Atorvastatin protects endothelial colony-forming cells against H_2O_2-induced oxidative damage by regulating the expression of annexin A2

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Abstract. Endothelial dysfunction and injury are central events in the pathogenesis of ischemic vascular disorders. Endothelial progenitor cells (EPCs) are mobilized from the bone marrow into the peripheral circulation, where they locate to sites of injured endothelium and are involved in endothelial repair and vascular regeneration. During these processes, EPCs are exposed to oxidative stress, a crucial pathological condition, which occurs during vascular injury and limits the efficacy of EPCs in the repair of injured endothelium. Statins are effective inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A reductase, and are commonly used to manage and prevent ischemic vascular disease by reducing plasma cholesterol levels. In addition to lowering cholesterol, statins have also been reported to exert pleiotropic actions, including anti-inflammatory and anti-oxidative activities. The present study aimed to investigate the ability of atorvastatin to protect endothelial colony-forming cells (ECFCs), a homogeneous subtype of EPCs, from hydrogen peroxide (H_2O_2)-induced oxidative damage, and to determine the mechanism underlying this protective action. MTT assay, acridine orange/ethidium bromide staining, reactive oxygen species assay, western blot analysis and tube formation assay were employed. The results demonstrated that H_2O_2 induced cell death and decreased the tube-forming ability of the ECFCs, in a concentration-dependent manner; however, these effects were partially attenuated following administration of atorvastatin. The reversion of the quantitative and qualitative impairment of the H_2O_2-treated ECFCs appeared to be mediated by the regulation of annexin A2, as the expression levels of annexin A2 were decreased following treatment with H_2O_2 and increased following treatment with atorvastatin. These results indicated that annexin A2 may be involved in the H_2O_2-induced damage of ECFCs, and in the protective activities of atorvastatin in response to oxidative stress.

Introduction

Endothelial injury and dysfunction are recognized as important contributors to a wide range of critical illnesses, and are considered to be major contributors to morbidity and mortality rates (1). Efficient repair of damaged endothelium and enhanced formation of new blood vessels are crucial for improvement of these pathological conditions. Maintenance and repair of the endothelium was originally considered to depend on angiogenesis, which occurs via the migration and proliferation of surrounding resident mature endothelial cells (2). However, this traditional concept has been challenged by the discovery of endothelial progenitor cells (EPCs), which are mobilized from bone marrow into the peripheral circulation, where they locate to sites of injured endothelium to promote vascular repair (3). Compared with angiogenesis, the formation of new blood vessels from the proliferation and differentiation of bone marrow-derived progenitor cells is defined as vasculogenesis (4). EPCs have been investigated extensively as biomarkers to assess the risk of cardiovascular disease, and as a potential cell therapeutic strategy for vascular regeneration (5-7). Evidence suggests that these putative EPCs encompass different cell populations, including cells of myeloid or endothelial origin, due to their lack of specific surface markers, and that they promote neovascularization via different mechanisms (8,9).

ECFCs, which are a subset of EPCs, are a homogenous cell population with a high proliferative capacity and the ability to

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form *de novo* blood vessels; therefore, ECFCS are considered true EPCs that are involved in new blood vessel formation (9-13). However, during the repair process of injured blood vessels, EPCs are exposed to oxidative stress, which limits their efficacy in neovascularization. Statins are pleiotropic compounds that participate in biochemical activities, and exert beneficial effects on the outcome of numerous diseases (14-18). Its protective activities have been reported to improve endothelial function by virtue of its anti-inflammatory and antioxidant effects (19,20). The present study aimed to investigate the protective action of atorvastatin on the oxidative stress-induced cell death and tube formation dysfunction of ECFCs, and to determine the mechanisms underlying these actions.

Annexin a2 is a highly conserved protein that is widely distributed in the nucleus, cytoplasm and extracellular surface of various eukaryotic cells. It is an important cellular redox regulatory protein and its redox activity is dependent on its reactive cysteine residue (Cys-8) in the N-terminus. Little is known regarding the correlation of annexin A2 and the dysfunction of EPCs in oxidative conditions and this protein with anti-oxidative activity of statins.

It was hypothesized that atorvastatin exerts protection against H_2O_2-induced oxidative damage by regulating the expression levels of annexin A2. This would provide a potential effective strategy, which may enhance the ability of ECFCs to form new blood vessels and treat vascular disorders.

Materials and methods

Drugs and chemicals. All reagents and chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA), unless stated otherwise.

ECFC cultures. ECFC cells (Lonza, Walkersville, MD, USA) were suspended in complete EGM-2 endothelial cell growth medium (Lonza), and were plated at a density of $3x10^4$ cells/cm^2 onto 75-cm^2 tissue culture flasks, which were pre-coated with type I rat tail collagen (BD Biosciences, Bedford, MA, USA). The cell cultures were maintained in a humidified atmosphere of 5% CO_2 at 37˚C, and the culture medium was replaced every 1-2 days.

Cell viability assay. Cell viability was evaluated using modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylt-etrazolium bromide (MTT), which is converted into blue formazan crystals in the presence of metabolically active cells. Briefly, the ECFCs were seeded at a density of $3x10^4$ cells/cm^2 onto 96-well plates pre-coated with type I rat tail collagen for 24 h at 37˚C. The cells were subsequently exposed either to purified water (controls), or exposed to various concentrations of H_2O_2 (100, 200 and 300 μM) for 3 h at 37˚C. MTT solution (5 mg/ml) was then added to the wells and the cells were incubated for 4 h at 37˚C. Following incubation, the culture medium was removed and 100 μl dimethyl sulfoxide was added to each well, in order to solubilize the formazan into a colored solution. The absorbance of the colored solution was measured at 570 nm using a microplate reader (Epoch; BioTek Instruments, Inc., Winooski, VT, USA). To examine the effects of atorvastatin on H_2O_2-induced cellular toxicity, the cells were pre-treated for 3 h at 37˚C with various concentrations of atorvastatin (0.001, 0.01,

0.1, 1, 10 μM), followed by co-incubation with 200 μM H_2O_2 for a further 3 h at 37˚C. Cell viability was assessed 3 h later by measuring the absorbance of the colored solution. Based on these results, 0.01 μM atorvastatin was used in all subsequent experiments.

Nuclear staining assay. The H_2O_2-induced changes in nuclear morphology were evaluated using acridine orange/ethidium bromide staining (AO/EB). ECFCs at a density of $3x10^4$ cells/cm^2 were plated onto 6-well plates and incubated at 37˚C in an atmosphere of 5% CO_2. Following 3 h pre-treatment with 0.01 μM atorvastatin, 200 μM H_2O_2 was added. Following co-incubation for 3 h, the cells were washed three times with phosphate-buffered saline (PBS), resuspended in PBS, followed by the addition of AO/EB (final concentration, 1 μg/ml). Following incubation for 10 min at room temperature, the cells were examined under a fluorescence microscope (IX71; Olympus Corporation, Tokyo, Japan). The cells with intact structures were stained green and were considered living cells, whereas the cells with condensed green nuclei were considered early apoptotic cells and those with condensed red-orange chromatin were considered late apoptotic cells. At least 300 cells were randomly observed, and the number of apoptotic cells was expressed as a percentage of the total cells counted.

Measurement of oxidative stress. Oxidative stress was measured using a reactive oxygen species (ROS) assay with 2'-7'-dichlorofluorescein diacetate (DCFH-DA), which is based on the ROS-dependent oxidation of DCFH-DA to fluorescent dichlorofluorescin (DCF). DCFH-DA readily crosses the membrane into cells, where it is converted into non-fluorescent DCFH by intracellular esterase. DCFH is then trapped within the cell and oxidized into highly fluorescent DCF by intracellular ROS; therefore, the fluorescence intensity reflects the levels of ROS production (21). Following treatment, the cells were incubated in bovine serum albumin-free Dulbecco's modified Eagle's medium with DCFH-DA at a final concentration of 20 μM for 30 min at 37˚C. Subsequently, the cells of each group were analyzed by flow cytometry (BD Biosciences) using the FL1 flow cytometer detection channels. The excitation wavelength was 485 nm, and the reading was performed at 530 nm.

Western blot analysis. Following treatment, the ECFCs were collected and lysed with cell lysis solution containing 4% sodium dodecyl sulfate (SDS), 2 mM EDTA and 50 mM Tris-HCl (pH 6.8). Protein concentration was determined using the Bradford method (GE Healthcare Life Sciences, Little Chalfont, UK). Equal quantities of proteins (40 μg) were separated by SDS-PAGE and transferred onto polyvinylidene fluoride membranes (GE Healthcare Life Sciences). The membranes were incubated in Tris-buffered saline/1% Tween buffer supplemented with 5% fat-free milk at room temperature for 1 h to block nonspecific binding. The membranes were then incubated with mouse monoclonal anti-human annexin a2 antibodies (1:1,000; BD Biosciences; cat. no. 610068) overnight at 4˚C, followed by incubation with horseradish peroxidase-conjugated anti-mouse secondary antibodies (1:1,000; Pierce Biotechnology, Inc., Rockford, IL, USA; cat. no. R-21455) for 2 h at room temperature. The blots were analyzed using an enhanced chemiluminescence system (GE Healthcare Life Sciences).
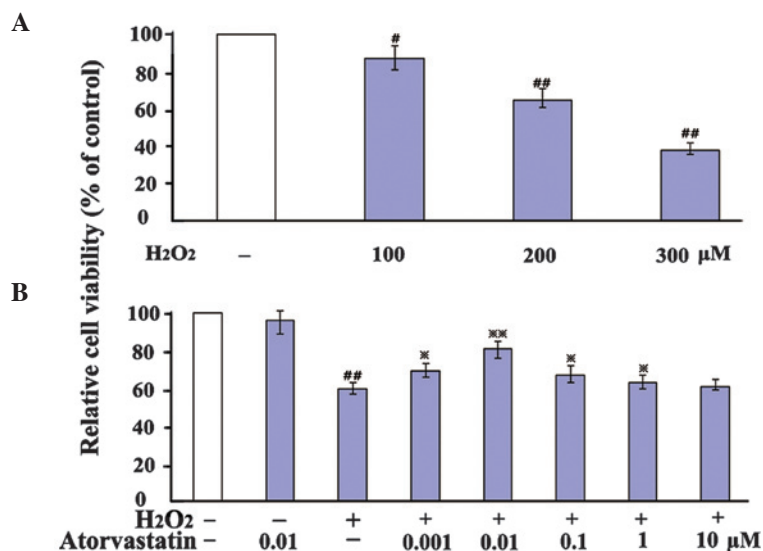
Figure 1. H_2O_2 reduces the cell viability of ECFCs, whereas atorvastatin attenuates H_2O_2-induced ECFC damage. (A) ECFCs were exposed to various concentrations of H_2O_2 (100, 200 and, 300 μM) for 3 h. (B) ECFCs were pre-treated with various concentrations of atorvastatin (0.001-10 μM) and then co-incubated with 200 μM H_2O_2 for 3 h. Cell viability was determined using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylt-etrazolium bromide assay. Data are presented as the mean ± standard error of the mean of three independent experiments, performed in six repeats. [#]P<0.05 and [##]P<0.01, vs. untreated control; [*]P<0.05 and [**]P<0.01, vs. H_2O_2-treated cells. ECFCs, endothelial colony-forming cells; H_2O_2, hydrogen peroxide.

Tube formation assay. The tube-forming capacity of ECFCs was measured using a Matrigel assay, as previously described (22,23). Briefly, following treatment, the ECFCs were trypsinized and seeded at a density of $3x10^4$ cells/cm^2 onto 96-well plates, which were pre-coated with 50 μl Matrigel™ (BD Biosciences), and incubated for 24 h at 37°C. The enclosed networks of complete tubes were then counted, and images were captured using an inverted microscope (IX71; Olympus Corporation). Each experiment was performed in triplicate.

Statistical analysis. Data are expressed as the mean ± standard error of the mean. Statistical analysis was performed using one-way analysis of variance, followed by Dunnett's multiple-comparisons test. Analyses were performed using SPSS version 15.0 (SPSS, Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

Atorvastatin attenuates H_2O_2-induced reductions in cell viability in ECFCs. H_2O_2-induced oxidative damage was investigated in the ECFCs using an MTT assay, which is a colorimetric assay used for measuring the activity of mitochondrial dehydrogenase in metabolically active cells. The cell viability of the ECFCs decreased following exposure to H_2O_2, in a dose-dependent manner. Following 3 h treatment, 100 μM H_2O_2 reduced cell viability to 87% of the control, whereas 200 and 300 μM H_2O_2 decreased cell viability to 63 and 37%, respectively (Fig. 1A). The reduced cell viability of the ECFCs was significantly attenuated by pre-treatment with 0.01 μM atorvastatin for 3 h (Fig. 1B). These results indicated that atorvastatin protected the ECFCs against H_2O_2-induced oxidative damage.

Atorvastatin attenuates H_2O_2-induced apoptosis in ECFCs. The protective role of atorvastatin in H_2O_2-induced apoptosis of ECFCs was measured using an AO/EB assay. Apoptosis is a type of programmed cell death, which is characterized by a series of distinct nuclear morphological changes that can be detected by AO/EB staining (24). Treatment with atorvastatin alone did not induce changes in the number of apoptotic cells, whereas treatment with H_2O_2 significantly increased the number of apoptotic cells, compared with the control group. Pre-treatment with 0.01 μM atorvastatin significantly decreased the number of H_2O_2-induced apoptotic cells (Fig. 2). These results suggested that atorvastatin may have an anti-apoptotic role in cells under oxidative conditions.

Atorvastatin inhibits H_2O_2-induced ROS production. The levels of ROS production were evaluated using DCFH-DA staining and flow cytometry, as previously described (25). DCFH-DA is a stable compound, which readily diffuses into cells, where it is converted into DCFH by intracellular esterase. DCFH is then trapped within the cells and oxidized to highly fluorescent DCF by intracellular ROS; therefore, the fluorescence intensity produced by DCF may reflect an intracellular oxidative state (21). Treatment with atorvastatin alone did not elicit changes in the levels of ROS production, compared with the control group. However, treatment with H_2O_2 induced a significant increase in the production of ROS in the ECFCs, which was markedly reversed by pre-treatment with atorvastatin (Fig. 3), further supporting an anti-oxidative role of atorvastatin.

Atorvastatin suppresses the H_2O_2-induced decrease in the expression of annexin A2 in ECFCs. To determine whether annexin A2 is involved in the death and dysfunction of ECFCs under oxidative conditions, the protein expression levels of annexin A2 were analyzed in H_2O_2-treated cells. The present study initially investigated whether H_2O_2 decreased the expression levels of annexin A2 in the ECFCs in a dose-dependent manner. ECFCs were exposed to various concentrations of
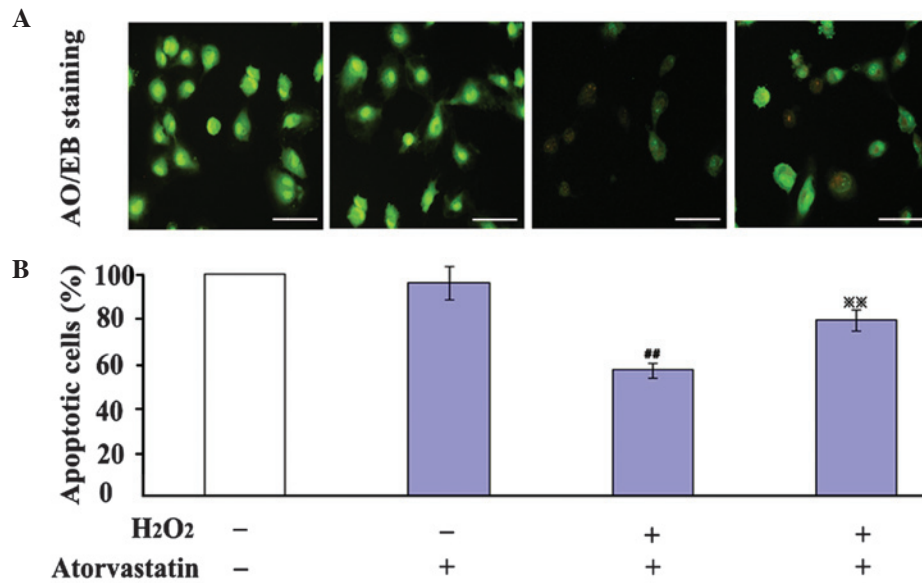
Figure 2. Treatment with atorvastatin inhibits H_2O_2-induced apoptosis of ECFCs. (A) ECFCs were pre-treated for 3 h with 0.01 μM atorvastatin, following which 200 μM H_2O_2 was added and the cells were analyzed under a microscope following AO/EB staining. The cells with intact structures were stained green and were considered to be viable cells, whereas the cells with condensed green nuclei were identified as early apoptotic cells and those with condensed red-orange chromatin were identified as late apoptotic cells. (B) Histograms of the percentage of apoptotic cells relative to the number of total cells. Data are presented as the mean ± standard error of the mean (n=3). [##]P<0.01, vs. untreated control; [**]P<0.01, vs. H_2O_2-treated cells. Scale bar=250 mm. ECFCs, endothelial colony-forming cells; H_2O_2, hydrogen peroxide; AO/EB, acridine orange/ethidium bromide.
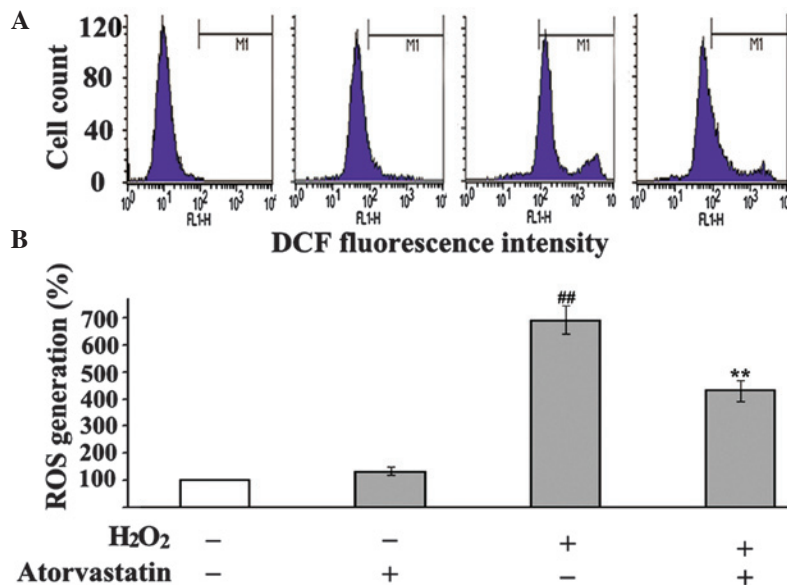

Figure 3. Atorvastatin inhibits H_2O_2-induced ROS production. Endothelial colony-forming cells were pre-treated with 0.01 μM atorvastatin for 3 h and then exposed to H_2O_2 for a further 3 h. (A) Production of ROS in the cells was measured using flow cytometry with 2'-7'-dichlorofluorescein diacetate. The shift of the curve to the right indicates an increase in ROS generation and a shift to the left indicates a reduction in ROS generation. (B) Graph of the relative levels of ROS, compared with the untreated control. Data are presented as the mean ± standard error of the mean (n=3). [##]P<0.01, vs. untreated control; [**]P<0.01, vs. H_2O_2-treated cells. ROS, reactive oxygen species; H_2O_2, hydrogen peroxide; DCF, dichlorofluorescin.

H_2O_2 (100, 200 and 300 μM) for 3 h, and the protein expression levels of annexin A2 were detected using western blot analysis. Consistent with the observed changes in cell viability, the expression levels of annexin A2 decreased in ECFCs treated with 100 μM H_2O_2, and decreased further following treatment with 200 and 300 μM H_2O_2 (Fig. 4A). These results suggested that annexin A2 was involved in ECFC oxidative damage. To examine the mechanism underlying the protective activity of atorvastatin, the present study investigated whether atorvastatin affected the expression of annexin A2 in H_2O_2-induced ECFCs. The administration of 200 μM H_2O_2 markedly decreased the expression levels of annexin A2, and these changes were markedly attenuated by pre-treatment with atorvastatin. Atorvastatin alone did not induce a change in the protein expression levels of annexin A2. These results suggested that regulation of the expression of annexin A2 is a potential molecular mechanism by which atorvastatin exerts its protective role against oxidative stress (Fig. 4B).
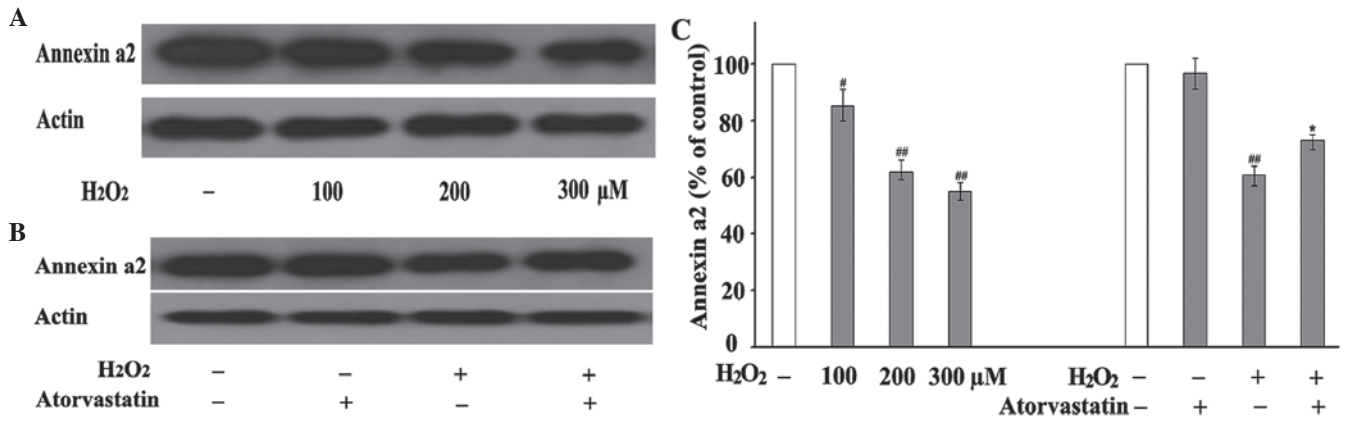
Figure 4. Atorvastatin attenuates the decreased expression levels of annexin A2 in H_2O_2-treated ECFCs. (A) ECFCs were treated with various concentrations of H_2O_2 (100, 200 and 300 μM). (B) ECFCs were pre-treated for 3 h with 0.01 μM atorvastatin, and then co-incubated with 200 μM H_2O_2. The protein expression levels of annexin A2 were determined using western blotting, with actin used as a loading control. (C) Histograms of the percentage of annexin a2 content relative to the content of the control. Data are presented as the mean ± standard error of the mean (n=3). [#]P<0.05 and [##]P<0.01, vs. the untreated control; [*]P<0.01, vs. H_2O_2-treated cells. ECFCs, endothelial colony-forming cells; H_2O_2, hydrogen peroxide.
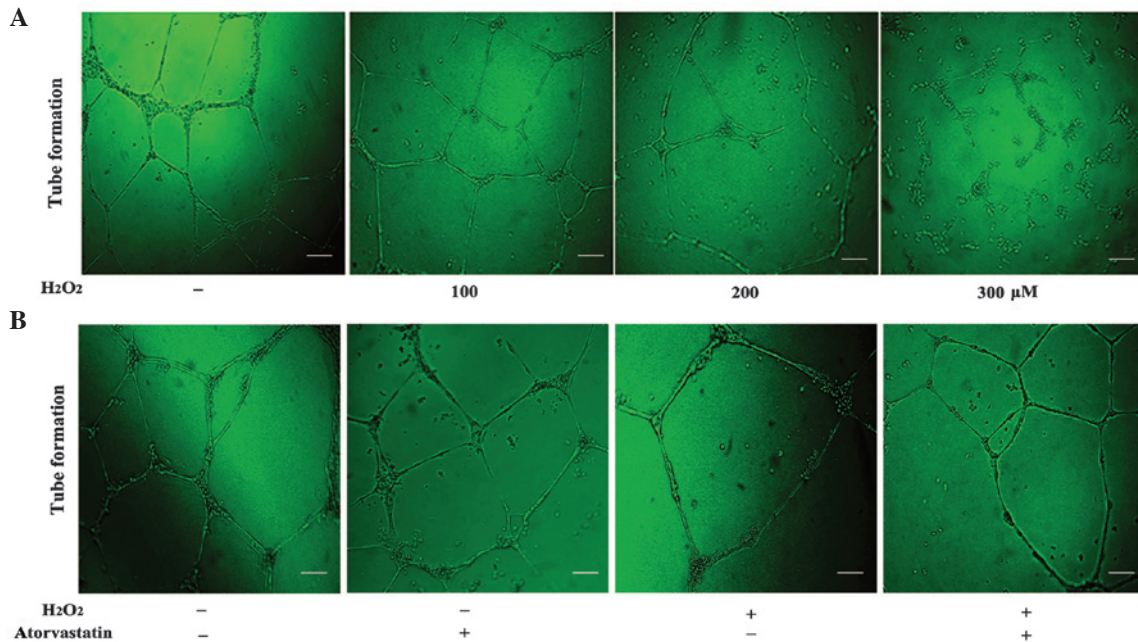

Figure 5. H_2O_2 reduces the tube-forming ability of ECFCs and atorvastatin attenuates the impaired tube-forming ability of ECFCs. (A) ECFCs were treated with increasing concentrations of H_2O_2 for 3 h. (B) Following pre-treatment with atorvastatin for 3 h, the ECFCs were exposed to 200 μM H_2O_2 for a further 3 h. The cells were seeded in Matrigel and incubated for 24 h, following which closed network formation was detected. No closed network formation was detected following treatment with 300 μM H_2O_2. Scale bar=250 mm. ECFCs, endothelial colony-forming cells; H_2O_2, hydrogen peroxide.

Atorvastatin attenuates the impairment in tube-forming ability of ECFCs under oxidative conditions. The most important function of ECFCs is tube formation, which can be evaluated using a Matrigel assay (26). To assess the dysfunction of ECFCs under oxidative condition, the cells were treated with various concentrations of H_2O_2 (100, 200 and 300 μM). The results revealed a decrease in the number of closed network units following exposure to H_2O_2, which occurred in a dose-dependent manner. No closed network unit formed following treatment with 300 μM H_2O_2 (Fig. 5A), suggesting that this concentration severely impaired the tube-forming capacity of the ECFCs under oxidative conditions. Pre-treatment with 0.01 μM atorvastatin markedly decreased the H_2O_2-induced impairment in ECFC tube

formation (Fig. 5B). These results indicated that atorvastatin attenuated the tube-forming dysfunction of ECFCs under oxidative conditions.

Discussion

Dysregulation of new vessel formation and inefficient repair of injured endothelium are common features of a wide range of disorders. EPCs are derived from the bone marrow, and circulate in the peripheral blood, locating to sites of injured endothelium, where they are involved in vascular regeneration and repair and may provide a potential cell therapy for the treatment of vascular diseases (3). However, oxidative stress during vascular damage limits the efficacy

of EPCs in the repair of injured endothelium (26); therefore, protection of EPCs against oxidative damage may promote EPC-mediated new blood vessel formation and the repair of injured blood vessels, which is beneficial for the treatment of vascular disorders. Statins are pleiotropic compounds, which were originally identified as effective agents in lowering lipid levels by inhibiting 3-hydroxy-methylglutaryl coenzyme A reductase through binding to the active site and inhibiting the substrate-product transition state of the enzyme (27,28). Previous evidence has revealed that statins are also effective in maintaining the integrity and function of endothelial cells and the stability of atherosclerotic plaques, inhibiting the thrombogenic response and in anti-oxidative stress and inflammation (29,30). The present study indicated that atorvastatin exerted a protective effect against oxidative damage in ECFCs, and this protective action may have been mediated by attenuating the H_2O_2-induced decreased expression levels of annexin A2.

ECFCs are a homogeneous subtype of EPCs with a high proliferative capacity, blood-forming activity and therapeutic potential in vascular injury (11-13). In addition to direct incorporation into the endothelial intima in the process of vasculogenesis, ECFCs release pro-angiogenic factors in a paracrine manner, which contribute to new blood vessel formation; therefore, this homogenous population of endothelial-like cells is important in postnatal neovascularization (12). In the process of new vessel formation, ECFCs are exposed to oxidative stress, which limits their efficacy, however, the molecular mechanisms underlying the quantitative and qualitative impairment of ECFCs under oxidative conditions remain to be fully elucidated. The results of the present study demonstrated that treatment with H_2O_2 significantly increased the number of apoptotic cells, in a dose-dependent manner, supporting the hypothesis that ECFCs are impaired under oxidative conditions. Notably, the expression levels of annexin A2 were also decreased in a dose-dependent manner in the H_2O_2-treated ECFCs, suggesting that annexin A2 may be involved in the oxidative stress-dependent ECFC death process. Annexin A2 is a multifaceted protein, which affects several cellular processes, including cell proliferation, survival, endocytosis, exocytosis and cytoskeletal reorganization (31-34). Changes in the expression levels of annexin A2 have been implicated in a wide range of pathogenetic events, responsible for a diverse number of disorders (35-37). Annexin A2 is present in cells in two forms: Monomeric and heterotetrameric. The heterotetramer is a stable complex, which comprises two subunits of A2 and two subunits of p11 (38-41). An *N*-terminal reactive cysteine residue is responsible for the redox regulatory function of annexin A2, as it can be oxidized by H_2O_2 and subsequently reduced by the thioredoxin system, enabling its involvement in several redox cycles (41). Therefore, the degradation of annexin A2 leads to cell vulnerability to oxidative attack, which may be partly responsible for the ECFC damage under oxidative conditions. In the present study, pre-treatment with atorvastatin repressed the degradation of annexin A2 and prevented apoptosis of the ECFCs treated with H_2O_2, indicating that annexin A2 was involved in oxidative damage to the ECFCs, and that the anti-oxidative role of atorvastatin may be mediated by regulating the expression of annexin A2.

Annexin A2 exerts diverse effects on new blood vessel formation; as a tissue plasminogen activator receptor on the cell surface of endothelial cells, annexin A2 catalyzes the conversion of plasminogen into plasmin, activating matrix metalloproteinases (MMPs) into active proteases (42-44). Once activated, MMPs cause extracellular matrix degradation, which is responsible for the formation of new blood vessels (44). Annexin A2 interacts directly with the vascular endothelial cadherin (VE-cad)-based complex, which is required for the maintenance of vascular endothelial integrity by forming VE-cad-dependent cell-cell contact formation, which leads to the disruption of endothelial cell-cell contacts (45,46). Annexin A2 also promotes vascular endothelial growth factor-mediated neovascularization (45). At present, the action of annexin A2 in EPC-mediated new vessel formation remains to be fully elucidated. The results of the present study revealed that treatment with H_2O_2 decreased the tube-forming ability of ECFCs in a concentration-dependent manner, and this was consistent with the decrease in the expression of annexin A2, indicating a correlation between the expression of annexin A2 and ECFC tube formation. Atorvastatin alleviated the decreased expression levels of annexin A2 and enhanced the tube-forming capacity of ECFCs under oxidative conditions, reinforcing the involvement of this protein in ECFC-mediated new vessel formation. However, the underlying mechanism responsible for the action of annexin A2 in ECFC tube formation remains to be elucidated. Actin is a key regulator in cytoskeletal remodeling, which is responsible for cell mobility. Various cell activities, including migration, morphological changes and polarity formation are regulated by actin filament dynamics, including actin filament disassembly, severing and reorganization (47,48). Previous studies have demonstrated that actin dysfunction results in impaired ECFC functions, including tubule formation (22,49). Annexin A2 is crucial in actin cytoskeletal rearrangements by binding the regions of free-barbed ends (50). Therefore, the involvement of annexin A2 in tube formation of ECFCs may be mediated by modulating actin activity. The regulatory role of statins in the expression of annexin A2 may be associated with the phosphatidylinositol 3-kinase (PI3K)/Akt survival pathways. Previous studies have revealed that statins exert their protective roles in the vascular system via modulation of the PI3K/Akt survival pathways (51,52). PI3K is an upstream signal of glycogen synthase kinase 3 (GSK-3), which is central in regulating the expression of annexin A2 (53). Therefore, the protective effects of statins may be mediated by the activation of PI3K, which inactivates the downstream signal protein, GSK-3β, thereby preventing the degradation of annexin A2. Further investigations are required to examine this pathway in the vasculature.

In conclusion, the present study demonstrated that the multifaceted annexin A2 protein was involved in ECFC oxidative damage and dysfunction, and atorvastatin attenuated the quantitative and qualitative impairment of the ECFCs under oxidative conditions. The protective action of atorvastatin may be partly mediated by alleviating the degradation of annexin A2 via the PI3K/Akt survival pathways involved in the GSK-3 cascade. Further investigations are required to precisely elucidate the mechanisms underlying the role of annexin A2 in ECFC function, and the regulatory role of

statins in the expression of annexin A2. These findings may assist in developing potential effective treatments for vascular disease by targeting oxidative stress-mediated ECFC damage.

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