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Original Article

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Atorvastatin protects cardiomyocytes from oxidative stress by inhibiting LOX-1 expression and cardiomyocyte apoptosis

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Abstract

Coronary artery disease (CAD) is a major health problem worldwide. The most severe form of CAD is acute coronary syndrome (ACS). Recent studies have demonstrated the beneficial role of atorvastatin in ACS; however, the mechanisms underlying this effect have not been fully clarified. Growing evidence indicates that activation of the lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1) plays an important role in oxidative stress-induced cardiomyocyte apoptosis during ACS. In this study, we examined whether atorvastatin inhibits H_2O_2-induced LOX-1 expression and H9c2 cardiomyocyte apoptosis, and investigated the underlying signaling pathway. Treatment of H9c2 cardiomyocytes with H_2O_2 resulted in elevated expression of LOX-1 mRNA and protein, as well as increased caspase-3 and -9 protein expression and cell apoptosis. H_2O_2-induced LOX-1 expression, caspase protein expression, and cardiomyocyte apoptosis were attenuated by pretreatment with atorvastatin. Atorvastatin activated H_2O_2-inhibited phosphorylation of Akt in a concentration-dependent manner. The Akt inhibitor, LY294002, inhibited the effect of atorvastatin on inducing Akt phosphorylation and on suppressing H_2O_2-mediated caspase up-regulation and cell apoptosis. These findings indicate that atorvastatin protects cardiomyocyte from oxidative stress via inhibition of LOX-1 expression and apoptosis, and that activation of H_2O_2-inhibited phosphorylation of Akt may play an important role in the protective function of atorvastatin.

Key words: atorvastatin, H_2O_2, lectin-like oxidized low-density lipoprotein receptor-1, cardiomyocyte, apoptosis, Akt

Introduction

Coronary artery disease (CAD) is a major health problem worldwide. The most severe form of CAD is acute coronary syndrome [ACS; i.e. myocardial infarction (MI) or unstable angina]. ACS refers to any group of symptoms attributed to the obstruction of coronary arteries which is an immediate threat of life and the leading cause of mortality. Although numerous mechanisms contribute to the pathogenesis of ACS, there is clear evidence in the literature indicating that increased production of reactive oxygen species (ROS), such as H_2O_2, leads to the development and progression of cardiomyocyte apoptosis, which plays a pivotal role in the pathogenesis of ischemic-reperfusion injury during ACS [1,2]. Adult cardiomyocytes are terminally differentiated cells that lack the ability to divide and/or proliferate, and accordingly it is important to protect cardiomyocytes through suppression of ROS-induced apoptosis during ACS.

Lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1) is a type II membrane protein of the C-type lectin family. It recognizes multiple classes of ligands, such as oxidized low-density lipoprotein (ox-LDL), acetylated LDL, platelets, apoptotic/aged cells, and bacteria [3]. LOX-1 is expressed in endothelial cells (ECs), macrophages, vascular smooth muscle cells (VSMCs), platelets, and cardiomyocytes and has been demonstrated to actively contribute to all stages of atherogenesis [4]. Recent evidence has indicated that LOX-1 plays an important role in apoptosis. In human and experimental atherosclerotic lesions, LOX-1 expression has been observed in apoptotic cells [5], and LOX-1 plays an important role in ox-LDL-induced apoptosis, both in artery ECs [6] and in VSMCs [7]. Inhibition of LOX-1 expression by an antisense oligonucleotide significantly decreased ox-LDL-induced apoptosis of human coronary artery ECs by 75% [6]. Furthermore, release of ROS activates LOX-1 and induces apoptosis in cardiomyocytes [8], and experiments using the LOX-1 receptor antagonist k-carrageenan have indicated that the increase in cardiomyocyte apoptosis is specifically mediated through the activation of LOX-1-dependent pathway [9]. *In vivo* studies have shown that LOX-1 up-regulation or activation promotes cardiomyocyte apoptosis in rat hearts subject to ischemia-reperfusion injury. In addition, LOX-1 inhibition or deletion can reduce cardiomyocyte apoptosis and infarct size both in MI and in ischemia-reperfusion injury experimental models [10–12]. The Akt signaling pathway plays key roles in the pathogenesis of various processes in the heart [13,14]. There is strong evidence suggesting that short-term activation of Akt has beneficial effects via the inhibition of apoptotic cell death [14]. Moreover, Akt plays a modulatory role in LOX-1 synthesis. Blocking Akt phosphorylation increases LOX-1 expression and activation of LOX-1 attenuates Akt phosphorylation [15].

In the past decade, extensive clinical use of 3-hydroxy-3-methylglutaryl-coenzyme A reductase inhibitors, or statins, for their cholesterol-lowering properties, has improved morbidity and mortality in patients with CAD. Studies indicated the multiple effects of atorvastatin, a member of the statin family, in atherosclerosis. Atorvastatin decreased LOX-1 expression and ox-LDL uptake in human coronary artery ECs [16], attenuated DC maturation, and reduced the inflammatory and oxidative stress responses [17]. Moreover, evidence obtained from the MIRACL [18], PROVE-IT [19], and IDEAL-ACS [20] studies has indicated that atorvastatin causes a significant reduction in recurrent ischemia and death or major cardiovascular events in patients with ACS. Current guidelines recommend initiating statin therapy before discharge, regardless of the baseline LDL level in patients with ACS [21]. However, the molecular mechanism of statins in myocardial protection during ACS has not been fully clarified. In this study, we investigated the effect of atorvastatin on H_2O_2-induced LOX-1 expression and apoptosis in H9c2 cardiomyocytes, and sought to clarify the identity of the Akt signaling pathway involved.

Materials and Methods

Materials

The rat cardiomyoblast cell line H9c2 was obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). H_2O_2 was purchased from Sigma (St Louis, USA) and prepared as a 600 μM solution in phosphate-buffered saline (PBS) immediately before use. Atorvastatin (Lipitor; Pfizer Pharmaceuticals, New York, USA) was dissolved in dimethylsulfoxide (DMSO). After storage at −30°C, the stock solution (100 mM) was diluted to 0.1, 1, and 10 μM, respectively. The CellTiter 96 Aqueous cell viability assay kit (MTS) was purchased from Promega (Madison, USA). The Annexin V/fluorescein isothiocyanate (FITC) kit was purchased from Bender MedSystems GmbH (Vienna, Austria). Antibodies against Akt [Phospho-Akt (Ser473)(D9E) XP Rabbit mAb #4060 and Akt (pan)(C67E7) Rabbit mAb #4691], anti-cleaved caspase-3, and anti-cleaved caspase-9 antibodies used for western blot analysis and the PI3K/Akt inhibitor, LY294002 (50 mM in DMSO), were obtained from Cell Signaling Technology Inc. (Beverly, USA). The anti-LOX-1 antibody was obtained from Abcam (Cambridge, UK).

Cell culture and treatment

H9c2 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum in a humidified incubator at 37°C with 5% CO_2 atmosphere. To examine the effect of atorvastatin, H9c2 cells were pretreated with atorvastatin at different concentrations (0, 0.1, 1, and 10 μM) for 3 h, and then incubated with 600 μM H_2O_2 or vehicle control for 24 h. In the other group, H9c2 cells were pretreated with the Akt inhibitor, LY-294002 (10 μM), for 30 min before incubation with H_2O_2 for 24 h. The cells were then processed in accordance with each assay. In addition, morphologic changes of treated H9c2 cells were monitored.

Cell viability assay

Cell viability was measured using CellTiter 96 Aqueous cell viability assay kit (MTS). Cells were seeded into 96-well plates at a density of 1×10^4 cells/well and allowed to attach overnight. After the indicated treatment, the assay was performed according to the manufacturer's instructions. In brief, 20 μl of MTS/PMS mixture was added into each well. Plates were incubated at 37°C for 1 h, and the absorbance at 490 nm was measured. Background absorbance was subtracted according to the readings from blank controls. Treatments were performed in triplicate and the results were expressed as the average percentage of total cell number.

Hoechst 33342/PI double staining

Morphological changes of the nuclei were observed by Hoechst 33342/PI staining. In brief, cultures were incubated with Hoechst 33342 (5 μg/ml; Invitrogen, Carlsbad, USA) for 15 min at 37°C, followed by propidium iodide (PI; 15 μg/ml; Invitrogen) for 10 min at 4°C. After extensive washing, Hoechst/PI-stained cells were examined under an inverted fluorescent microscope IX81 (Olympus, Tokyo, Japan) equipped with epifluorescence illumination.

Flow cytometry analysis

To quantify apoptotic cells, flow cytometry was used after Annexin V binding and PI staining. After treatment as indicated, the cells were washed with ice-cold PBS, and stained with FITC-coupled Annexin V protein and PI for another 20 min. Flow cytometry was performed with a 488-nm laser coupled to a cell sorter (FacsCalibur; BD Biosciences, San Jose, USA). Cells stained with Annexin V alone were considered early apoptotic, and cells stained with both PI and Annexin V were considered late apoptotic.

Western blot analysis

After treatment, the cultured cells were washed with cold PBS and then lysed in a buffer containing 50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM Na_2EDTA, 1% Triton-X 100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 mM NaF, 1 μg/ml leupeptin, and 1 mM phenylmethanesulfonyl fluoride. Protein concentration was quantified using a BCA protein assay kit. Proteins (50 μg/

lane) were separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred onto a polyvinylidene difluoride membrane (Millipore, Milford, USA). Membranes were blocked in blocking buffer (PBS with 7.5% non-fat dry milk, 2% BSA, and 0.1% Tween-20) for 1 h and incubated with primary antibodies (1 : 1000) overnight at 4°C. Membranes were washed subsequently in Tris-buffered saline with 0.1% Tween-20 washing buffer and incubated with horseradish peroxidase-conjugated secondary antibodies at room temperature for 1 h. Membranes were then detected by enhanced chemiluminescence detection reagents.

Quantitative RT-PCR

Quantitative RT-PCR was performed by LightCycler Technology (Roche Molecular Biochemicals, Mannheim, Germany) using SYBR Green I detection reagents. In all assays, cDNA was amplified in a standard program (10 s denaturing step; 55 cycles of 5 s at 95°C, 15 s at 65°C, and 15 s at 72°C; melting point analysis in 0.1°C steps; final cooling step). Each LightCycler capillary was loaded with 1.5 μl of DNA Master Mix, 1.8 μl of $MgCl_2$ (25 mM), 10.1 μl of H_2O, and 0.4 μl of each primer (10 μM). The final amount of cDNA per reaction was reversely transcribed from 2 ng RNA. Relative quantification of target genes was performed using a mathematical model recommended by Roche Molecular Biochemicals. The following primers were used: LOX-1_f (5′-gtggaggagctcttcaggga-3′) and LOX-1_r (5′-aggcacccagggtgatgcaa-3′). The primers used for the housekeeping gene, which was used for normalization, were as follows: GAPDH_f (5′-accacgtccatgccatcac-3′) and GAPDH_r (5′-tcc accaccctgttgctgta-3′).

Statistical analysis

All experiments were repeated three times. The results were expressed as the mean ± SD. Statistical significance was analyzed by one-way analysis of variance. A P-value of <0.05 was considered statistically significant.

Results

Atorvastatin inhibits H_2O_2-induced morphology and viability changes in H9c2 cells

Morphological observations were carried out during the experiments. H9c2 cells were treated with H_2O_2 (600 μM) in the presence or absence of 0.1, 1, and 10 μM atorvastatin, or with 10 μM atorvastatin only for 24 h. After H_2O_2 treatment, a large fraction of cells exhibited apoptosis-like changes, such as detachment and cytoplasmic shrinkage leading to rounding. However, the proportion of cells with abnormal morphology decreased with increasing atorvastatin dosage, indicating a dose-dependent preventive effect (**Fig. 1A**). Cell viability was measured using CellTiter 96 Aqueous cell viability assay kit (MTS). H_2O_2 treatment significantly decreased the viability of H9c2 cells. However, in the presence of different doses of atorvastatin, the induction of cell death was blocked in a dose-dependent manner (**Fig. 1B**).

Atorvastatin blocks H_2O_2-induced H9c2 cell apoptosis

The anti-apoptotic effects of atorvastatin were detected by both Hoechst 33342/PI staining and flow cytometry analysis. Morphological changes in the nuclei were observed by Hoechst 33342/PI staining in H9c2 cells (**Fig. 2**). Treatment with H_2O_2 increased the number of apoptotic cells with nuclear condensation, and the addition of atorvastatin (0.1–10 μM) reduced the number of apoptotic cells induced by H_2O_2 in a concentration-dependent manner. Cells treated with atorvastatin alone displayed nuclei with normal morphology. In addition, to obtain a definitive quantification of the effect of atorvastatin on H_2O_2-induced H9c2 cell apoptosis, the percentage of apoptotic cells was determined by flow cytometry analysis. As shown in **Fig. 3**, after 24 h of H_2O_2 (600 μM) treatment, the apoptosis index of H9c2 cells was markedly increased compared with that of control group ($P < 0.01$). Interestingly, pretreatment with atorvastatin for 3 h at different concentrations (0.1, 1, and 10 μM) significantly decreased the apoptosis index compared with the H_2O_2-treated group ($P < 0.01$). These results demonstrated that H_2O_2 treatment significantly increased H9c2 cell apoptosis, in the presence of different doses of atorvastatin, and apoptosis was significantly blocked in a dose-dependent manner.

Atorvastatin suppresses H_2O_2 induction of LOX-1 mRNA and protein levels in H9c2 cells

Recent studies have indicated that the activation of LOX-1 by oxidative stress plays an important role in cardiomyocyte apoptosis [9]. Hence, we examined the expression of LOX-1 at the mRNA and protein levels by RT-PCR and western blot analysis, respectively. Treatment of H9c2 cells with H_2O_2 for 24 h resulted in increased levels of LOX-1 mRNA and protein, while pretreatment of cells with atorvastatin markedly suppressed this effect in a dose-dependent manner (**Fig. 4**).

Atorvastatin activates H_2O_2-suppressed phosphorylation of Akt in H9c2 cells

Akt has been found to modulate LOX-1 synthesis [22]. To gain further insight into the potential molecular mechanisms involved in the above-mentioned experiments, the effect of H_2O_2 and/or atorvastatin treatment on the Akt pathway was examined. Western blot analysis indicated that H_2O_2 treatment resulted in a decrease in the phosphorylated forms of Akt compared with non-H_2O_2-treated cells, and atorvastatin reversed this effect in a dose-dependent manner. Total amounts of Akt protein were not influenced by individual or combined treatment with H_2O_2 and atorvastatin (**Fig. 5A**). The effect of LY-294002 (10 μM), on Akt phosphorylation was also investigated. Western blot analysis indicated that the ability of atorvastatin to activate phosphorylation of Akt was abolished by LY294002 (**Fig. 5B**).

Atorvastatin suppresses H_2O_2-induced caspase-3 and caspase-9 up-regulation and apoptosis through activating the Akt signal pathway

Caspase-3 and caspase-9 are important molecules in the apoptosis pathway, and their expression is positively correlated with cell apoptosis. The expression levels of caspase-3 and caspase-9 were examined by western blot analysis. Our results showed that H_2O_2 (600 μM) significantly increased caspase-3 and caspase-9 expression, whereas the addition of 10 μM atorvastatin to the culture system significantly suppressed H_2O_2-induced caspase-3 and -9 expression. To determine whether the Akt signal pathway activation was responsible for the protective effect of atorvastatin, the effect of blocking the Akt pathway was observed. In the presence of LY294002 (10 μM), the suppressive effects of atorvastatin on H_2O_2-induced caspase up-regulation were significantly reversed (**Fig. 6**). Flow cytometry analysis also showed that atorvastatin significantly decreased the percentage of H_2O_2--induced apoptotic H9c2 cells. Pretreatment with LY294002 reduced the protective effect of atorvastatin against H_2O_2-induced apoptosis in

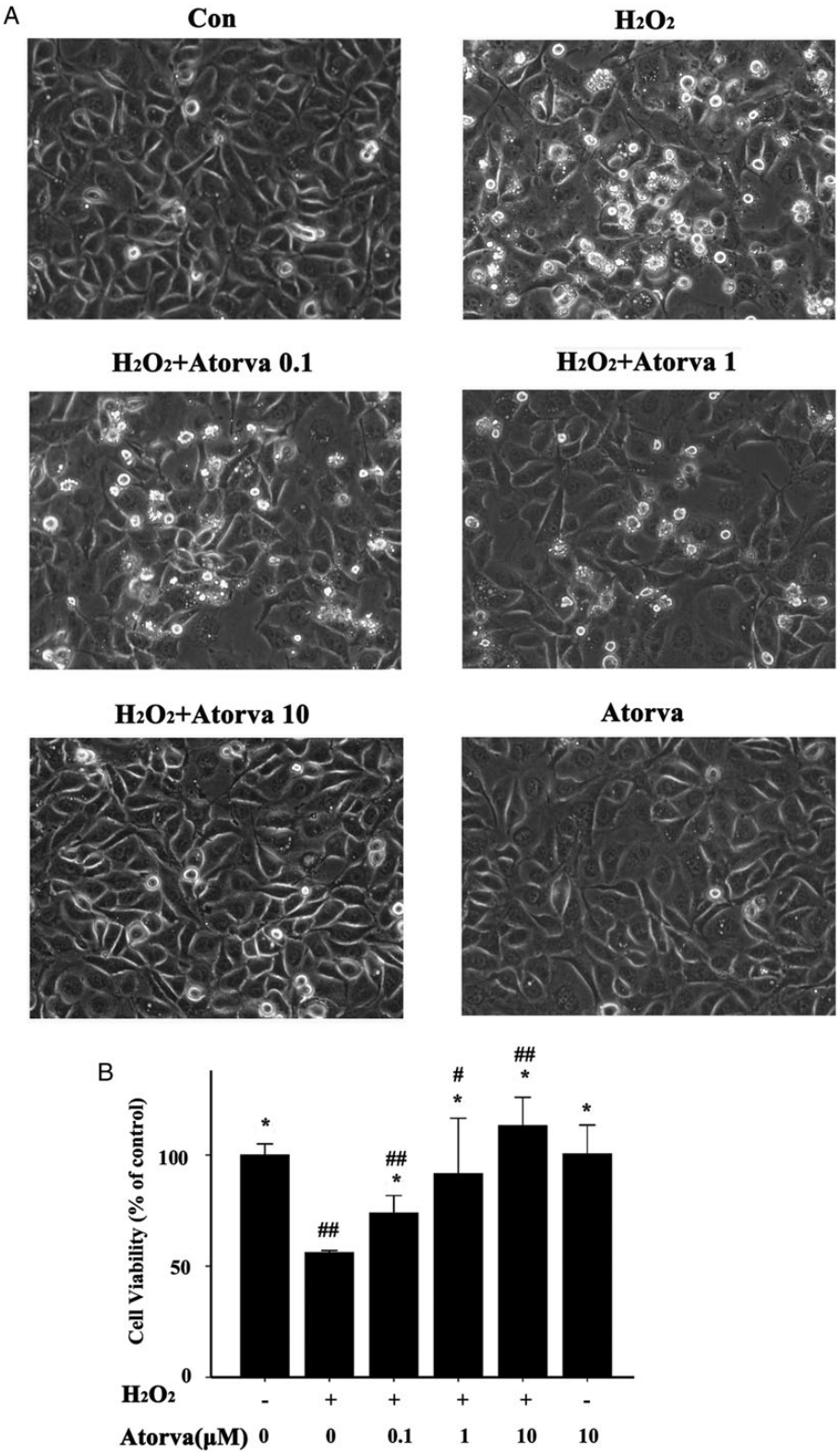

Figure 1. Atorvastatin inhibits changes of cell morphology and viability induced by H_2O_2 in H9c2 cells (A) Treated with H_2O_2 (600 μM) for 24 h, a large fraction of H9c2 cells exhibited apoptosis-like changes such as detachment and cytoplasmic shrinkage leading to rounding. The proportion of cells with abnormal morphology induced by H_2O_2 decreased in parallel with the concentration of atorvastatin. Con: vehicle-treated cells, H_2O_2: H_2O_2 (600 μM) alone, H_2O_2 + Atorva 0.1: H_2O_2 + atorvastatin (0.1 μM), H_2O_2 + Atorva 1: H_2O_2 + atorvastatin (1 μM), H_2O_2 + Atorva 10: H_2O_2 + atorvastatin (10 μM), and Atorva: atorvastatin (10 μM) alone. Magnification: ×10. (B) Cell viability was measured using CellTiter 96 Aqueous cell viability assay kit (MTS). H_2O_2 treatment significantly decreased the viability of H9c2 cells. In the presence of different doses of atorvastatin, the induction of cell death was blocked in a dose-dependent manner (5×10^4/well). Data are presented as the mean ± SD of three independent experiments. $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ compared with normal control; $^{*}P < 0.01$ compared with H_2O_2 treatment alone.

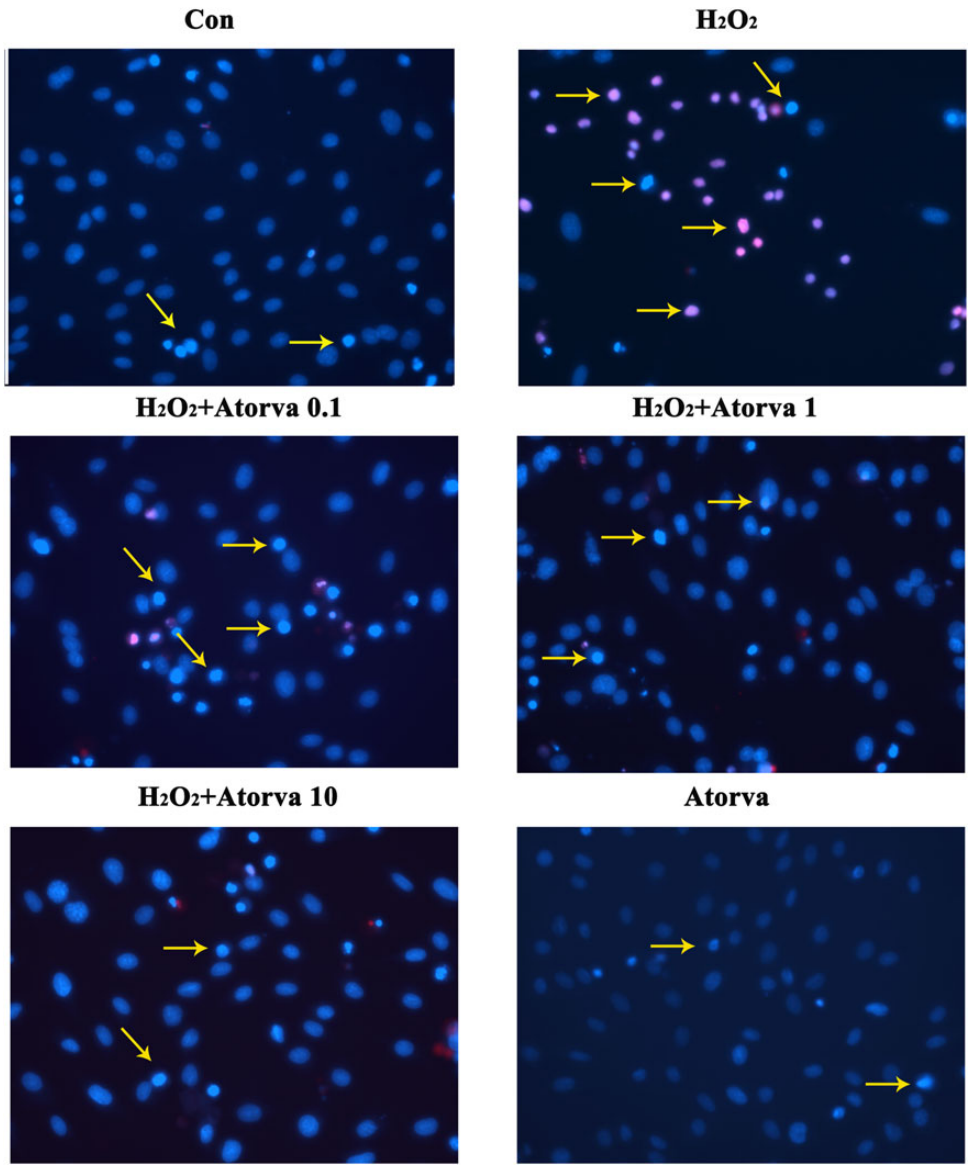

Figure 2. Atorvastatin blocks H_2O_2-induced morphological changes in the H9c2 cell nuclei observed by Hoechst 33342/PI staining Vehicle-treated cells displayed nuclei with normal morphology. Apoptotic cells are characterized by nuclear condensation and stained color as indicated. Treatment with H_2O_2 increased the number of apoptotic cells, and the addition of atorvastatin reduced the number of apoptotic cells induced by H_2O_2 in a concentration-dependent manner. Cells treated with atorvastatin alone displayed nuclei with normal morphology. Con: vehicle-treated cells; H_2O_2: H_2O_2 (600 μM) alone, H_2O_2+Atorva 0.1: H_2O_2 + atorvastatin (0.1 μM), H_2O_2+Atorva 1: H_2O_2 + atorvastatin (1 μM), H_2O_2+Atorva 10: H_2O_2 + atorvastatin (10 μM), and Atorva: atorvastatin (10 μM) alone.

H9c2 cells (**Fig. 7**). Therefore, the Akt signaling pathway is involved in the anti-apoptotic effect of atorvastatin.

Discussion

This study shows the protective effects of atorvastatin on H_2O_2-induced LOX-1 expression and cell apoptosis via the Akt pathway in H9c2 cardiomyocytes. The cardiomyoblast cell line H9c2, derived from embryonic rat heart, possesses many characteristics of cardiomyocytes and is one of the most appropriate *in vitro* cardiomyocyte models [23]. Our results indicated that H_2O_2 up-regulates the expression of LOX-1, caspase-3, and caspase-9 and induces apoptosis of H9c2 cardiomyocytes. Atorvastatin inhibits H_2O_2-mediated LOX-1 expression, caspase up-regulation and apoptosis in H9c2 cardiomyocytes. Mechanistically, we have shown that atorvastatin activates H_2O_2-suppressed phosphorylation of Akt. The ability of atorvastatin to induce Akt phosphorylation and anti-apoptosis is abolished by LY294002. Our findings support the hypothesis that inhibition of LOX-1 expression in H9c2 cardiomyocytes via the Akt pathway plays an important role in the protective effects of atorvastatin against oxidative stress-induced cardiomyocyte apoptosis. Our results also support the cardioprotective advantage of atorvastatin in human and animal models.

Accelerated cardiomyocyte apoptosis has been shown to play a pivotal role in the pathogenesis of ischemic injury and reperfusion injury during ACS [2]. Recent studies have indicated that the activation of LOX-1 by oxidative stress plays an important role in cardiomyocyte apoptosis [9]. LOX-1 inhibition or deletion can significantly reduce cardiomyocyte apoptosis [10,11]. Moreover, LOX-1 activation can induce

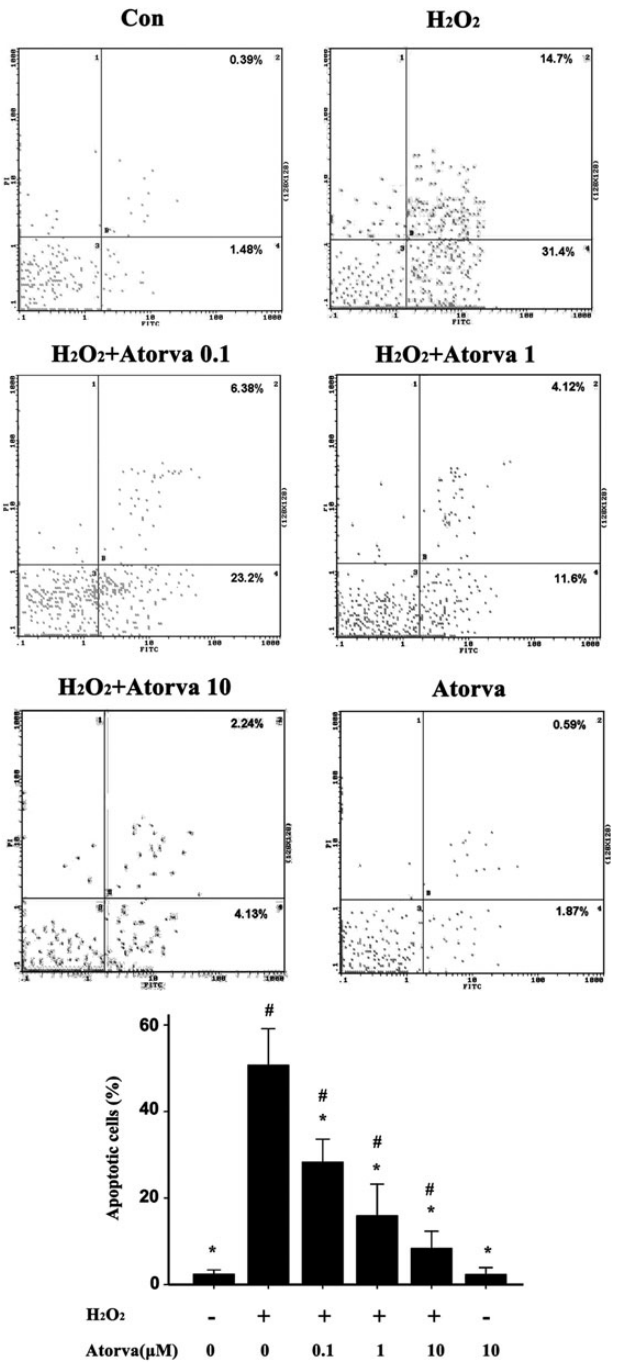

Figure 3. Atorvastatin blocks H_2O_2-induced H9c2 cell apoptosis Apoptotic cells detected by FITC-Annexin V/PI double staining flow cytometry. Region UR shows the late apoptotic cells ($FITC^+/PI^+$), region LL shows the viable cells ($FITC^-/PI^-$), and region LR shows the early apoptotic cells ($FITC^+/PI^-$). H_2O_2 treatment significantly increased H9c2 cell apoptosis. In the presence of atorvastatin, apoptosis was significantly blocked in a dose-dependent manner. Con: vehicle-treated cells; H_2O_2: H_2O_2 (600 μM) alone; H_2O_2 + Atorva 0.1: H_2O_2 + atorvastatin (0.1 μM); H_2O_2 + Atorva 1: H_2O_2 + atorvastatin (1 μM); H_2O_2 + Atorva 10: H_2O_2 + atorvastatin (10 μM); Atorva: atorvastatin (10 μM) alone. Data are presented as the mean ± SD of three independent experiments. $^{\#}P < 0.01$ compared with normal control; $^{*}P < 0.01$ compared with H_2O_2 treatment alone.

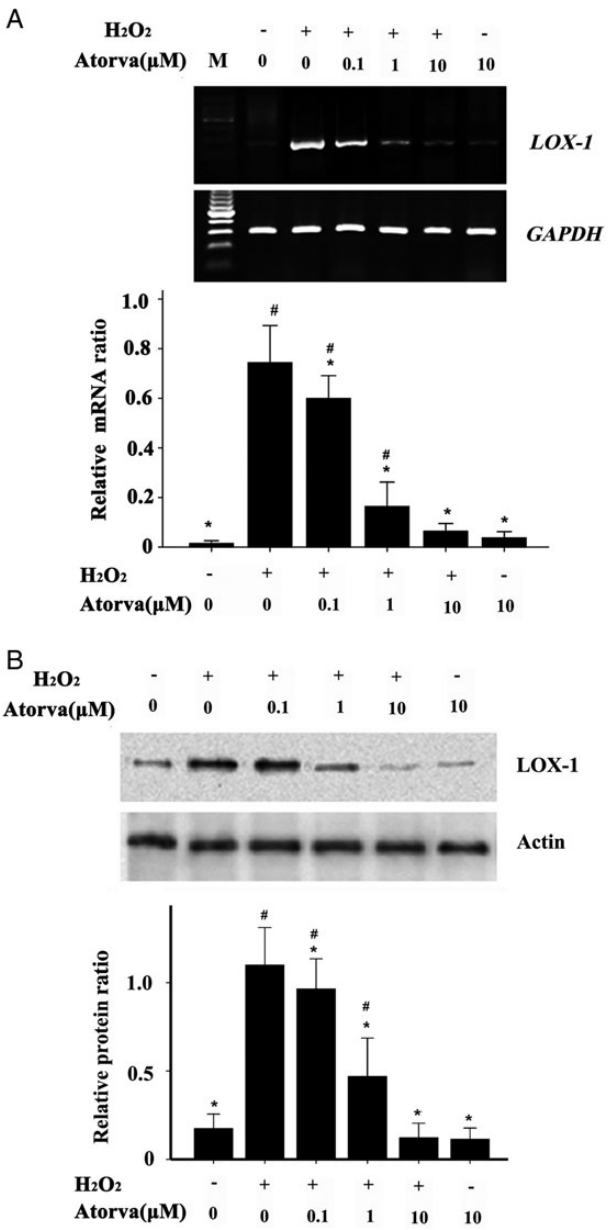

Figure 4. Pretreatment of H9c2 cells with atorvastatin inhibits H_2O_2-induced LOX-1 expression in a dose-dependent manner Data are presented as the mean ± SD of three independent experiments. $^{\#}P < 0.01$ compared with normal control; $^{*}P < 0.01$ compared with H_2O_2 (600 μM) treatment alone. (A) mRNA level of *LOX-1* in treated H9c2 cells was detected by quantitative RT-PCR. (B) Protein expression of LOX-1 in treated H9c2 cells was detected by western blot analysis.

oxidative stress [8]. It indicates the existence of a positive feedback loop between oxidative stress and LOX-1 expression. In this study, we showed that H_2O_2 induces an increase in the number of apoptotic cardiomyocytes and caspase protein expression, an increase in the expression levels of LOX-1 mRNA and protein, but a decrease in H9c2 cell viability. These results suggested that H_2O_2 induces cardiac myocyte apoptosis through the activation of a LOX-1-dependent pathway.

Evidence obtained from clinical studies indicates that atorvastatin provides significant protection against major cardiovascular events in patients with ACS. The protective effects of statins in ACS may include prevention of endothelial dysfunction [24], enhanced plaque stability, anti-inflammatory effects, and correction of prothrombotic tendencies [25]. Statin therapy during the peri-infarct period significantly

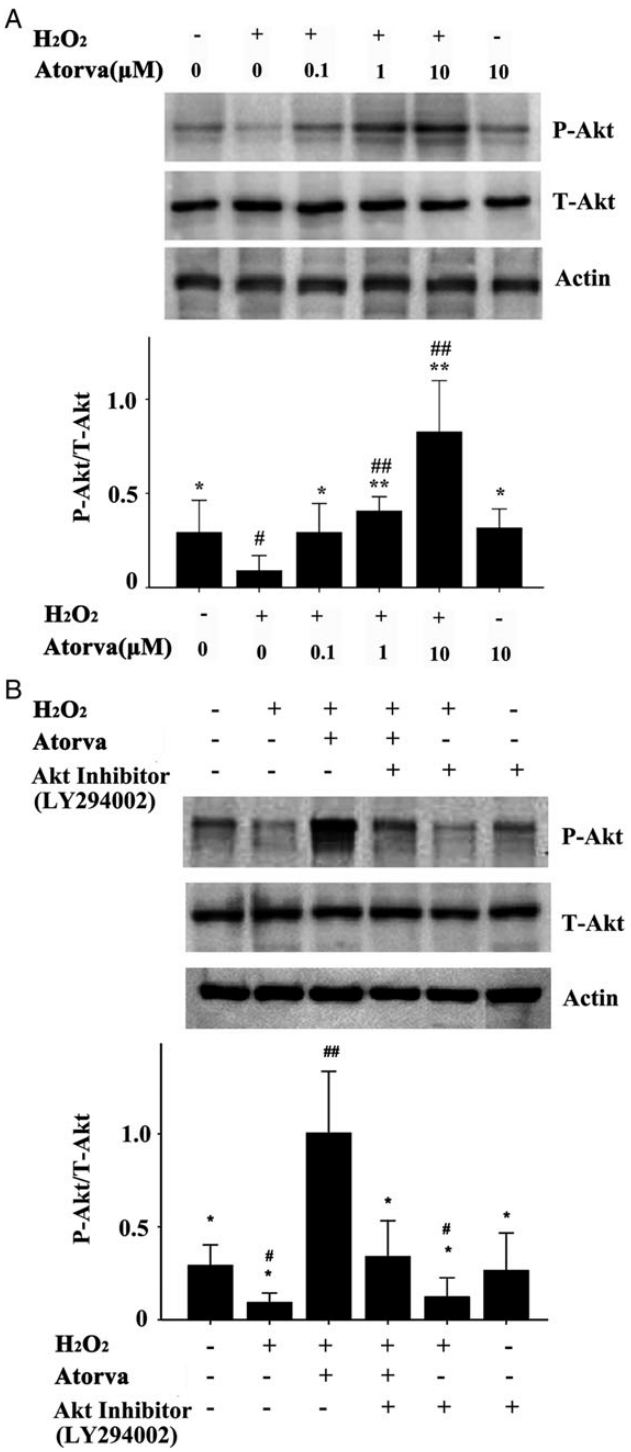

Figure 5. Atorvastatin activates H_2O_2-suppressed phosphorylation of Akt in H9c2 cells (A) Western blot analysis showed that atorvastatin activated H_2O_2-suppressed phosphorylation of Akt in a dose-dependent manner. Data are presented as the mean ± SD of three independent experiments. $^{\#}P<0.05$ and $^{\#\#}P<0.01$ compared with normal control; $^{*}P<0.05$ and $^{**}P<0.01$ compared with H_2O_2 treatment alone. (B) The atorvastatin induced phosphorylation of Akt was suppressed by the Akt inhibitor LY294002 in H_2O_2-treated H9c2 cells. $^{\#}P<0.05$ and $^{\#\#}P<0.01$ compared with normal control; $^{*}P<0.01$ compared with H_2O_2 (600 μM) + atorvastatin (10 μM) treatment.

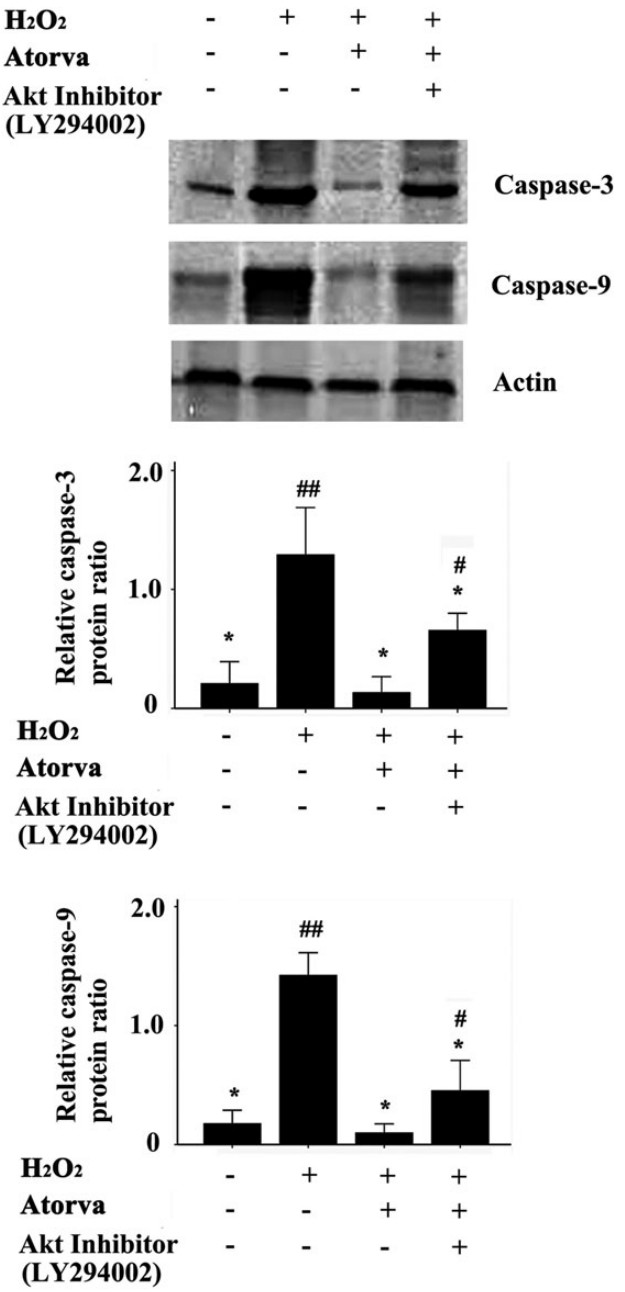

Figure 6. Atorvastatin suppresses H_2O_2-induced caspase-3 and caspase-9 up-regulation through activating Akt signal pathway in H9c2 cells Protein expression of caspase-3 and caspase-9 in treated H9c2 cells was detected by western blot analysis. Atorvastatin (10 μM) suppressed H_2O_2 (600 μM)-induced caspase-3 and caspase-9 up-regulation. The Akt inhibitor, LY294002 (10 μM), inhibited the suppressive effects of atorvastatin. Data are presented as the mean ± SD of three independent experiments. $^{\#}P<0.01$ compared with normal control; $^{*}P<0.01$ compared with H_2O_2 treatment alone.

decreased myocardial apoptosis and improved left ventricular function in the experimental animal model of MI [26,27]. However, the molecular mechanism of atorvastatin in myocardial protection during ACS has not been fully clarified. In this study, H9c2 cardiomyocytes were used to investigate the protective effects of atorvastatin on H_2O_2--induced LOX-1 expression and cell apoptosis. To our knowledge, this

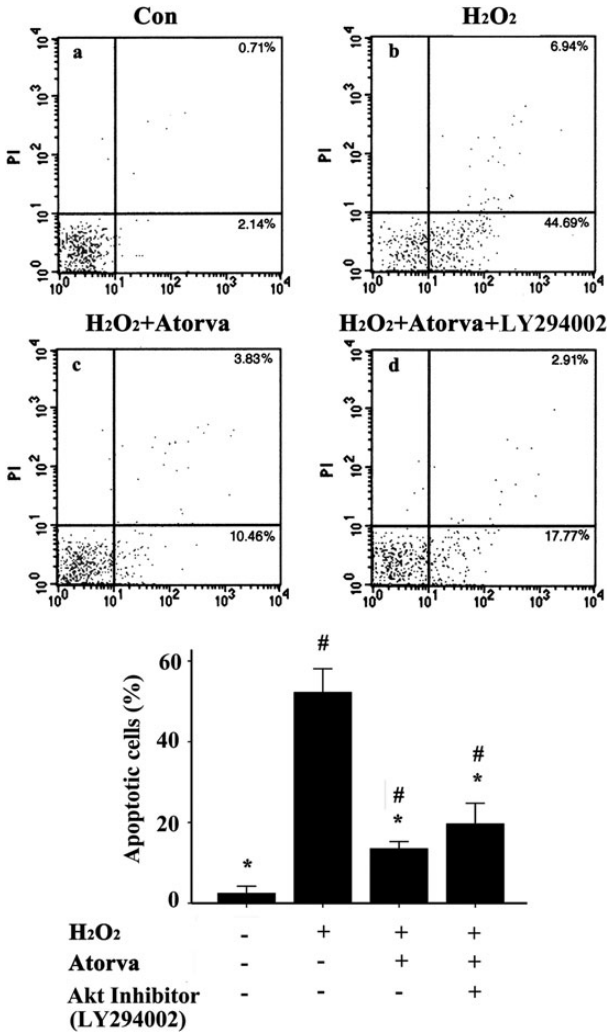

Figure 7. Atorvastatin suppresses H_2O_2-induced apoptosis through activating Akt signal pathway in H9c2 cells Flow cytometry analysis showed that atorvastatin blocks H_2O_2-induced H9c2 cell apoptosis. The Akt inhibitor, LY294002, reduced the protective effect of atorvastatin against apoptosis. Con: vehicle-treated cells; H_2O_2: H_2O_2 (600 μM) alone; H_2O_2 + Atorva: H_2O_2 + atorvastatin (10 μM); H_2O_2 + Atorva + LY294002: H_2O_2 + atorvastatin (10 μM) + LY294002 (10 μM).

study is the first report to demonstrate that atorvastatin attenuates H_2O_2-induced apoptosis of H9c2 cardiomyocytes via a LOX-1-dependent pathway in a concentration-dependent manner. It establishes a possible mechanistic basis for the beneficial effects of statins in ACS. Our results are supported by previous studies of statins demonstrating protection against atherosclerosis through the LOX-1 pathway in other cell lines. Ox-LDL uptake by ECs induces endothelial activation, dysfunction and loss of integrity, and alterations in cell secretory function. These changes in endothelial biology provide an early event in atherosclerosis [16]. Statins inhibit the expression of LOX-1 elicited by ox-LDL and, subsequently, attenuate the uptake of ox-LDL, decrease the expression of adhesion molecules [28], reverse the reduction in PKB activity [16], and modulate inflammatory activation [29] in ECs. Other studies have shown that statins down-regulated LOX-1 expression in macrophages [30], aortic SMCs [30], and platelets [31,32] to influence foam cell formation and platelet activation.

The Akt pathway plays key roles in the pathogenesis of various processes in the heart [13,14]. There is strong evidence in the literature suggesting that short-term activation of Akt has beneficial effects via the inhibition of apoptotic cell death [14]. Moreover, Akt plays a modulatory role in LOX-1 synthesis. Blocking Akt phosphorylation increases LOX-1 expression and activation of LOX-1 attenuates Akt phosphorylation [15]. It is well known that statins can inhibit the synthesis of isoprenoid intermediates such as farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP) downstream of the mevalonate pathway [33]. FPP and GGPP participate in protein prenylation, a posttranslational modification of many proteins, including small GTPases, such as Rho, Rac1, and Ras, which enables their correct localization and participation in signal transduction processes [33,34]. It was reported that Rho A can inhibit the PI3K/Akt pathway [35]. As statins can inhibit GTPases, they can also activate the PI3K/Akt pathway [36]. Consistent with this idea, our study encompassed the examination of Akt targets to explore the possible underlying mechanism of the attenuation of H_2O_2-induced LOX-1 expression and H9c2 cell apoptosis by atorvastatin. Our results showed that after 24 h treatment with H_2O_2, phosphorylation of Akt was inhibited in H9c2 cardiomyocytes, and atorvastatin inhibited this effect in a dose-dependent manner. The specific Akt inhibitor, LY294002, abolished the ability of atorvastatin to increase phosphorylation of Akt and protect H9c2 cardiomyocytes against H_2O_2-induced apoptosis. Caspase-3 and caspase-9 are important molecules in the apoptosis pathway, and their expressions are positively correlated with cell apoptosis. In the presence of LY294002, the suppressive effects of atorvastatin on H_2O_2-induced caspase up-regulation were significantly reversed. These results suggested that atorvastatin may prevent cardiovascular disease by attenuating cardiomyocyte LOX-1 expression and apoptosis via interference with Akt during oxidative stress. Data from an *in vivo* study demonstrated that activation of Akt reduced cardiomyocyte apoptosis, preserved cardiac function, and enhanced cardiac myocyte survival after myocardial ischemia-reperfusion injury [37]. Given that atorvastatin protects cardiomyocytes by interfering with Akt signaling during oxidative stress, further experiments are required to elucidate the complexity of the feedback regulatory pathways involved in this process.

In conclusion, this study demonstrates that atorvastatin protects cardiomyocytes from oxidative stress by inhibiting LOX-1 expression and cell apoptosis, and activation of H_2O_2-inhibited phosphorylation of Akt may play an important role in the protective effect of atorvastatin. Our results confirm and expand the body of evidence describing the cardioprotective advantage of statins in humans and animal models, and establish a possible mechanistic basis for the beneficial effects of statins in ACS.

Supplementary Data

Supplementary data are available at *ABBS* online.

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