

Original Article

Atorvastatin attenuates homocysteine-induced apoptosis in human umbilical vein endothelial cells via inhibiting NADPH oxidase-related oxidative stress-triggered p38MAPK signaling

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Aim: To examine the effect of atorvastatin on homocysteine (Hcy)-induced reactive oxygen species (ROS) production and apoptosis in human umbilical vein endothelial cells (HUVECs).

Methods: HUVECs were cultured with Hcy (0.1–5 mmol/L) in the presence or absence of atorvastatin (1–100 $\mu\text{mol/L}$) or various stress signaling inhibitors, including the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase inhibitor diphenylene iodonium (DPI, 10 $\mu\text{mol/L}$), the p38 mitogen-activated protein kinase (p38 MAPK) inhibitor SB203580 (10 $\mu\text{mol/L}$) and antioxidants N-acetyl cysteine (NAC, 1 mmol/L). Cell apoptosis was evaluated by Annexin V/propidium iodide staining and flow cytometry. ROS were detected by 2',7'-dichlorodihydrofluorescein diacetate ($\text{H}_2\text{DCFH-DA}$). NADPH oxidases were evaluated with lucigenin-enhanced chemiluminescence. Hcy-induced expression of p38MAPK protein was measured by Western blotting analysis.

Results: Atorvastatin inhibited endothelial cell apoptosis induced by 1 mmol/L Hcy in a dose-dependent manner and the maximal inhibitory effect was reached at 100 $\mu\text{mol/L}$. Atorvastatin (10 $\mu\text{mol/L}$) significantly suppressed Hcy (1 mmol/L for 30 min) induced ROS accumulation (3.17 ± 0.33 vs 4.34 ± 0.31 , $P < 0.05$). Atorvastatin (10 $\mu\text{mol/L}$) also antagonized Hcy (1 mmol/L for 30 min) induced activation of NADPH oxidase (2.57 ± 0.49 vs 3.33 ± 0.6 , $P < 0.05$). Furthermore, atorvastatin inhibited Hcy-induced phosphorylation of p38 MAPK (1.7 ± 0.1 vs 2.22 ± 0.25 , $P < 0.05$), similar effects occurred with DPI, NAC and SB203580.

Conclusion: Atorvastatin may inhibit Hcy-induced ROS accumulation and endothelium cell apoptosis through an NADPH oxidase and/or p38MAPK-dependent mechanisms, all of which may contribute to atorvastatin-induced beneficial effect on endothelial function.

Keywords: atorvastatin; homocysteine; apoptosis; reactive oxygen species; NADPH oxidase; p38MAPK; human umbilical vein endothelial cells

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Introduction

Hyperhomocysteinemia (HHcy) is recognized as an independent cardiovascular risk factor^[1–3], but only little of the pathogenetic mechanisms involved in its vascular actions have been unveiled. Extensive literature^[4, 5] shows that a key event in the vascular pathobiology associated with HHcy is the induction of endothelial dysfunction. Prolonged exposure of endothelial cells to homocysteine impairs the production of nitric oxide and endothelium-dependent vasodilatation. They combine with low-density lipoprotein cholesterol to produce aggregates that are taken up by vascular macrophages in the arterial intima (foam cells), produce aggregatory effects on the

platelets, and decrease endothelial antithrombotic activity due to changes in the thrombomodulin function. Endothelial dysfunction not only impairs the regulation of permeability and vasoactivity, but also affects the regulation of cell adhesion, inflammatory responses and of cell growth within the vessel wall^[6].

Reactive oxygen species (ROS)-mediated apoptosis of endothelial cells^[7] has been proved crucial in endothelial dysfunction and early atherogenesis. Recently it has been shown that exposure of human umbilical vein endothelial cell (HUVECs)^[8, 9] to homocysteine (Hcy) leads to endothelial apoptosis accompanied by increased level of ROS, together with an increased level of caspase3 expression and activation. Hcy primes human neutrophils for an increased production of ROS and induces ROS production in endothelial cells^[10, 11]. Hcy seems to promote the formation of ROS primarily by a

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biochemical mechanism involving endothelial nitric oxide synthase and increased endothelial lipid peroxidation^[12]. Hcy also increases intracellular reactive oxygen species by NADPH oxidase (Nox) activation^[11, 13], as shown by the membrane translocation of its p47 (phox) and p67 (phox) subunit^[11, 14]. The increased ROS lead to increased oxidant stress and decreased nitric oxides bioactivity in the vasculature and the activation of redox-sensitive signaling pathway, all of which promote endothelial dysfunction.

Atorvastatin is a synthetic 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitor. Apart from its lipid-lowering effects, atorvastatin has pleiotropic effects including anti-atherogenic and anti-inflammatory actions^[15]. Atorvastatin has been found to inhibit the *in vitro* oxidation of LDL and to reduce various oxidative stress markers^[16]. Atorvastatin exerts cellular antioxidant effects by decreasing mRNA expression of essential NADPH oxidase subunit nox1^[17]. The NADPH oxidase system is the main source of ROS production in the vessel wall. New evidence shows that endothelial Nox4 overexpression and formation of an active complex with p22 (phox) enhance superoxide anion formation and phosphorylation of p38 MAPK^[18]. The p38 MAPK, a stress-activated serine/threonine protein kinase, is a downstream target of proinflammatory cytokines and oxidative stress^[19]. In addition, activation of p38 MAPK has been also implicated in induction of cell apoptosis^[20]. Therefore, in the present study, we test the hypothesis that Hcy-induced endothelial cell death is initiated by oxidative stress, which activates p38 MAPK and induces cell apoptosis. The underlying protective effects of atorvastatin involve reducing oxidative stress and modulating p38 MAPK activation in HUVECs.

Materials and methods

Materials

Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were obtained from Gibco (Gaithersburg, MD, USA). Cell culture materials were from Costar (Corning, NY, USA). Homocysteine, DPI, SB203580, NADPH, β -actin and lucigenin was obtained from Sigma-Aldrich (St Louis, MO, USA). NAC and H₂DCF-DA were purchased from Calbiochem (Darmstadt, Germany). Atorvastatin was purchased from the National Institute for the Control of Pharmaceutical and Biological Products. Anti-phospho-p38 MAPK was purchased from Cell Signaling Technology (Danvers, MA, USA). Other reagents were indicated in the text.

Cell cultures

HUVECs were provided by Cell Bank of Institute of Cellular Biology in Shanghai, Chinese Academy of Science. Cells were grown in DMEM supplemented with 10% FBS and penicillin/streptomycin (100 U/mL and 100 μ g/mL, respectively) at 37 °C, in a humidified atmosphere containing 95% air and 5% CO₂. Experiments were performed with cells grown to a confluency of 80%. The sub-confluent cells were made quiescent by incubation in FBS-free DMEM for 24 h before stimulation.

Cellular apoptosis detected by flow cytometry

HUVECs were seeded in 12-well plates and were treated with various concentrations of Hcy in the presence or absence of atorvastatin, DPI, NAC and SB203580 for 24 h, respectively. The cells were then collected and treated as the protocol in Annexin V-FITC Apoptosis Detection Kit (Merck, Germany) and the percentages of apoptotic cells were determined by a flow cytometer (BD, USA). Cells were measured with a FACS Calibur (Becton Dickinson, San Jose, CA, USA). Results were analyzed by Cell Quest Pro software (Becton Dickinson).

Measurement of ROS production

To evaluate ROS production by HUVECs, the membrane permeable indicator H₂DCF-DA was employed. The cells were loaded with 10 μ mol/L H₂DCF-DA in serum-free DMEM at 37 °C for 30 min and then washed twice with phosphate buffered saline (PBS). After pre-incubated with atorvastatin (10 μ mol/L), NAC (1 mmol/L) or DPI (10 μ mol/L) for 30 min, Cells were stimulated with Hcy (1 mmol/L) for 30 min, then immediately monitored with flow cytometer (BD, USA) at a excitation wavelength of 488 nm and an emission wavelength of 525 nm. ROS was determined by comparing the changes in fluorescence intensity with that of the control.

Determination of NADPH oxidase activity

The lucigenin-derived enhanced chemiluminescence assay was used to determine NADPH oxidase activity in HUVECs as previously described by Henrik^[21]. HUVECs were starved by serum deprivation for 24 h, washed twice with ice-cold PBS and harvested. After low spin centrifugation, the pellet was resuspended in ice-cold buffer containing 1 mmol/L ethylene glycol tetraacetic acid, protease inhibitors, and 150 mmol/L sucrose. Cells were lysed, and then the total protein concentration was determined by using a Bradford assay and adjusted to 1 mg/mL. 200 μ L protein sample including 5 μ mol/L lucigenin were measured over 6 min in quadruplicate using NADPH (100 μ mol/L) as a substrate in a luminometer counter (Berthold luminometer centro LB 960 Germany). Data were collected at 2 min intervals in order to measure relative changes in NADPH oxidase activity.

Western blotting analysis

Following treatment, cells were washed with ice-cold PBS and lysed as described previously^[22]. The protein concentration was determined by the Bradford method. 10 μ L of protein was loaded in each lane and subjected to 9% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and then transferred to a polyvinylidene difluoride (PVDF) membrane. The blots were probed with antibodies against phospho-p38 MAPK. Horseradish peroxidase-conjugated secondary antibodies were used in conjunction with an ECL chemiluminescence detection system (Amersham, UK). Staining was quantified by scanning densitometry.

Statistical analysis

The obtained data were presented as means \pm standard deviation.

tions for replicate experiments. One-way ANOVA and Dunnett in SPSS 13.0 software were used for statistical analysis and $P < 0.05$ was considered as statistically significant.

Results

Atorvastatin inhibited endothelial cell apoptosis induced by Hcy in a dose-dependent manner

The endothelial cell apoptosis induced by Hcy was examined in the presence of different concentrations of Hcy (0, 0.1, 0.5, 1 and 5 mmol/L) for 24 h (Table 1) and in the presence of 1 mmol/L Hcy for 0, 12, 24, and 48 h (Table 2). It is obvious that Hcy induced the endothelial cell apoptosis in a time- and dose-dependent manner. To observe the effects of atorvastatin on Hcy-induced endothelial cell apoptosis, different concentrations of atorvastatin (1, 10, 100 $\mu\text{mol/L}$) were added 30 min before 1 mmol/L Hcy stimulation for 24 h. As shown in Table 3, atorvastatin inhibited endothelial cell apoptosis induced by 1 mmol/L Hcy in a dose-dependent manner and the maximal inhibitory effect was reached at 100 $\mu\text{mol/L}$.

Atorvastatin inhibited endothelial cell apoptosis induced by Hcy involving ROS

To determine whether the inhibitory roles of atorvastatin in Hcy-induced endothelial cell apoptosis involve ROS, we observed the effects of the NADPH oxidase inhibitor (DPI, 10 $\mu\text{mol/L}$) and the free radical scavenger (NAC, 1 mmol/L) on endothelial cell apoptosis induced by Hcy (1 mmol/L) (Figure 1A). Further, we measured intracellular ROS levels using the ROS-sensitive probe $\text{H}_2\text{DCF-DA}$, by a flow cytometry. Compared with controls, 1 mmol/L Hcy increased DCF fluorescence in a time-dependent manner (Figure 1B). ROS generation increased significantly at 20 min and then further increased until a plateau was reached at 30 min. Atorvastatin

(10 $\mu\text{mol/L}$) markedly blunted the production of ROS induced by Hcy (1 mmol/L, 30 min) (Figure 1C and 1D) and similar inhibitory effects were observed with DPI (10 $\mu\text{mol/L}$) and NAC (1 mmol/L) (Figure 1C).

Atorvastatin inhibited Hcy-induced activation of NADPH oxidase

To explore the underlying mechanism by which atorvastatin suppressed intracellular oxidative stress, we measured NADPH oxidase activity with lucigenin-enhanced chemiluminescence. Stimulation with 1 mmol/L Hcy led to a time-dependent increase of NADPH oxidase activity to $275\% \pm 47\%$ at 20 min and $333\% \pm 60\%$ at 30 min (Figure 2A). As demonstrated in Figure 2B, pretreatment of the cells with atorvastatin (10, 100 $\mu\text{mol/L}$) reduced Hcy-dependent NADPH oxidase activation.

Atorvastatin inhibited endothelial cell apoptosis induced by Hcy involving MAPK signaling

To explore whether a p38 MAPK signaling event was involved in the inhibitory effects of atorvastatin on Hcy-stimulated apoptosis, we observed the role of the p38 MAPK inhibitor, SB203580 in Hcy-induced apoptosis (Figure 3A). We found that SB203580 partially blocked the effects of Hcy, suggesting that p38 MAPK was involved in endothelial cell apoptosis. Further, we measured the activation of p38 MAPK by Western blotting. Hcy rapidly induced p38 MAPK phosphorylation in a time- and dose-dependent manner. 10 mmol/L Hcy increased phosphorylation of p38 MAPK at maximal levels (Figure 3B) and the peak of phosphorylation was reached 30 min following exposure to 1 mmol/L Hcy (Figure 3C). The activation of p38 MAPK induced by Hcy was significantly suppressed by 10 $\mu\text{mol/L}$ atorvastatin. Similar effects were seen on DPI, NAC (Figure 3D), suggesting that the activation of p38 MAPK was dependent on oxidative stress.

Table 1. Apoptosis rate of HUVECs incubated with different concentrations of Hcy for 24 h. Mean \pm SD. $^{\circ}P < 0.01$ vs 0 mmol/L Hcy group. Hcy, homocysteine.

| Hcy (mmol/L) | 0 | 0.1 | 0.5 | 1.0 | 5.0 |
|--------------------|-----------------|---------------------------|-----------------------------|-----------------------------|-----------------------------|
| Apoptosis rate (%) | 1.36 \pm 0.25 | 7.3 \pm 1.15 $^{\circ}$ | 10.89 \pm 1.69 $^{\circ}$ | 15.14 \pm 1.02 $^{\circ}$ | 21.06 \pm 0.89 $^{\circ}$ |

Table 2. Apoptosis rate of HUVECs incubated with 1 mmol/L Hcy for different time. Mean \pm SD. $^{\circ}P < 0.01$ vs 0 hour. Hcy, homocysteine.

| Time (h) | 0 | 12 | 24 | 48 |
|--------------------|-----------------|-----------------------------|-----------------------------|-----------------------------|
| Apoptosis rate (%) | 1.36 \pm 0.25 | 11.08 \pm 2.17 $^{\circ}$ | 15.14 \pm 1.02 $^{\circ}$ | 23.63 \pm 3.92 $^{\circ}$ |

Table 3. Apoptosis rate of HUVECs incubated with different concentrations of atorvastatin induced by 1 mmol/L Hcy. Mean \pm SD. $^{\text{b}}P < 0.05$, $^{\text{c}}P < 0.01$ vs 0 $\mu\text{mol/L}$ atorvastatin group. Hcy, homocysteine.

| Atorvastatin ($\mu\text{mol/L}$) | 0 | 1.0 | 10 | 100 |
|------------------------------------|------------------|--------------------------------|--------------------------------|-------------------------------|
| Apoptosis rate (%) | 15.14 \pm 1.02 | 12.18 \pm 1.51 $^{\text{b}}$ | 10.77 \pm 1.69 $^{\text{c}}$ | 7.28 \pm 0.83 $^{\text{c}}$ |

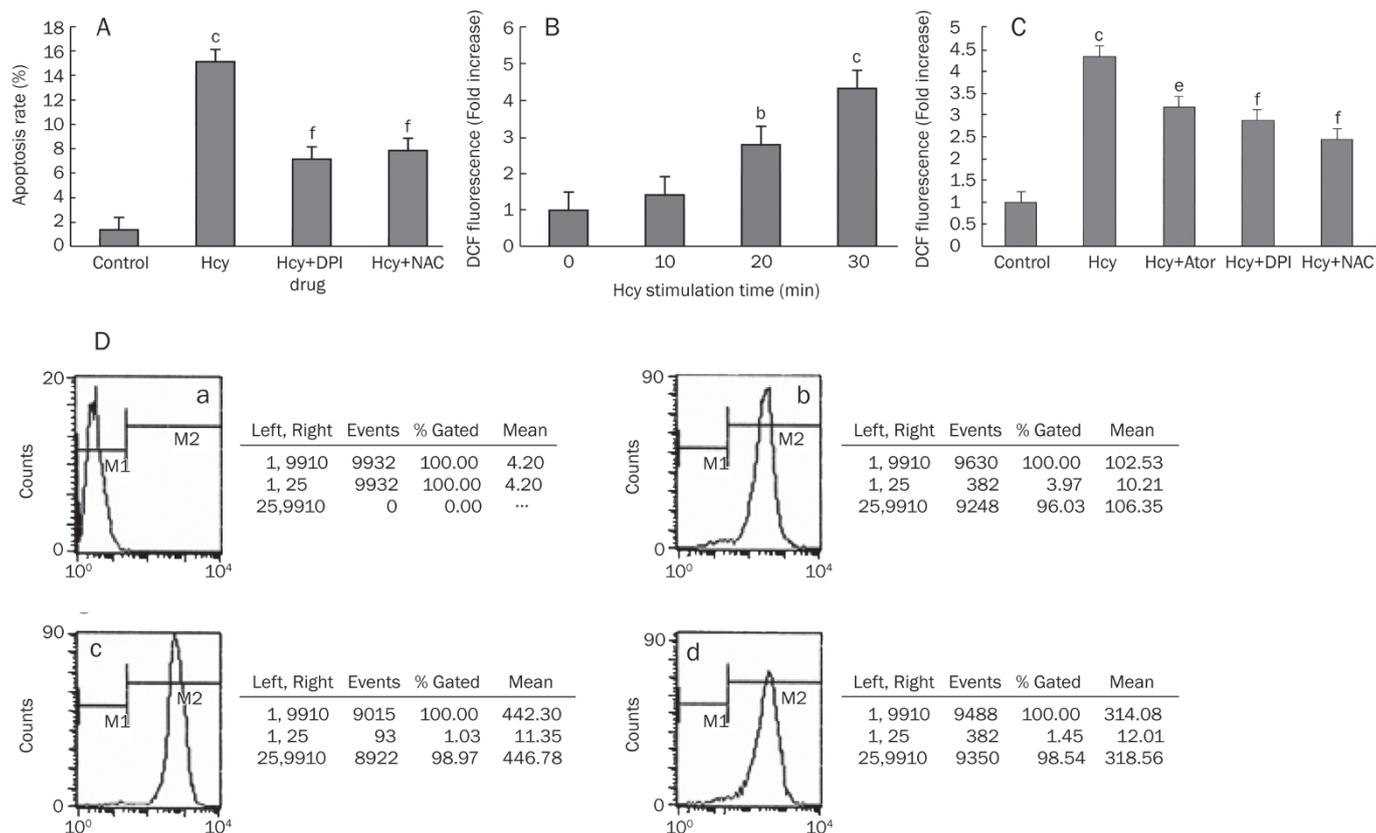


Figure 1. Atorvastatin inhibited HUVECs apoptosis induced by Hcy involving ROS. (A) Cells were pre-incubated with DPI (10 μ mol/L) and NAC (1 mmol/L) for 30 min before the addition of 1 mmol/L Hcy for 24 h and apoptotic cells were counted. (B) Cells were incubated at 37 °C with 10 μ mol/L H₂DCF-DA for 30 min then stimulated with 1 mmol/L Hcy for 10, 20, 30 min. (C) Effects of atorvastatin, DPI and NAC on Hcy-induced ROS within HUVECs. Cells were incubated with 10 μ mol/L H₂DCF-DA for 30 min at 37 °C and pre-incubated with 10 μ mol/L atorvastatin, 10 μ mol/L DPI and 1 mmol/L NAC for 30 min before the addition of 1 mmol/L Hcy for 30 min. (D) The representative experiment of the Hcy induced ROS production reduced by atorvastatin. The fluorescence intensity was measured with a flow cytometer. The relative fluorescence intensity of cells was calculated relative to untreated control cells. (a) negative control(without H₂DCF-DA); (b) blank control(without Hcy);(c) 1mol/L Hcy stimulation for 30 min; (d) 1 mol/L Hcy stimulation for 30 min+pre-incubated with atorvastatin for 30 min. Results are the means \pm SD from three independent experiments. ^b*P*<0.05, ^c*P*<0.01 vs untreated cells. ^e*P*<0.05, ^f*P*<0.01 vs 1 mmol/L Hcy-treated cells. Ator: atorvastatin; Hcy, homocysteine.

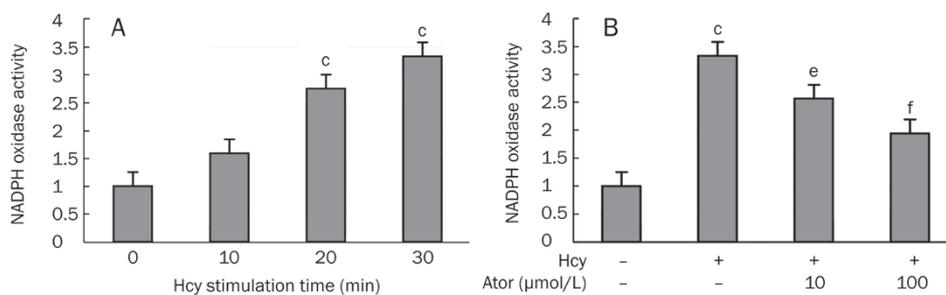


Figure 2. Atorvastatin suppressed Hcy-induced activation of NADPH oxidase. (A) Hcy (1 mmol/L) increased NADPH oxidase activity in a time-dependent manner. (B) Pretreatment of cells with atorvastatin 30 min prior to 1 mmol/L Hcy abolished NAD(P)H oxidase activation at concentrations of 10 μ mol/L and 100 μ mol/L. NAD(P)H oxidase activity was assessed as lucigenin-enhanced chemiluminescence using NADPH as a substrate. Results are the means \pm SD from three independent experiments. ^c*P*<0.01 vs untreated cells. ^e*P*<0.05, ^f*P*<0.01 vs only 1 mmol/L Hcy-treated cells. Ator: atorvastatin; Hcy, homocysteine.

Discussion

Numerous clinical and epidemiological studies have indicated that HHcy is an independent risk factor for vascular

disease^[1-3]. However, the underlying mechanisms responsible for endothelial cell injury with increased plasma concentration of homocysteine still remain incompletely elucidated. Many

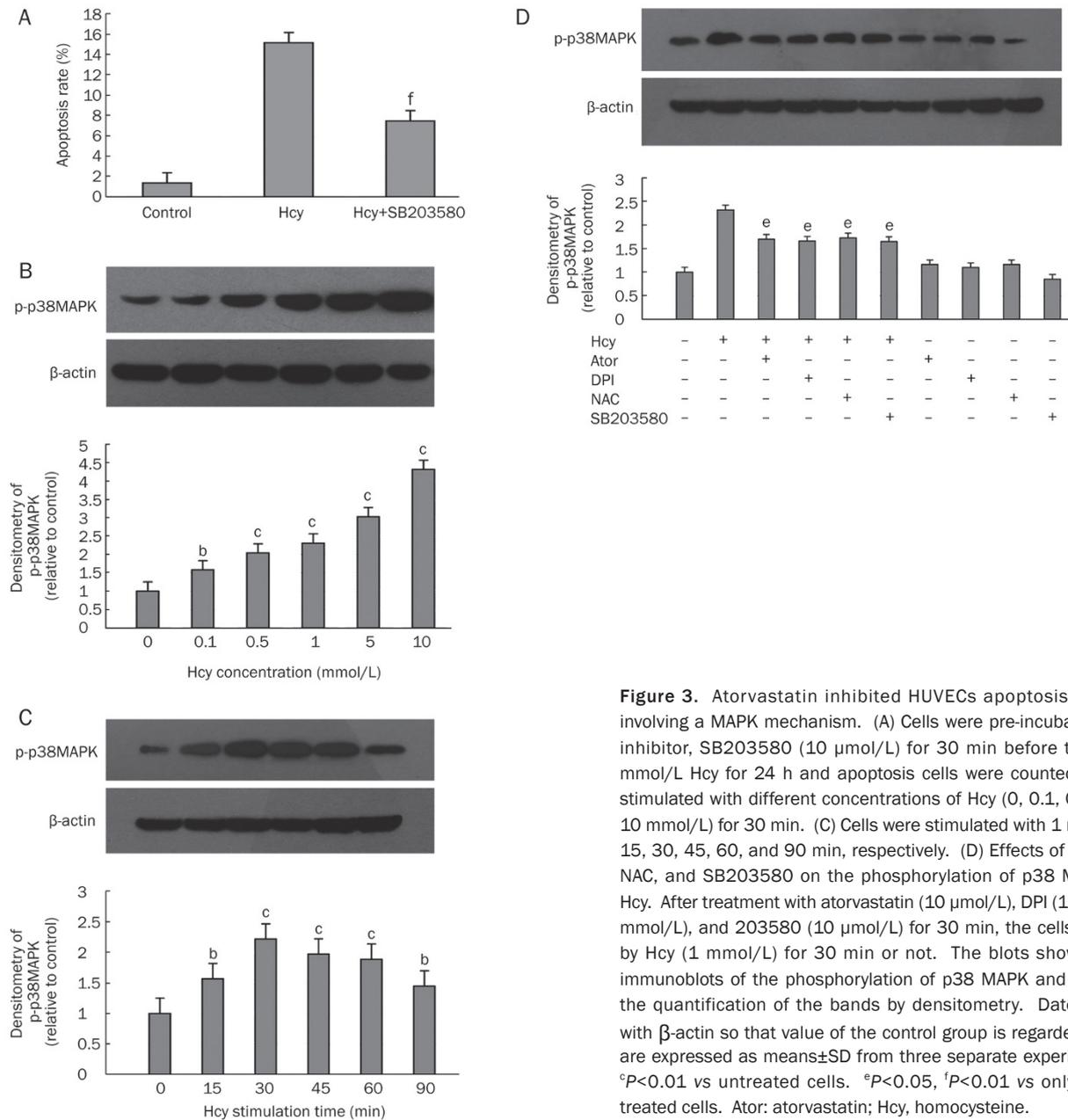


Figure 3. Atorvastatin inhibited HUVECs apoptosis induced by Hcy involving a MAPK mechanism. (A) Cells were pre-incubated with the p38 inhibitor, SB203580 (10 μ mol/L) for 30 min before the addition of 1 mmol/L Hcy for 24 h and apoptosis cells were counted. (B) Cells were stimulated with different concentrations of Hcy (0, 0.1, 0.5, 1.0, 5.0, and 10 mmol/L) for 30 min. (C) Cells were stimulated with 1 mmol/L Hcy for 0, 15, 30, 45, 60, and 90 min, respectively. (D) Effects of atorvastatin, DPI, NAC, and SB203580 on the phosphorylation of p38 MAPK induced by Hcy. After treatment with atorvastatin (10 μ mol/L), DPI (10 μ mol/L), NAC (1 mmol/L), and 203580 (10 μ mol/L) for 30 min, the cells were stimulated by Hcy (1 mmol/L) for 30 min or not. The blots show representative immunoblots of the phosphorylation of p38 MAPK and the graphs show the quantification of the bands by densitometry. Data are normalized with β -actin so that value of the control group is regarded as 1.0. Values are expressed as means \pm SD from three separate experiments. ^b P <0.05, ^c P <0.01 vs untreated cells. ^e P <0.05, ^f P <0.01 vs only 1 mmol/L Hcy-treated cells. Ator: atorvastatin; Hcy, homocysteine.

reports have shown that Hcy can promote necrosis and apoptosis^[23, 24]. In our study, we observed the apoptosis potential of HUVECs induced by Hcy in the presence of 0.1–5 mmol/L Hcy and found that Hcy induced endothelial cell apoptosis in a time- and dose-dependent manner. We also found that atorvastatin inhibited endothelial cell apoptosis induced by Hcy in a dose-dependent manner.

Several mechanisms have been explained that cytotoxicity of Hcy on vascular endothelial cells is closely associated with accumulation of ROS, which is believed to play a key role in homocysteine-induced endothelial apoptosis^[25, 26]. Hcy seems to promote the formation of ROS primarily by a biochemical mechanism involving NADPH oxidase (Nox), endothelial nitric oxide synthase (eNOS) and endothelial lipid peroxida-

tion^[11–13], which lead to an increase of ROS including hydrogen peroxide (H_2O_2) and superoxide anion (O_2^-) and a decrease in nitric oxide (NO) bioavailability that play a critical role in endothelial cell damage and function. A result of eNOS uncoupling is the transfer of one electron to molecular oxygen instead of five electrons oxidation of L-arginine, resulting in O_2^- generation. At higher levels, O_2^- will react with NO to form a cytotoxic peroxynitrite ($ONOO^-$), a main component of nitrooxidative stress. ROS generation can induce death by apoptosis and this programmed cell death can be prevented by a variety of antioxidants. In cells treated with Hcy we can easily detect different apoptosis characteristics such as internucleosomal DNA fragmentation, cytochrome *c* release or caspase-3 activation^[24–26]. In agreement with previous stud-

ies^[25, 26], our results showed that ROS are strongly involved in Hcy induced apoptosis, since radical scavengers NAC reduced apoptotic cell death by 48.15%.

Statins have been shown to improve endothelial function by lowering serum LDL as well as by mechanisms unrelated to HMG-CoA reductase inhibition, including upregulation of eNOS expression and reduced O₂⁻ formation. Atorvastatin is very popular in clinical settings and recently has been found to have anti-oxidative effects^[17]. We also found that atorvastatin (10 μmol/L) significantly suppressed Hcy (1 mmol/L for 30 min) induced ROS accumulation (3.17±0.33 vs 4.34±0.31, P<0.05). However, the mechanism by which atorvastatin down-regulates Hcy-induced intracellular ROS levels is not known. Therefore, we focused our interest on the possible pathway of atorvastatin inhibiting ROS. Our results that DPI reduced Hcy-induced ROS production strongly suggest that Hcy-induced ROS might occur via NADPH oxidase activation. The NADPH oxidase system is the main source of ROS production in the vessel wall. The Nox family of NADPH oxidases has seven members, including Nox1, Nox2, Nox3, Nox4, Nox5, Duox1, and Duox2. The NADPH oxidase is an enzyme complex composed of two membrane-bound Cytochrome (gp91phox, p22phox), three cytosolic factors (p47phox, p67phox, p40phox), and the small GTPase, Rac2. The activation of enzyme complex, cytosolic proteins translocate to the membrane catalyzes the transfer of one electron from NADPH to molecular oxygen, resulting in the formation of superoxide. In our lucigenin-enhanced chemiluminescence assay, Hcy increased NADPH oxidase activity leading to the increase of intracellular superoxide quickly, which was reduced by atorvastatin. Atorvastatin may inhibit Hcy-induced activation of NADPH oxidase and exert cellular antioxidant effects through decreased mRNA expression of essential NADPH oxidase subunit Nox1^[17] and inhibited endothelial Nox4 overexpression and formatting an active complex with p22 (phox) which enhance superoxide anion formation and phosphorylation of p38 MAPK^[18].

It is well known that the stress-activated kinases p38MAPK is closely associated with apoptosis in many cell lines. Our results that inhibition of p38MAPK using the selective inhibitor SB235080 rescued cells from Hcy-mediated cell apoptosis, consistent with the hypothesis that activation of p38MAPK is an important effector of apoptosis in HUVECs cells. It has been reported that ROS increase the phosphorylation of tyrosine or/and serine of MAPK^[18, 19]. Our results that DPI and NAC inhibited p38 MAPK activation demonstrated that the p38 MAPK signaling pathway is ROS-dependent. This conclusion was further confirmed by previous study^[27], which found that siRNA against NADPH oxidase suppress the phosphorylation of p38 MAPK. Oxidative stress can directly or indirectly modulate the functions of p38 MAPK and downstream transcription factors, which ultimately result in gene expression changes that influence cells to survive or die^[19, 20]. Hcy is known to induce oxidative stress by increasing ROS levels by autoxidation and by interfering with the activity and expression of pro- and antioxidant enzymes, while Nox4 is the

dominant ROS source in endothelial cells. A recent publication^[28] has located endogenous Nox4 to the nucleus of human umbilical endothelial cells (HUVECs), which pointed to the nucleus as an intracellular site of ROS production in endothelial cells. The nuclear localization of Nox4 suggests that it might regulate gene expression, and activate serine/threonine protein kinase through production of ROS. Hcy caused dose and time dependent HUVECs apoptosis accompanied by a transient increase in ROS via NADPH oxidase, which leads to downstream activation of p38MAPK. All of those can be attenuated by atorvastatin. Therefore, we believe that the inhibitory effect of atorvastatin on the activation of p38 MAPK might be related to its down-regulation of intracellular ROS levels.

Serum levels of statins in humans range between 0.002 and 0.2 μmol/L for atorvastatin (10 to 80 mg/d)^[29]. However, in our *in vitro* studies, anti-apoptosis effects were observed at concentrations between 1 and 100 μmol/L, whereas anti-oxidative and anti-phosphorylation of p38 MAPK effects were observed at 10 μmol/L atorvastatin. The difference in doses may result from the distinctiveness between *in vitro* and *in vivo* studies. Our *in vitro* studies are acute experiments and pretreatment time of atorvastatin is only 30 min. Moreover, HUVEC is immortal cell line, which is not susceptible to drugs. Further *in vivo* studies are needed to satisfy the possible clinical translation.

In summary, our findings strongly suggested that atorvastatin exerted its antiapoptosis activity through inhibiting NADPH oxidase-ROS-p38 signaling *in vitro*. However, the influence of atorvastatin on other sources of ROS, such as xanthine oxidase, mitochondria respiration, lipoxygenase and nitric oxide synthase needs to be further clarified. Furthermore our results also suggest that ROS are not the only factor that accounts for the initiation of apoptosis. Additional signaling pathways leading to Hcy induced apoptosis may be involved in the endoplasmic reticulum stress through activation of the unfolded protein response^[30], which needs to be elucidated furthermore.

Author contribution

Xiao-mei BAO designed the project, performed the research, analyzed the data, and wrote the paper. Chun-fang WU cultured cell. Guo-ping LU partly designed the project and revised the paper.

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