS. In another paralleled experiment, RAW264.7 macrophages transfected with silence mock siRNA were treated with 10 μmol/L SnPP (inhibitor of HO catalytic activity) for 0.5 h before treatment with 100 μmol/L atorvastatin, and co-cultured with atorvastatin and LPS (100 ng/mL) for 6 h. The TNF-α concentration in culture supernatants was measured by ELISA. The HO-1 protein expression level measured by Western blot was effectively inhibited by the HO-1 siRNA and the HO-1 activity suppressed by SnPP or the HO-1 expression inhibited by siRNA could significantly decrease the attenuation effects of atorvastatin on TNF-α expression and secretion (Figure 4) in LPS-treated RAW264.7 macrophages, suggesting that atorvastatin can regulate LPS-induced TNF-α secretion in RAW264.7 macrophages via the HO-1-dependent pathway.

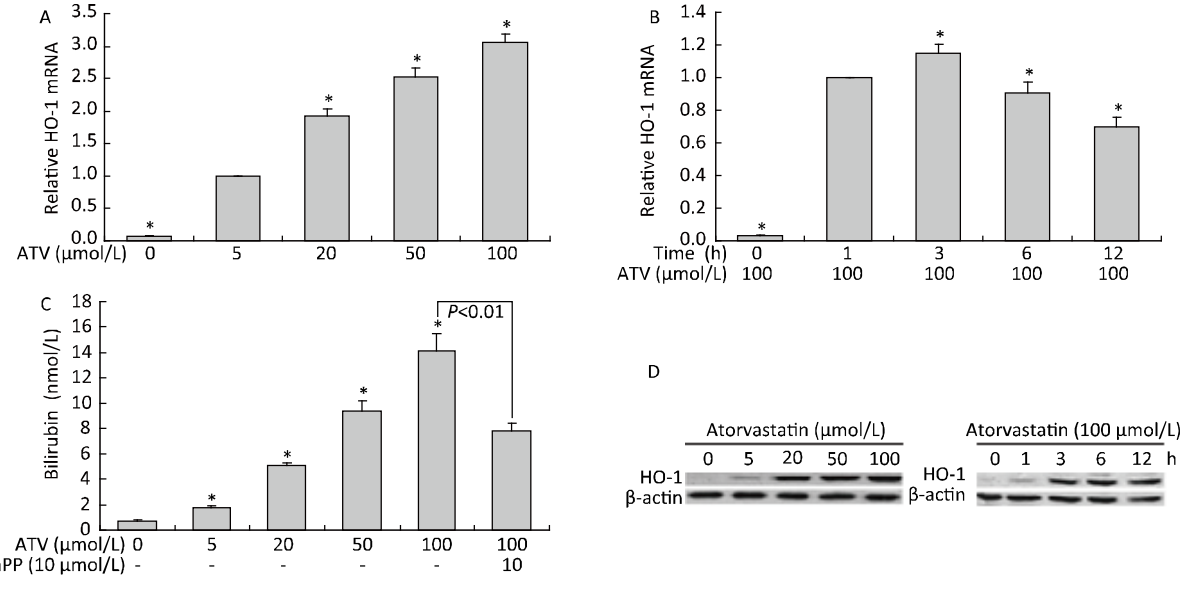

Figure 2. Effects of atorvastatin on HO activity and HO-1 expression in RAW264.7 macrophages. HO enzyme activity was assayed by bilirubin generation as previously described[22]. After treatment, supernatant proteins (1 mg) were incubated in the dark with 200 μL reaction mixture containing mouse liver cytosol (1 mg protein), 50 μmol/L hemin, 1 mmol/L NADPH, 2 mmol/L glucose-6-phosphate, and 0.2 unit glucose-6-phosphate dehydrogenase in 100 mmol/L potassium phosphate buffer, pH 7.4, at 37 °C for 1 h. Bilirubin was extracted and quantitated with a double-beam spectrophotometer. HO-1 activity was expressed as nanomoles of bilirubin formed per mg protein per hour. Dose-dependent and time-dependent effects of atorvastatin on HO-1 expression (A, B, D) and activity (C) were evaluated. $^{*}P<0.01$ *vs.* basic value (Figure 2C) or control value (Figure 2A, 2B).

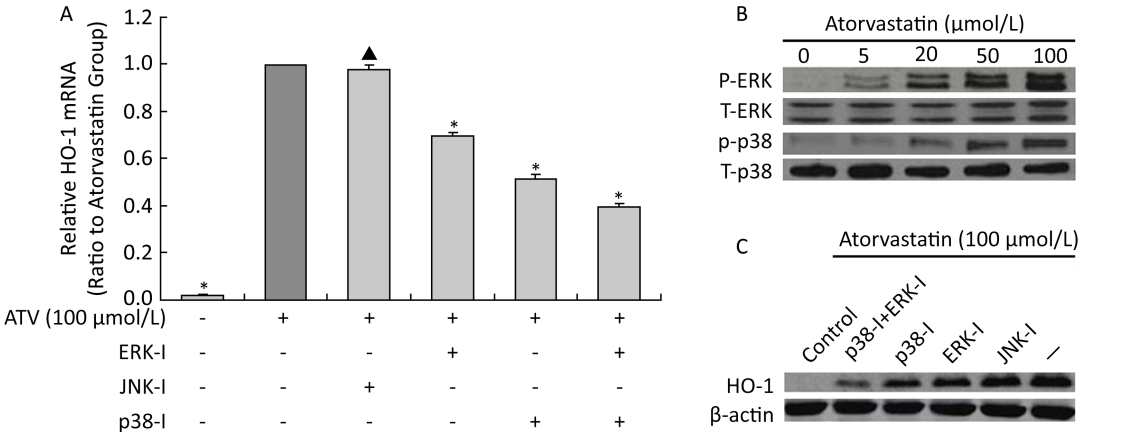

Figure 3. Role of MAPK pathway in atorvastatin-activated HO-1 expression in RAW264.7 macrophages. A: Effects of MAPK pathway inhibitors on HO-1 mRNA expression in RAW264.7 macrophages. B: Effects of atorvastatin on ERK and p38 MAPK pathways protein expression in RAW264.7 macrophages. C: Effects of MAPK pathway inhibitors on HO-1 protein expression in RAW264.7 macrophages. ERK-I=ERK inhibitor, JNK-I=JNK inhibitor, p38-I=p38 inhibitor. $^{*}P<0.01$ and $^{\blacktriangle}P>0.05$ *vs.* atorvastatin group.

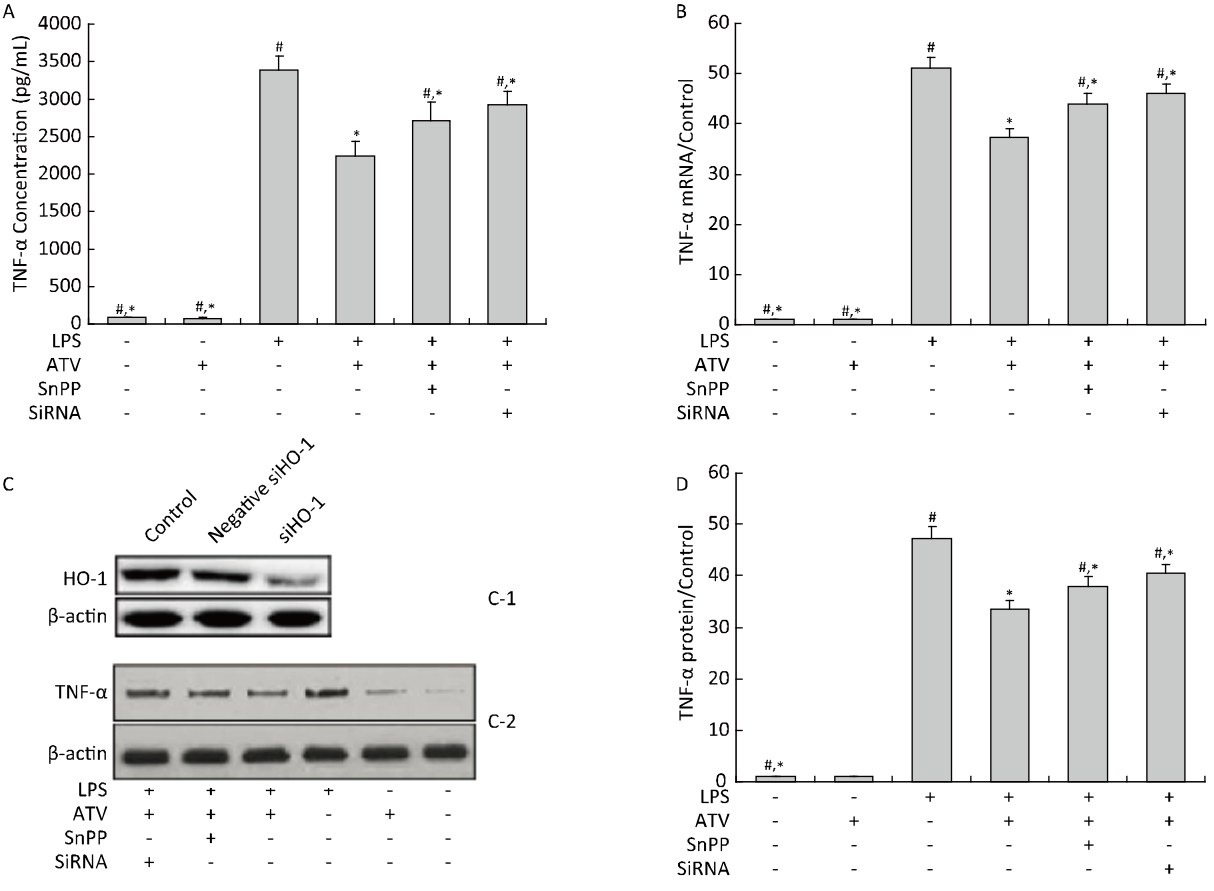

Figure 4. Role of HO-1 in TNF-α secretion in LPS-treated RAW264.7 macrophages. After treatment, mRNA and protein were extracted and supernatants were collected. The effects of atorvastatin on TNF-α secretion (A) and expression (B, C-2, D) were detected by PCR, Western-blot, and ELISA, respectively. Inhibition efficiency of siHO-1 on protein expression was assessed by Western blot (C-1). $^{*}P<0.01$ *vs.* single LPS treatment, $^{\#}P<0.01$ *vs.* LPS+atorvastatin treatment.

DISCUSSION

Sepsis is a systemic inflammatory response that can induce severe organ damage and result in a high mortality. When Gram-negative bacteria invade the blood, LPS is released into circulation, and then LPS-binding protein (LBP) transfers LPS to CD14, which concentrates LPS and increases the sensitivity of LPS signaling to mediate inflammatory response[23]. LPS, a component of the outer membrane of Gram-negative bacteria, is well accepted to be the most potent activator of inflammatory cells such as monocytes and macrophages. LPS-induced macrophage activation results in the secretion of pro-inflammatory mediators, such as TNF-α, which plays a pivotal role in initiating inflammatory response and septic shock. In addition, TNF-α also participates in other inflammatory diseases such as asthma and other pulmonary diseases[24-26], rheumatic arthritis[27], atherosclerotic diseases[28-29], etc.

Heme oxygenases (HO) are several rate-limiting enzymes in heme catabolism, responsible for catalyzing the breakdown of heme into biliverdin, free iron, and carbon monoxide (CO). The mechanism of HO-1-catalyzed degradation of heme has been comprehensively reviewed[30]. Three mammalian HO subtypes have been identified, of which, in most tissues, HO-1 is either expressed at low levels or not expressed at all. It is now well established that HO-1 expression protects cells from physical, chemical, and biologic stress[31-34]. Presently, HO-1 and its products are commonly regarded as the protective effector molecules with anti-inflammatory and pro-inflammation-resolution effects. Accordingly, HO-1 is deemed to play an important role in treating sepsis and other inflammation-related diseases[35-38].

In the present study, atorvastatin could significantly suppress TNF-α production in LPS-stimulated RAW264.7 macrophages, and remarkably increased the HO-1 mRNA and protein expression via the ERK and p38 MAPK pathways. In addition, it suppresses HO-1 activity by SnPP or inhibits HO-1 expression by siRNA which could greatly decrease the attenuation effects of atorvastatin on TNF-α mRNA expression and secretion in LPS-stimulated RAW264.7 macrophages, suggesting that atorvastatin attenuates inflammatory response by activating the HO-1 pathway via the ERK and p38 MAPK pathways, similar to the results from Chen et al.[39]. Although there are controversial points regarding the value of statins for sepsis, it has been demonstrated that continuation of atorvastatin is associated with improved survival[14-16]. The results from this study also suggest that atorvastatin inhibits inflammatory diseases including sepsis by reducing inflammatory media such as TNF-α production especially in patients with cardiovascular diseases accompanying sepsis. However, no study is available on the value of statins in patients with cardiovascular diseases and sepsis. More multi-center randomized controlled trials are needed to further clarify the potential clinical benefits of statins.

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