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Atorvastatin prevents angiotensin II-induced high permeability of human arterial endothelial cell monolayers via ROCK signaling pathway

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ABSTRACT

Intracranial aneurysm, as a common cause of cerebral hemorrhage, is often discovered when the aneurysm ruptures, causing subarachnoid hemorrhage. Unfortunately, the formation of cerebral aneurysm, which is associated with endothelial damage and macrophage migration, still cannot be prevented now. Tight junctions (TJs) open due to the disappearance of TJ proteins occludin and zona occludens-1 (ZO-1) in damaged endothelia, thus allowing macrophage migration and forming cerebral aneurysm. Therefore, cerebral aneurysm formation can be prevented by increasing TJs of the artery endothelium. Interestingly, statin, which can reduce saccular aneurysm, may prevent aneurysm formation through acting on different steps, but the underlying mechanism remains unclear. In this study, angiotensin II (Ang II) significantly increased the permeability of human arterial endothelial cell (HAEC). Moreover, the distribution of ZO-1 in cell-cell junction area and the total expression in HAECs were significantly decreased by Ang II treatment. However, the abnormal distribution and decreased expression of ZO-1 and hyperpermeability of HAECs were significantly reversed by pretreatment with atorvastatin. Furthermore, Ang II-induced phosphorylations of MYPT1, LIMK and MLC2 were significantly inhibited with atorvastatin or Rho kinase (ROCK) inhibitor (H1152) pretreatment. Knockdown of ROCK-II probably abolished Ang II-induced abnormal ZO-1 distribution and expression deficiency and hyperpermeability of HAECs. In conclusion, atorvastatin prevented Ang II-induced rupture of HAEC monolayers by suppressing the ROCK signaling pathway. Our results may explain, at least in part, some beneficial effects of statins on cardiovascular diseases such as intracranial aneurysm.

1. Introduction

Cerebral aneurysm, also known as intracranial or intracerebral aneurysm, is a thin or weak spot on a blood vessel in the brain that balloons out and fills with blood. Upon possible rupture, blood is split into surrounding tissues (called hemorrhage). Although cerebral aneurysms can occur anywhere in the brain, most of them are located along a loop of arteries that run between the brain underside and the skull base. The formation of cerebral aneurysms is associated with macrophage migration and endothelial damage. In the very early stage before aneurysm formation, the expressions of occludin and zona occludens-1 (ZO-1) are reduced in injured endothelial cell junctions with gaps. During aneurysm progression, their expressions are further reduced and correlated with macrophage migration, forming cerebral aneurysms as a result [1,2]. Increasing occludin and ZO-1 expressions can inhibit macrophage exudation and aneurysm formation [2]. Therefore, increasing tight junctions (TJ) of the artery endothelium may be able to prevent cerebral aneurysm formation.

Many previous studies have linked angiotensin II (Ang II) with aneurysm formation [3–5], although Ang II-induced inflammatory response is considered as aneurysm formation in hypertensive patients. The destruction of TJs may facilitate macrophage migration and cerebral-aneurysm formation in a rat model [2]. Ang II increases the monolayer permeability of human arterial endothelial cells (HAECs) through downregulation of occludin and ZO-1 and activation of the Rho kinase (ROCK) pathway [6,7]. ROCK is upregulated at the aortic aneurysm lesions in humans as well as at the Ang II-induced ones in mice [8]. As an important kinase related with cytoskeleton rearrangement, ROCK regulates inflammatory

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cell infiltration, TJ protein disassembly and elevates vascular permeability [9]. Fasudil, a selective ROCK inhibitor, reduces the development of Ang II-induced aortic aneurysm formation [10]. Therefore, aortic aneurysm formation may be controlled by inhibiting the ROCK signaling way.

Hydroxymethylglutaryl-coenzyme A reductase inhibitors (statins), as widely used anti-hypercholesterolemic agents, show pleiotropic effects such as improvement of vascular endothelium function as well as suppression of inflammation and oxidative stress in animals [11]. Interestingly, statins may have protective effects on aneurysm development [12], but the underlying mechanism is still unclear. Till now, many studies have reported that statin prevented the hyperpermeability of endothelial cells by inhibiting RhoA/ROCK signaling [13,19,20]. Hence, we hypothesized that atorvastatin may be involved in inhibition of endothelial cell monolayers' permeability by suppressing aortic aneurysm formation. In this study, Ang II was used to increase the permeability of HAEC monolayers, and atorvastatin inhibited such hyperpermeability via the ROCK signaling pathway.

2. Material and methods

2.1. Regents

Angiotensin II was purchased form AnaSpec Inc (San Jose, CA, USA). Dimethyl sulfoxide (DMSO) and Atorvastatin were acquired from SIGMA. Phospho-Myosin Light Chain 2, Myosin Light Chain 2, Phospho-Cofilin, Cofilin, β-Actin, Occludin, Anti-rabbit IgG (H + L) 488 and goat anti-rabbit IgG HRP-conjugated antibody were all purchased from Cell Signaling Technology (Berverly, MA). ZO-1 Antibody was purchased from Santa Cruz Biotechnology.

2.2. Cell culture and transfection

Primary human coronary artery endothelial cells (HCAECs) were obtained from ATCC (Cat. No. PCS-100-020). HCAECs were grown in Vascular Cell Basal Medium (Cat. No. PCS-100-030), Endothelial Cell Growth Kit (Cat. No. PCS-100-040) and supplemented with 5% fetal bovin serum. Cultured HCAECs were used at passage 6-7 in all experiments. The cells were transfected with the indicated plasmids using Lipofectamine® LTX with Plus™ Reagent (Life Technologies, Cat No. 15338100) cell transfection reagent according to the manufacturer's protocol.

2.3. Endothelial permeability assays

Permeability across the HCAEC monolayer was studied in an *In Vitro* Vascular Permeability system (Cat. No. ECM642, Chemicon, Darmstadt, Germany), which has 0.4 μm pore size. HCAECs were seeded into the upper chamber of transwells at 5×10^5 cells/mL and grown in the EGM-2 medium. Cells reached 80%-90% confluence after 5-7 days, and were then treated with different concentrations of Atorvastatin (0.1, 0.3 and 1 μM) or with DMSO as controls for 12 hours. Then HCAECs were incubated for 24 h with 0.2 μM angiotensin II. After treatment, the same amount of FITC-Dextran tracers (final concentration 20 μg/mL) were added into the upper chamber and incubated at 37 °C for another 20 min at room temperature. The amount of tracer that penetrated through the cell monolayer into the lower chamber was measured with the use of a LS 50B Luminescence Spectrometer (PerkinElmer, Beaconsfield, United Kingdom; excitation wavelength, 485 nm; emission wavelength, 530 nm) as the index of monolayer permeability of endothelial cells.

2.4. Immunofluorescence staining

After treatment with Angiotensin II, HCAECs were fixed in 4% formaldehyde solution for 20 min at room temperature, and permeabilized with 0.3% Triton X-100 in phosphate buffered saline with Tween-20 (PBS-T) for 10 min at room temperature. After blocking with 2% bovine serum albumin (BSA) in PBS, the cells were incubated with rabbit anti-ZO-1 (diluted 1:100; Santa Cruz Biotechnology) to visualize the distribution of ZO-1. After incubation with the primary antibody and fluorescence-tagging with a secondary antibody, slides were then analyzed using immunofluorescence microscopy (Olympus, Japan).

2.5. Western blot analysis

Cultured HCAECs were treated with different concentrations of Atorvastatin or DMSO for 12 hours, and then incubated into Angiotensin II for 24 hours. The treatment was halted by washing cells twice with cold phosphate-buffered saline. Total proteins were collected from cell lysates. 30 μg of lysate protein was loaded for Western blot analysis. Total cellular protein was separated by 10% SDS-polyacrylamide gel electrophoresis and then transblotted onto Immun-Blot PVDF Membrane (Bio-Rad Laboratories, Inc, Hercules, CA). After blocking the membrane with 5% nonfat dried milk, the membrane was probed with the respective primary antibodies against ZO-1, claudin-1, occludin, MLC2 and cofilin, respectively, over night in 4 °C. The membranes were washed 3 times with 0.1% Tween 20-TBS and then incubated in the Anti-rabbit IgG, HRP-linked secondary antibody for 1 hour at room temperature. The immunoreactive bands were detected by enhanced chemiluminescent (ECL) plus reagent kit. The band density was measured with the use of Quantity One 4.2.1 (Bio-Rad Laboratories, Inc, Hercules, CA) and normalized to β-actin expression.

2.6. Statistical analysis

Data were expressed as the mean ± SD. Comparisons were analyzed with the Student *t* test. A P value <0.05 was considered statistically significant.

3. Results

3.1. Atorvastatin prevented Ang II-induced endothelial permeability

Ang II can increase the permeability of endothelial cell monolayers [14]. HAECs were herein treated with 0.2 μM Ang II for 24 h. Endothelial permeability measured by FITC-dextran and monolayers stained with cell stain dye indicated that Ang II significantly raised endothelial permeability (Fig. 1B).

Atorvastatin suppressed Ang II-induced aortic aneurysm formation, suggesting that statins may have protective effects on aneurysm development [12], but the underlying mechanism remains elusive. Given that statin reversed the hyperpermeability of endothelial cells [13], we initially investigated the effect of atorvastatin concentration on the permeability of HAEC monolayers. HAECs were firstly pretreated with atorvastatin for 12 h and then incubated with Ang II for another 24 h Fig. 1C and D shows that atorvastatin prevents Ang II-increased endothelial permeability.

3.2. Atorvastatin prevented Ang II-decreased ZO-1 expression

Endothelial TJ regulates the paracellular permeability of endothelial cells, and treatment of Ang II can significantly reduce TJ proteins (e.g. ZO-1) in endothelial cells [15]. In this study, less ZO-1 was distributed in the cell-cell junction area after 24 h of incubation

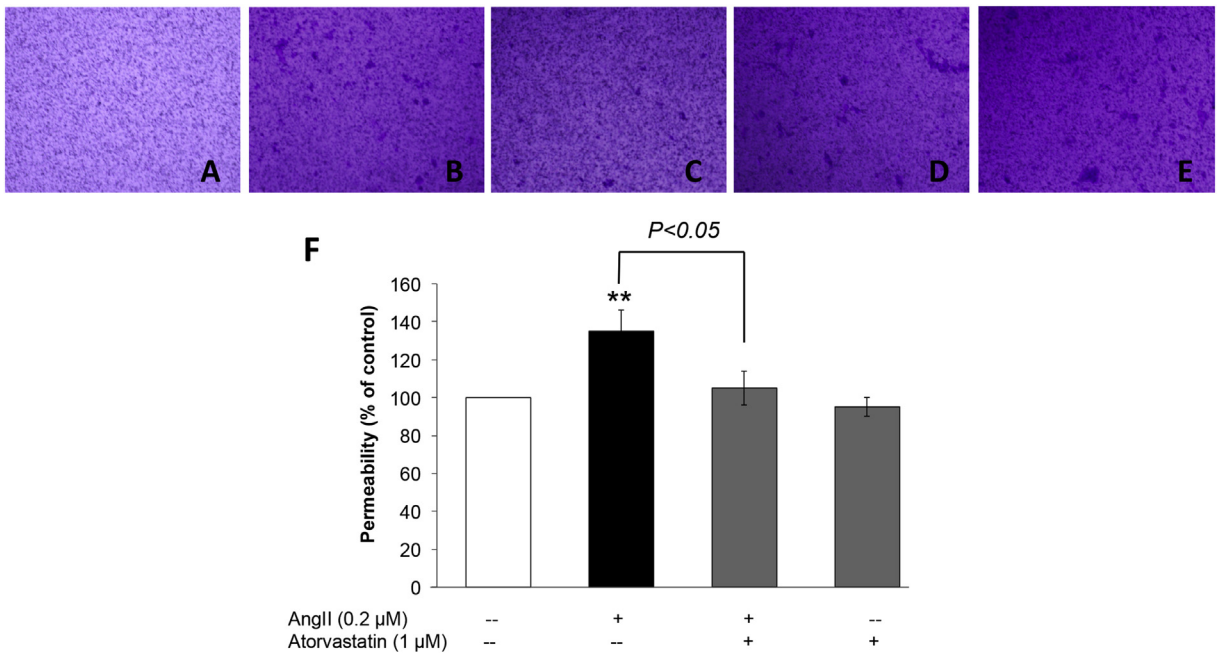

Fig. 1. Atorvastatin prevented Ang II-induced hyperpermeability in HAECs monolyer. HAECs were treated with 0.2 μM Ang II for 24 hours prior to treatment with 1 μM atorvastatin for 12 hours. (A) No seed cell served as blank group. (B) Control; (C) Ang II. (D) Atorvastatin + Ang II. (E) Atorvastatin. HAECs monolayers were stained with Cell stain dye. (F) Permeability of HUVEC monolayers measured by 40 kDa weight FITC-dextran. Each point represents the mean (±s.d.) of three independent experiments, each performed in triplicate. *$P < 0.05$ *vs.* control.

with Ang II (Fig. 2B, B′). To further investigate the changes in endothelial cells, we next treated the cells with the same way as mentioned above and extracted proteins for Western blotting. Obviously, the expression of ZO-1 in Ang II-treated group was significantly decreased compared with that in the cells treated with 0.1% DMSO. Interestingly, the abnormal distribution and decreased expression of ZO-1 were significantly reversed by pretreatment with atorvastatin (Fig. 2).

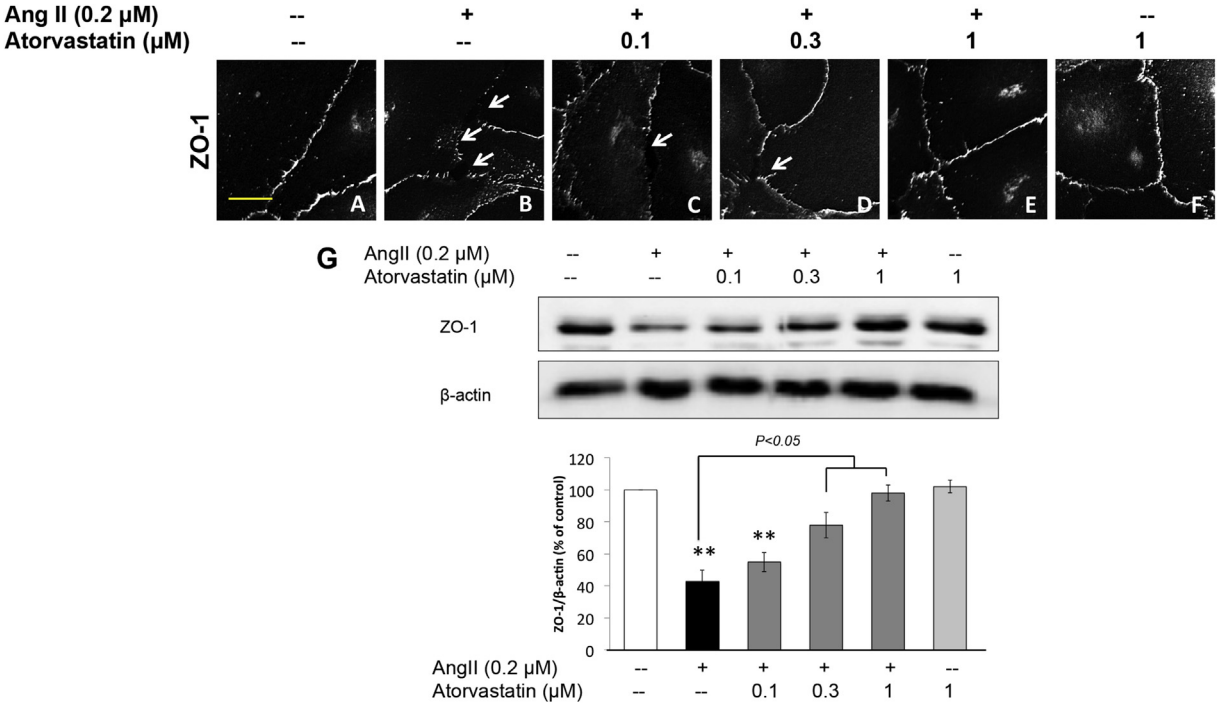

Fig. 2. Atorvastatin prevented Ang II-induced ZO-1 abnormal destruction and expression deficiency. HAECs were treated with 0.2 μM Ang II for 24 hours prior to treatment with 0.1, 0.3 and 1 μM atorvastatin for 12 hours. (A) Control. (B) Ang II. (C–E) Ang II + atorvastatin (0.1, 0.3, 1 μM). (F) Atorvastatin (1 μM). ZO-1 expression in intercellular junction was examined by an immunofluorescence. Arrows indicate points where intercellular junction was disrupted. (G) Western blot analysis for ZO-1 was performed. β-Actin was served as the loading control. Quantitative analysis was performed by measuring protein expression relative to the control. Each point represents the mean (±*s.d.*) of three independent experiments, each performed in triplicate. *$P < 0.05$, **$P < 0.01$ *vs.* control.

3.3. Atorvastatin reduced Ang II-induced Rho/ROCK activation

Since statins can protect Ang II-induced rupture of adherens junctions in endothelial cells by inhibiting the ROCK signaling pathway [16], we determined the role of this pathway in Ang II-induced endothelial permeability. MYPT1, LIMK and MLC2, as direct downstream factors of ROCK, regulate endothelial barrier function. It has previously been demonstrated that endothelial barrier dysfunction is mediated via the ROCK signaling pathway that enhances MYPT1, LIMK and MLC2 phosphorylations [17,18]. Treating HAECs with 0.2 μM Ang II (24 h) induced the phosphorylations of MYPT1, LIMK and MLC2 (Fig. 3), which were significantly inhibited by pretreatment with atorvastatin or ROCK inhibitor (H1152) for 12 h.

3.4. Atorvastatin prevented Ang II-induced hyperpermeability via the ROCK signaling pathway

To clarify whether Ang II-induced hyperpermeability was related with the ROCK signaling pathway, HAECs were pretreated with H1152. Fig. 4 indicates that H1152 prevents Ang II-induced abnormal ZO-1 distribution (loss in the cell–cell junction area) and expression deficiency. Thus, atorvastatin prevented Ang II-induced hyperpermeability of HAECs, which was mediated by inhibition of the ROCK signaling pathway.

4. Discussion

Given that atorvastatin suppressed Ang II-induced aortic aneurysm formation, statins may have protective effects on aneurysm development [12], following an undefined mechanism though. Many studies have indicated that statin reversed the hyperpermeability of endothelial cells [13,19,20]. Besides, in the early stage before aneurysm formation, the expressions of TJ proteins are reduced in injured endothelial cell junctions exhibiting gaps, thus promoting aneurysm progression by facilitating macrophage migration. Increased TJ protein expressions are associated with inhibition of macrophage exudation and aneurysm formation [1,2]. Accordingly, we hypothesized that atorvastatin may suppress aortic aneurysm formation through inhibiting the permeability of endothelial cell monolayers.

Endothelial TJ regulates the paracellular permeability of endothelial cells, and Ang II can increase the permeability of endothelial cell monolayers [14]. Furthermore, ZO-1 and TJ protein are reduced with Ang II treatment. Meanwhile, ZO-1 expression and intercellular junctions are markedly decreased in endothelial cells [15]. In this study, Ang II significantly increased HAECs permeability (Fig. 1), and the distribution of ZO-1 in cell-cell junction area and the total expression in HAECs were significantly decreased (Fig. 2). However, these outcomes were significantly reversed by being pretreated with atorvastatin. In the meantime, Ang II-induced hyperpermeability of HAECs was also prevented by atorvastatin treatment (Figs. 1 and 2).

Peng H et al. reported that statin prevented high glucose- or hyperglycemia-induced hyperpermeability by inhibiting RhoA/ROCK signaling in endothelial cells and in db/db mice [13]. Hypoxia-reoxygenation augments the permeability of endothelial monolayers by activating RhoA, which can be compromised by inhibiting RhoA/ROCK signaling with RhoA or ROCK inhibitors [21]. Activation of RhoA by bradykinin (BK) is associated with TJ protein disassembly, and an increase in blood-tumor barrier permeability in rat brain microvascular endothelial cells (RBMECs). ROCK inhibitor (Y-27632) and ROCK siRNA manage to inhibit endothelial leakage in RBMECs. Additionally, Y-27632 inhibits BK-induced relocation of TJ proteins from cellular borders into the cytoplasm as well as stress fiber formation in RBMECs. BK increases the phosphorylation of ROCK downstream factors, such as myosin light chain (p-MLC) and phosphorylated cofilin (p-cofilin) which are also inhibited by Y-27632 [9]. In this study, Ang II-induced phosphorylations of MYPT1, LIMK and MLC2 were significantly inhibited with atorvastatin or ROCK inhibitor (H1152) pretreatment (Fig. 3). Moreover, Knockdown ROCK-II abolished Ang II-induced abnormal ZO-1 distribution and expression deficiency and hyperpermeability in HAECs monolyer. Obviously, atorvastatin prevented Ang II-induced hyperpermeability via the ROCK signaling pathway.

In conclusion, activating ROCK signaling by using Ang II disrupted and decreased ZO-1 translocation and expression, thereby increasing cell permeability. Atorvastatin prevented Ang II-induced hyperpermeability and dysregulation of ZO-1 by suppressing ROCK signaling. These results suggest a potential molecular mechanism of atorvastatin in prevention of aortic aneurysm formation. Atorvastatin may enhance TJs by inhibiting macrophage migration and

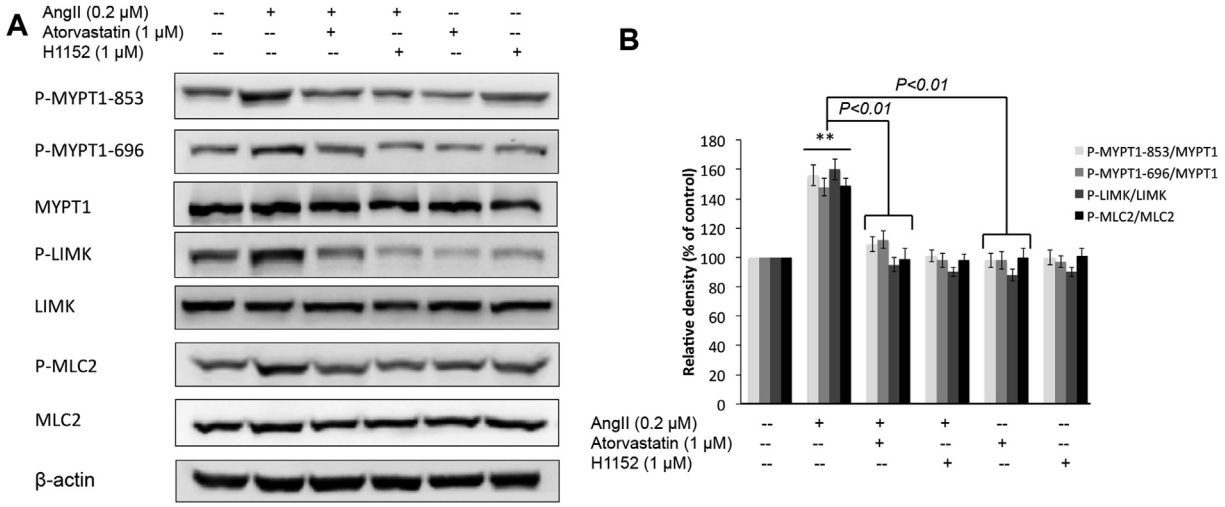

Fig. 3. Atorvastatin or H1152 inhibited Ang II-stimulated ROCK signaling pathway. HAECs were treated with 0.2 μM Ang II for 24 hours prior to treatment with 1 μM atorvastatin or 1 μM H1152 for 12 hours. (A) Western blot analysis for p-MYPT1853/696, p-LIMK/LIMK, p-MLC2/MLC was performed. β-Actin was served as the loading control. (B) Quantitative analysis was performed by measuring protein expression relative to the control. Each point represents the mean (±*s.d.*) of three independent experiments, each performed in triplicate. $^{*}P < 0.05$, $^{**}P < 0.01$ *vs.* control.

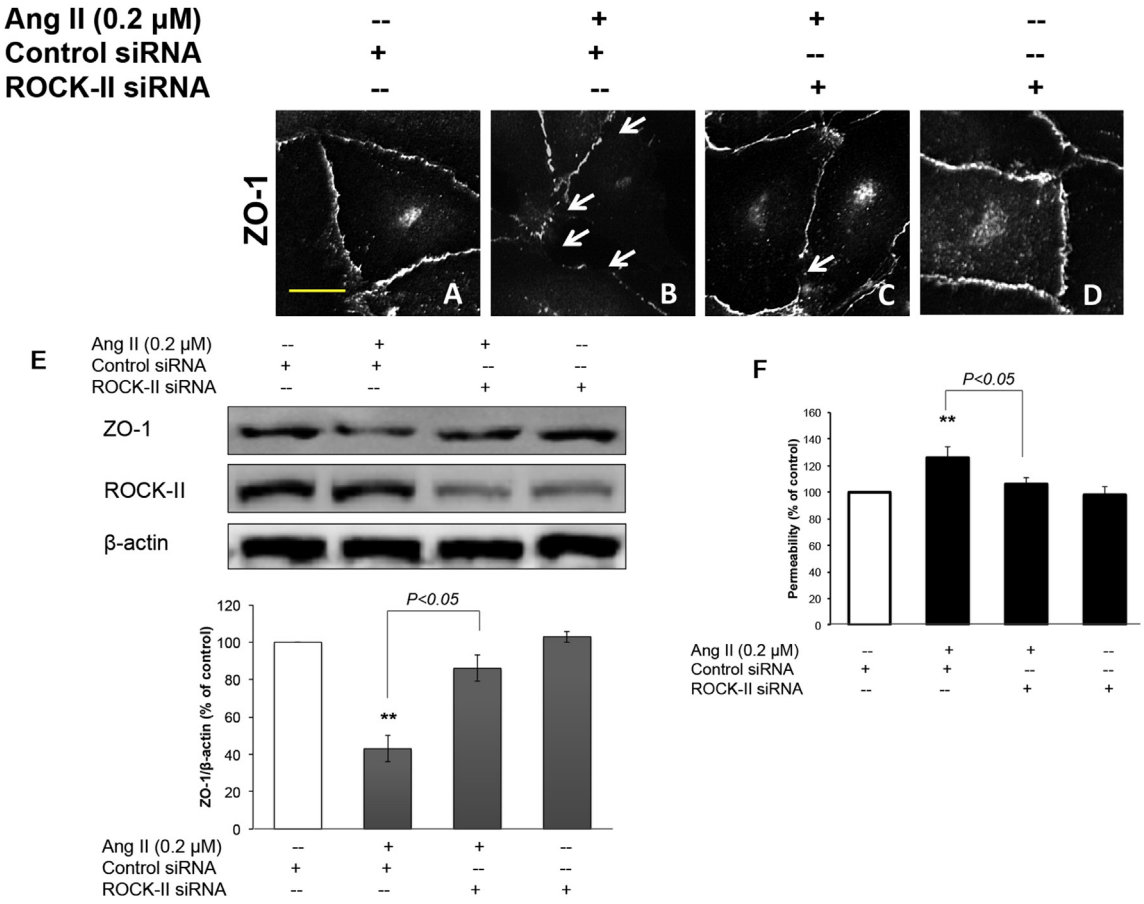

Fig. 4. Knockdown of ROCK-II prevented Ang II-induced ZO-1 abnormal destruction, expression deficiency and hyperpermeability in HAECs monolyer. Transfected HAECs were treated with 0.2 μM Ang II for 24 hours prior to treatment with 1 μM atorvastatin for 12 hours. (A) Control siRNA. (B) Ang II + Control siRNA. (C) Ang II + ROCK-II siRNA. (D) ROCK-II siRNA. ZO-1 expression in intercellular junction was examined by an immunofluorescence. Arrows indicate points where intercellular junction was disrupted. (E) Western blot analysis for ZO-1 was performed. β-Actin was served as the loading control. Quantitative analysis was performed by measuring protein expression relative to the control. Each point represents the mean (±*s.d.*) of three independent experiments, each performed in triplicate. *$P < 0.05$, **$P < 0.01$ *vs.* control.

aneurysm formation. However, more *in vivo* experimental studies are still in need to verify the therapeutic effects of statin.

Conflict of interest

None.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.02.076.

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