

Effects of various doses of atorvastatin on vascular endothelial cell apoptosis and autophagy *in vitro*

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Received August 23, 2016; Accepted January 26, 2018

DOI: 10.3892/mmr.2019.9828

Abstract. Atorvastatin (Lipitor™) is a lipid-lowering agent that is widely used in the treatment of cardiovascular diseases. Previous research has largely focused on its cholesterol-lowering effects; however, a limited number of studies have investigated the actions of atorvastatin on vascular endothelial cells. In the present study, the effects of various doses of atorvastatin were investigated on human umbilical vein endothelial cells (HUVECs). HUVECs were treated with various concentrations of atorvastatin in serum-free or serum-containing medium, and alterations in HUVEC morphology were observed. Cell survival and necrosis rates were evaluated using sulforhodamine B and lactate dehydrogenase assays, respectively. In addition, the protein expression levels of cellular apoptosis and autophagy markers were detected using western blot analysis. The results revealed that HUVEC morphology was altered following treatment with various concentrations of atorvastatin. In addition, autophagy was demonstrated to be induced by atorvastatin treatment at all concentrations, whereas high concentrations appeared to induce apoptosis and suppress the survival of HUVECs. In conclusion, the results of the present study suggested that various doses of atorvastatin may exert differential effects on HUVECs, and high doses may suppress angiogenesis. Therefore, atorvastatin may present a novel potential anti-tumor therapeutic strategy. However, further studies are required to fully elucidate the association between the dose of atorvastatin and its clinical outcome.

Introduction

Atorvastatin (Lipitor™) is the most commonly prescribed statin for decreasing cholesterol levels in patients with cardiovascular

disorders, including hyperlipidemia, atherosclerosis and arterial plaques (1). Atorvastatin has been demonstrated to significantly limit the occurrence of cardiovascular events in patients with average and high serum cholesterol levels (2). In addition, atorvastatin has been reported to suppress oxidative stress and platelet activation, and thus prevent or modulate coronary thrombosis (3). The protective effects of atorvastatin are mediated by molecular mechanisms that may include promoting microvascular formation, anti-inflammatory effects, and promoting endothelial progenitor cell (EPC) homing in ischemic tissues (4,5). High doses of atorvastatin have been reported to exert beneficial effects in cardiovascular disease in clinical practice. For instance, high doses facilitated EPC mobilization in patients that had undergone percutaneous coronary intervention, which may limit the extent of endothelial injury (6,7). In addition, a high dose of atorvastatin was revealed to prevent contrast-induced nephropathy following carotid artery stenting (8).

Vascular endothelial cells (VECs) form the barrier between circulating blood in the lumen of the vessel and the vessel wall. Endothelial dysfunction has been implicated in the pathogenesis of cardiovascular diseases (9). Low concentrations of atorvastatin have been reported to protect endothelial cells from apoptosis (10); however, the effects of atorvastatin on VECs at higher doses have yet to be elucidated.

Autophagy is an evolutionarily conserved process, which serves to degrade intracellular components, including abnormal protein aggregates and damaged organelles (11). Aberrant autophagy has been associated with a variety of pathological conditions, including cancer, neurodegenerative and cardiovascular disorders (12). Previous studies suggest that, under different conditions, autophagy may promote cell survival or cell death, and may therefore be implicated in the regulation of cell apoptosis (13,14). Statins have been demonstrated to exert regulatory effects on autophagy in tumor cells, VECs and myocardial cells; however, the effects of atorvastatin on the regulation of VEC apoptosis via autophagy-associated signaling pathways have yet to be elucidated (15).

In the present study, human umbilical vein endothelial cells (HUVECs), which are widely used as an *in vitro* model for the study of cardiovascular diseases, were treated with various doses of atorvastatin. The molecular mechanisms underlying the effects of atorvastatin on autophagy and apoptosis were

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Key words: atorvastatin, vascular endothelial cell, apoptosis, autophagy

then investigated. The results provide preliminary evidence of the molecular mechanisms that may be involved in the action of atorvastatin in VECs.

Materials and methods

Reagents. Atorvastatin was purchased from Pfizer, Inc. (New York, NY, USA) and was diluted with anhydrous ethanol to 0.7, 7, 35 and 70 μ M. Dulbecco's modified Eagle's medium (DMEM) was obtained from Gibco (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Fetal bovine serum (FBS) was from HyClone (GE Healthcare Life Sciences, Logan, UT, USA). Radioimmunoprecipitation assay (RIPA) lysis buffer was purchased from Beyotime Institute of Biotechnology (Haimen, China). The protease inhibitor cocktail was obtained from Sigma-Aldrich; Merck KGaA (Darmstadt, Germany). The lactate dehydrogenase (LDH) assay kit was purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

Antibodies. Antibodies against neuron-specific enolase (NSE; #w10278) and glial fibrillary acidic protein (GFAP; #w10836) were purchased from Wanleibio (Shenyang, China). Antibodies against poly (ADP-ribose) polymerase-1 (PARP-1; #9542), Cleaved PARP-1 (#5625), caspase-3 (#9662), cleaved caspase-3 (#9664) β -actin (#3700), microtubule-associated protein 1A/1B-light chain 3 (LC3; #4108) and Beclin1 (#3738), and horseradish peroxidase-conjugated secondary antibodies were obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA).

Cell culture. HUVECs were purchased from the Cell Bank of Chinese Academy of Sciences (Shanghai, China) and cultured in DMEM supplemented with 10% FBS, 100 U/ml penicillin and 100 μ g/ml streptomycin (Invitrogen; Thermo Fisher Scientific, Inc.). Cells were maintained in a humidified incubator at 37°C in a 5% CO₂ atmosphere.

Semi-quantitative reverse transcription polymerase chain reaction (RT-PCR). HUVECs were seeded in 6-cm dishes. When the cells were 80% confluent, the medium was replaced with serum-free low-glucose DMEM and cells were treated with 0, 0.7, 7, 35 or 70 μ M atorvastatin at 37°C for 24 h. Cells treated with an equal volume of anhydrous alcohol served as the control group and cells cultured with 10% serum-containing medium served as the normal group. Total RNA was extracted according to the manufacturer's protocol using TRIzol[®] reagent (Invitrogen; Thermo Fisher Scientific, Inc.). Then, 1 μ g total RNA was reverse transcribed into cDNA according to the manufacturer's protocol, by the PrimeScript[™] RT reagent kit (RR037Q; Takara Biotechnology Co., Ltd., Beijing, China). The 20 μ l RT reaction solution consisted of 4 μ l 5X PrimeScript Buffer, 1 μ l PrimeScript RT Enzyme Mix I, 1 μ l Oligo(dT) Primer, 1 μ l Random 6 mers, 1 μ g Total RNA, and RNase Free dH₂O to 20 μ l. The RT reaction procedure: 37°C for 15 min, followed by 5 sec at 85°C, and then 4°C for 10 min. PCR was performed using the following primers: NSE, forward, 5'-ACCTGACCTCTTGCTGTCTC-3' and reverse, 5'-CTATGCACAGTTCACGGCTC-3'; neurofilament light polypeptide (NF-L), forward, 5'-TGGGTGTGGAGATTGTGTTAGGA-3' and reverse, 5'-TAGGACACCAAC

CTGCTGTG-3'; β -actin, forward, 5'-AAGATCAAGATCATTGCTCCTC-3' and reverse, 5'-GGACTCATCGTACTCTG-3'. PCR was performed using SYBR Premix Ex Taq II kit (#DR039A; Takara Biotechnology Co., Ltd., Dalian, China) and carried out in a ABI 7500 system (Applied Biosystems, Carlsbad, CA, USA). The PCR solution consisted of 12.5 μ l 2X Premix, 1 μ l Forward Primer (10 μ M), 1 μ l Reverse Primer (10 μ M), 2 μ l cDNA template and 8.5 μ l RNase-free dH₂O. PCR procedure: 95°C for 5 min, followed by 40 cycles of 5 sec at 95°C, 45 sec at 60°C, and then 72°C for 1 min. The PCR products were resolved by 2.0% agarose gel electrophoresis and stained by GelRed (A616697; Sangon Biotech Co., Ltd., Shanghai, China). The ImageJ program (version 1.44p; National Institutes of Health, Bethesda, MD, USA) was used to densitometry analysis for semi-quantitation of the bands obtained by ChemiDoc[™] XRS+ System. Relative target gene expression was normalized to β -actin expression.

Evaluation of cell survival. A total of 2,000 HUVECs were seeded in 96-well plates. Following treatment with 0, 0.7, 7, 35 or 70 μ M atorvastatin for 24 h, at 37°C in a 5% CO₂ atmosphere, and cells treated with an equal volume of anhydrous alcohol served as the control group, and cells cultured with 10% serum containing medium served as the normal group. Then cells were stained with 4% sulforhodamine B (SRB) at room temperature for 15 min. The absorbance of each sample was subsequently measured at 570 nm using a microplate reader.

Evaluation of cell necrosis. When the cells were 80% confluent, the medium was replaced with serum-free low-glucose DMEM and HUVECs were treated with 0, 0.7, 7, 35 or 70 μ M atorvastatin for 24 h at 37°C. Cells treated with an equal volume of anhydrous alcohol served as the control group and cells cultured with 10% serum-containing medium served as the normal group. Following treatment, the cell media were collected. The cell necrosis rate was evaluated using an LDH assay kit, according to the manufacturer's protocols. Subsequently, the absorbance of each sample was measured at 440 nm using a microplate reader.

Western blot analysis. Following treatment detailed above, HUVECs were washed twice with PBS and lysed with RIPA lysis buffer containing 1 mM phenylmethylsulfonyl fluoride on ice for 15 min. The lysates were collected and centrifuged at 12,000 x g at 4°C for 15 min. Equal quantities of extracted protein (20-40 μ g) were separated by 12% SDS-PAGE and transferred onto polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA). The membranes were blocked in 5% (w/v) nonfat dry milk dissolved in Tris-buffered saline containing 0.05% Tween-20 (TBST) for 1 h at room temperature, and then incubated with primary antibodies (PARP-1, 1:500; cleaved PARP-1, 1:500; caspase-3, 1:200; cleaved caspase-3, 1:500; β -actin, 1:3,000; LC3, 1:400; and Beclin1, 1:200) at 4°C overnight. The membranes were subsequently washed three times in TBST, and incubated with the corresponding horseradish peroxidase-conjugated secondary antibodies (#7074, 1:5,000; Cell Signaling Technology, Inc.) for 1 h at room temperature. TBST was used to wash the membranes three times, and the protein bands were visualized

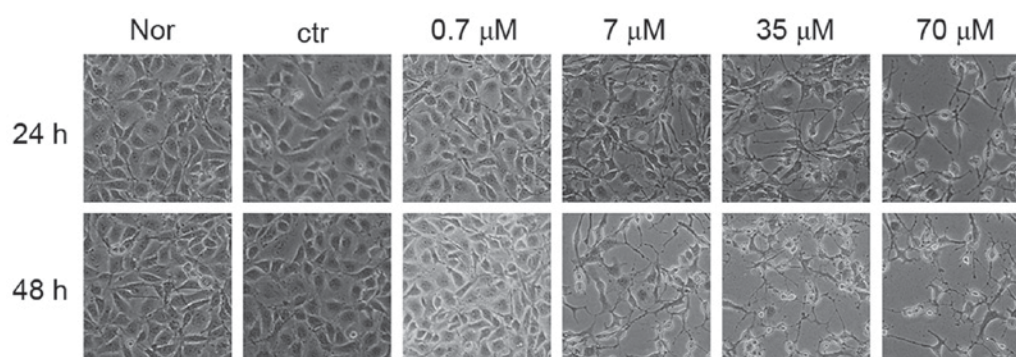


Figure 1. Alterations in HUVEC morphology following treatment with atorvastatin. HUVECs were seeded in 6-cm dishes. When the cells were 80% confluent, the medium was replaced with serum-free low-glucose Dulbecco's modified Eagle's medium and cells were treated with 0, 0.7, 7, 35 or 70 μ M atorvastatin at 37°C for 24 or 48 h. Cells treated with an equal volume of anhydrous alcohol served as the ctr group, and cells cultured with serum containing medium served as the Nor group. Cell morphology was observed under an inverted microscope, and photomicrographs were captured (magnification, x100). HUVEC, human umbilical vein endothelial cell; Nor, normal; ctr, control.

using an enhanced chemiluminescence kit (Thermo Fisher Scientific, Inc.). β -actin was used as the loading control. Blots were semi-quantified using ImageJ software (version 1.44p; National Institutes of Health, Bethesda, MD, USA).

Statistical analysis. The results are expressed as the mean \pm standard error of the mean. Data were collected from at ≥ 3 independent experiments. Data were then analyzed with the GraphPad Prism 5 software (version 5.01; GraphPad Software, Inc., La Jolla, CA, USA). Differences among groups were assessed using one-way analysis of variance followed by a post hoc Holm-Šidák test for multiple comparisons. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Alterations in cell morphology following treatment with atorvastatin. Following treatment with increasing concentrations of atorvastatin for 24 h, HUVECs gradually exhibited a long and thin, neuron-like cell morphology, which was particularly pronounced at 70 μ M atorvastatin (Fig. 1). Following treatment for 48 h, these morphological alterations were more pronounced, when compared with the same concentration at 24 h (Fig. 1).

Atorvastatin does not induce HUVEC trans-differentiation into neuron-like cells. Since treatment with atorvastatin induced a neuronal-like morphology in HUVECs, the potential of atorvastatin to induce HUVEC trans-differentiation into neuron-like cells was investigated. The neuronal markers, NSE and NF-L, were detected using semi-quantitative RT-PCR, and NSE and GFAP were detected using western blot analysis. As presented in Fig. 2, the mRNA and protein expression levels of these markers remained unaltered following treatment with all concentrations of atorvastatin. Therefore, these results indicate that atorvastatin does not induce the trans-differentiation of HUVECs into neuron-like cells.

High doses of atorvastatin decrease the viability and increase necrosis in HUVECs. The effects of atorvastatin on cell survival and necrosis were investigated in HUVECs using SRB staining and an LDH assay, respectively. The results revealed

that, following treatment with 7, 35 and 70 μ M atorvastatin, the cell survival rate significantly decreased in a dose-dependent manner (Fig. 3A). In addition, a significant increase in necrosis was observed following treatment of HUVECs with 70 μ M atorvastatin when compared with the control cells (Fig. 3B). These results suggest that high doses of atorvastatin may exert cytotoxic effects on HUVECs.

Atorvastatin promotes apoptosis in HUVECs. As a high dose of atorvastatin was revealed to decrease the survival rate of HUVECs, the authors investigated the possibility that apoptosis may have been responsible for these observed effects. To explore this hypothesis, the expression levels of apoptosis-associated proteins were determined by western blot analysis. The protein expression levels of cleaved caspase-3, which is a widely-used marker of apoptosis, were significantly upregulated in HUVECs following treatment with 35 μ M atorvastatin, in the presence and absence of serum (Fig. 4). Consistent with these observations, the protein expression levels of an additional marker of apoptosis, cleaved PARP-1, were significantly upregulated in HUVECs treated with 35 μ M atorvastatin in the presence and absence of serum when compared with control cells (Fig. 4).

Atorvastatin promotes autophagy in HUVECs. In order to investigate the effect of atorvastatin on cell autophagy, the protein expression levels of LC3 and Beclin1, which are common markers of autophagy, were analyzed. The results demonstrated that the expression levels of LC3 and Beclin1 were significantly upregulated following treatment with atorvastatin in the absence of serum, regardless of the concentration that was used (Fig. 5). By contrast, LC3 and Beclin1 protein expression levels were significantly reduced following treatment with 0.7 μ M atorvastatin in the presence of serum (Fig. 5). These results suggested that under different conditions, such as with or without serum, atorvastatin may exert varying effects on HUVECs.

Discussion

Dyslipidemia is one of the primary risk factors for the development of atherosclerosis (2). Atorvastatin is a widely-used agent

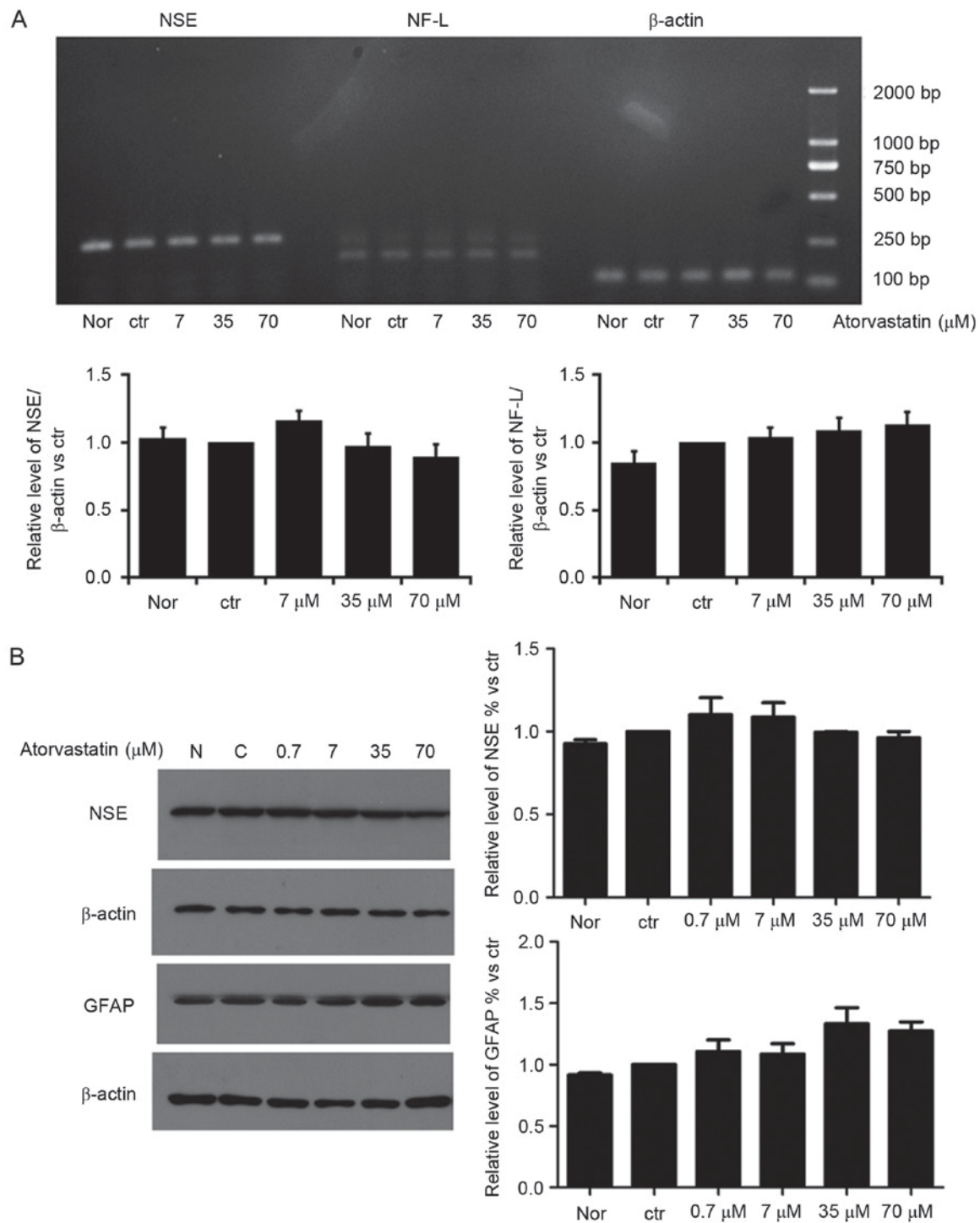


Figure 2. Expression of neuronal markers in HUVECs was unaltered following treatment with atorvastatin. When the cells were 80% confluent, the medium was replaced with serum-free low-glucose Dulbecco's modified Eagle's medium, and the cells were treated with 0, 0.7, 7, 35 or 70 μM atorvastatin for 24 h at 37°C. Cells treated with an equal volume of anhydrous alcohol served as the ctr group. (A) Semi-quantitative reverse transcription polymerase chain reaction analysis was performed to assess the mRNA expression levels of NSE and NF-L. (B) The protein expression levels of NSE and GFAP were detected by western blot analysis. The results are expressed as the mean ± standard error (n=3). HUVEC, human umbilical vein endothelial cell; NSE, neuron-specific enolase; NF-L, neurofilament light polypeptide; GFAP, glial fibrillary acidic protein; Nor, normal; ctr, control.

with lipid-lowering abilities; however, its effects on VECs have not yet been fully elucidated. In the present study, the effects of various doses of atorvastatin on VEC apoptosis and autophagy were investigated. The results demonstrated that low doses of atorvastatin promoted autophagy, whereas they did not affect apoptosis *in vitro*. Conversely, intermediate and high doses of atorvastatin were revealed to potentially induce apoptosis and

promote autophagy in HUVECs. To the best of the authors' knowledge, this is the first report demonstrating the differential effects of various doses of atorvastatin on VECs *in vitro*.

Previous studies have reported that atorvastatin exerts beneficial effects on the vascular wall by promoting the differentiation of monocytes to macrophages and EPCs to endothelial cells (16-18). The results of the present study demonstrated that

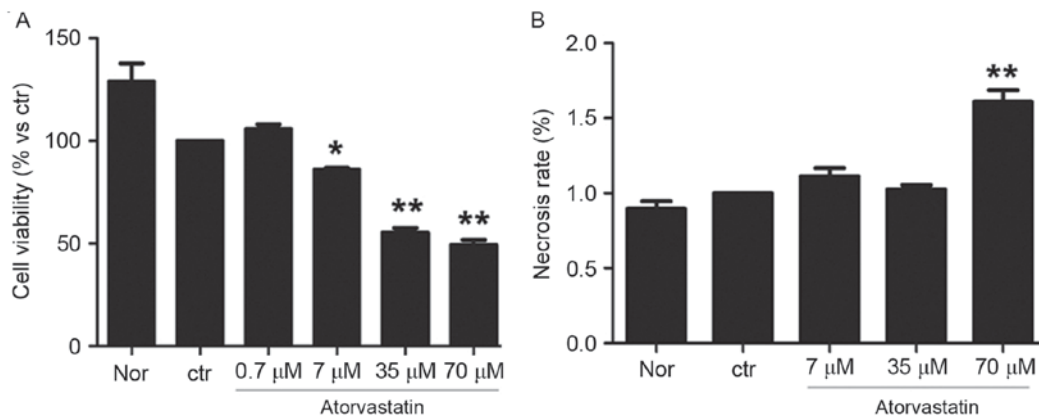


Figure 3. High doses of atorvastatin decrease HUVEC viability and promote necrosis. When the cells were 80% confluent, the medium was replaced with serum-free low-glucose Dulbecco's modified Eagle's medium and HUVECs were treated with 0, 0.7, 7, 35 or 70 μ M atorvastatin for 24 h at 37°C. Cells treated with an equal volume of anhydrous alcohol served as the ctr group and cells cultured with serum containing medium served as the Nor group. Following treatment, (A) cell viability was assessed by staining cells with sulforhodamine B and (B) the activity of lactate dehydrogenase was detected to measure the level of cellular necrosis. Data are expressed as the mean \pm standard error (n=3). *P<0.05 and **P<0.01 vs. ctr group. HUVEC, human umbilical vein endothelial cell; Nor, normal; ctr, control.

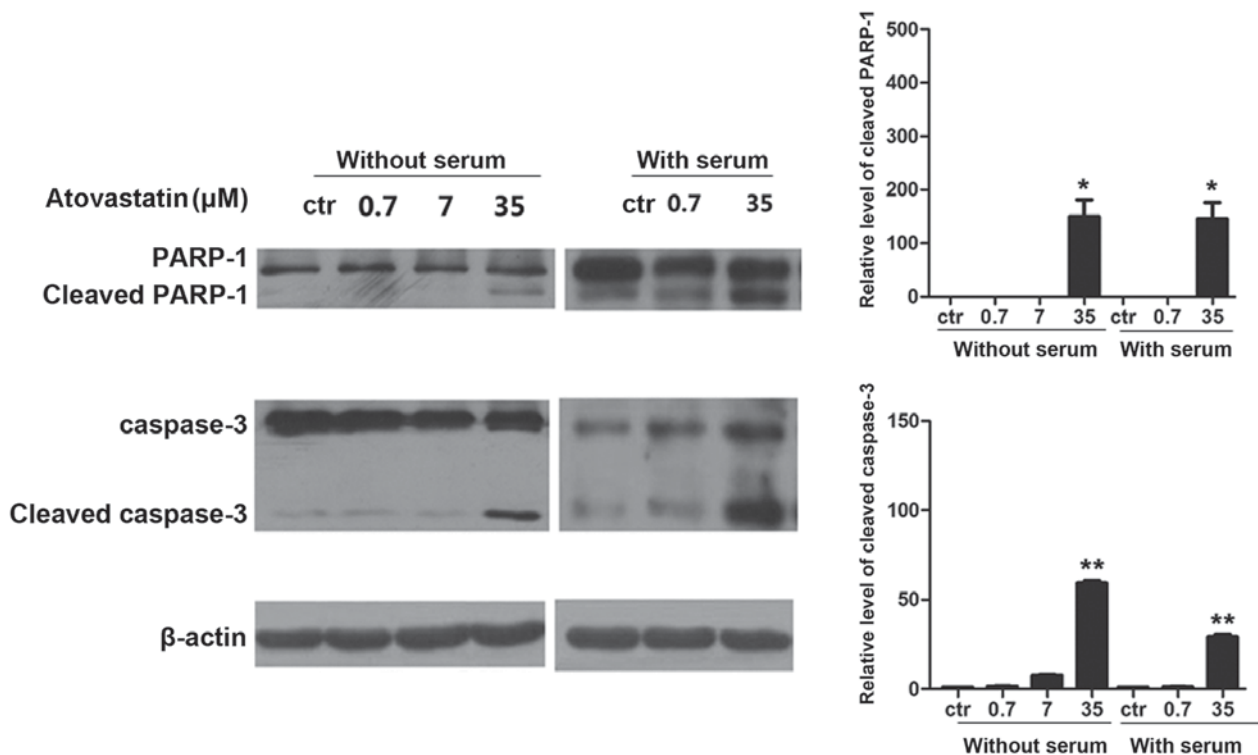


Figure 4. High doses of atorvastatin may promote HUVEC apoptosis. When the cells were 80% confluent, cells were treated with 0, 0.7, 7 or 35 μ M atorvastatin in the presence or absence of 10% serum, and incubated at 37°C for 24 h. Cells treated with an equal volume of anhydrous alcohol served as the ctr group and cells cultured with serum containing medium served as the Nor group. Following treatment, the protein expression levels of PARP-1, C-PARP-1, CASP-3 and C-CASP3 apoptosis markers were detected by western blot analysis. The results are expressed as the mean \pm standard error (n=3). *P<0.05 and **P<0.01 vs. ctr. HUVEC, human umbilical vein endothelial cell; C-PARP-1, cleaved-poly (ADP-ribose) polymerase-1; C-CASP-3, cleaved caspase-3; ctr, control.

HUVEC morphology was altered following treatment with atorvastatin, particularly at higher concentrations. However, the mRNA and protein expression levels of neuronal markers remained unaltered, indicating that atorvastatin did not promote the differentiation of VECs to neuronal cells *in vitro*.

Autophagy serves a dual role in cell survival (19). Previous studies have reported that atorvastatin exerts opposing effects on autophagy under various conditions (19,20). In the present study, treatment with a low dose of atorvastatin (0.7 μ M)

appeared to enhance the survival rate of HUVECs when compared with control cells; however, intermediate and high concentrations demonstrated the opposite effect. The apparent increase in cell viability was in accordance with the increase in autophagy that was observed following treatment with 0.7 μ M atorvastatin, which suggests that the promotion of autophagy may underlie the protective roles of atorvastatin in the maintenance of microcirculation. Of particular note, this dose effect has been demonstrated in animal trials (21,22).

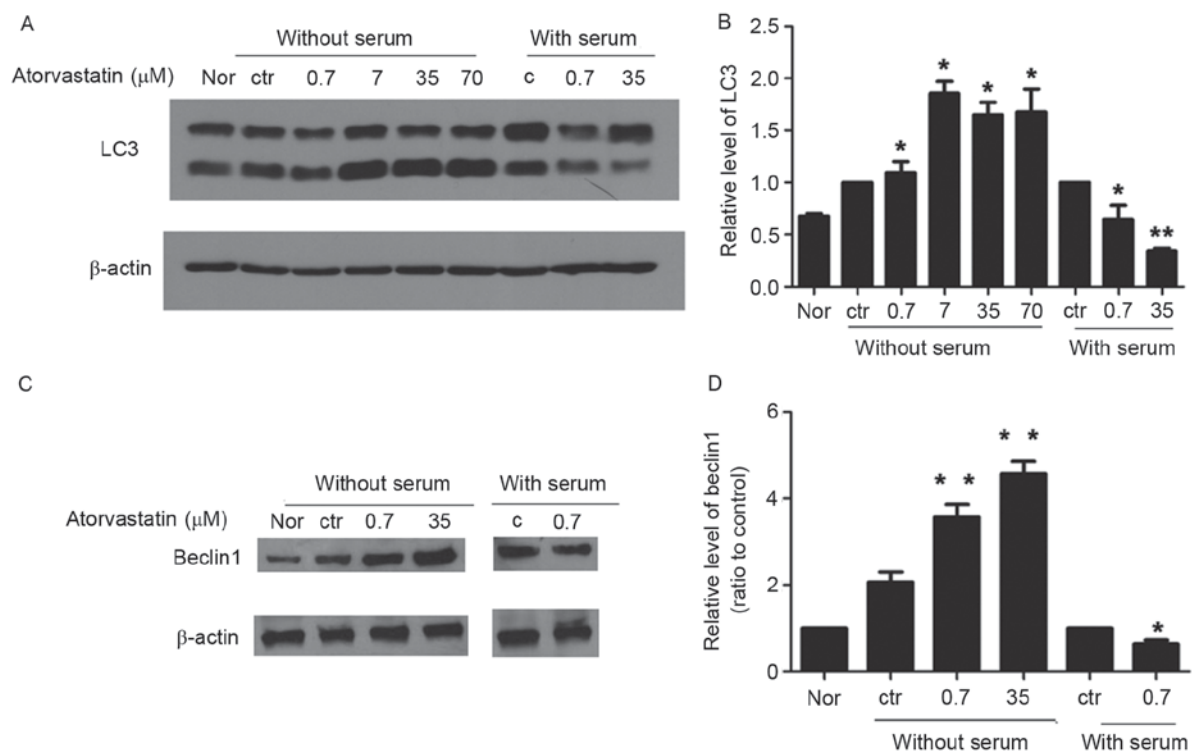


Figure 5. Treatment of HUVECs with atorvastatin increased the protein expression levels of autophagy markers. When the cells were 80% confluent, HUVECs were treated with 0.7, 7, 35 and 70 μ M atorvastatin in 10% serum-containing or serum-free low-glucose medium, and incubated at 37°C for 24 h. Cells treated with an equal volume of anhydrous alcohol served as the ctr group and cells cultured with serum containing medium served as the Nor group. The protein expression levels of the (A) LC3 marker of autophagy were determined by western blot analysis and (B) quantified relative to β -actin expression. The protein expression levels of the (C) Beclin1 marker of autophagy were determined by western blot analysis and (D) quantified relative to β -actin. Data are expressed as the mean \pm standard error of the mean of 3 independent experiments. * $P < 0.05$ and ** $P < 0.01$ vs. ctr. HUVEC, human umbilical vein endothelial cell; LC3, microtubule-associated protein 1A/1B-light chain 3; Nor, normal; ctr, control.

High doses of atorvastatin have been associated with short-term protective effects on the endothelium; however, the long-term clinical effects of high-dose treatment have yet to be elucidated (23). In the present study, high doses of atorvastatin were revealed to suppress the viability and promote the necrosis of HUVECs *in vitro*, as well as induce cell apoptosis. These results are in accordance with a previous study, which associated high-dose atorvastatin treatment with an increased risk of hepatotoxicity (24).

The development of tumor vasculature is critical for the supply of oxygen and nutrients to rapidly proliferating cancer cells (25). Tumor angiogenesis serves a vital role during tumor growth, and is thus considered to be a promising therapeutic target for the development of anticancer treatments (26). Atorvastatin exhibits anticancer effects in several types of human cancer, through the suppression of angiogenesis and the inhibition of autophagy (27). Therefore, the combination of anti-angiogenic agents with conventional chemotherapeutic drugs, at the appropriate doses, may present a novel therapeutic strategy to increase the efficacy of anticancer treatments.

In conclusion, the results of the present study suggest that various doses of atorvastatin may exert differential effects on VECs *in vitro*. However, further studies are required to explore the molecular mechanisms that underlie the actions of atorvastatin, and to fully elucidate the association between apoptosis and autophagy, as well as the role of atorvastatin in these cellular processes.

Acknowledgements

The authors thank Professor Shangli Zhang and the staff of School of Life Science at Shandong University for useful comments on the manuscript and Dr Minglei Wang of Shandong Tumor Hospital for his help with elaboration of figures.

Funding

The present study was supported by the Natural Science Foundation of China (grant nos. 31070999, 31371158 and 81800161) and the Key Scientific and Medical Project of Shandong Province Health Department (grant no. 2011QZ016), and the Science and Technology Developmental Project of Shandong Province (grant nos. 2016GSF201035, 2016GSF201042 and ZR2014CM030).

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

WBZ and JZ designed the project and analyzed the data; FC and FL performed most experiments and prepared the figures; HF, JL and JW performed experiments, prepared the figures and movies; WBZ wrote the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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