

Angiotensin II inhibits the protein expression of ZO-1 in vascular endothelial cells by downregulating VE-cadherin

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Abstract. Angiotensin II (Ang II) is reported to be involved in the development of various cardiovascular diseases by disrupting microvessel permeability, however, the underlying mechanism remains to be elucidated. The present study aimed to investigate the mechanism by which Ang II disrupts microvascular permeability. Rat endothelial cells were subjected to primary culture and identification. Cells in passages 4-7 were then used for the following experiments. The cells were divided into control, Ang II, and Ang II + valsartan groups, and reverse transcription-quantitative polymerase chain reaction and western blot analyses were performed to evaluate the expression of zonula occludens-1 (ZO-1) and vascular endothelial (VE)-cadherin in the cells. The distribution of ZO-1 protein was also detected using immunofluorescence assays. It was found that, compared with the control group, lower expression levels of ZO-1 and VE-cadherin were present in the Ang II group ($P < 0.01$). ZO-1 was also irregularly distributed at the periphery of the cells. In addition, the overexpression of VE-cadherin reversed the effect of Ang II on the expression and distribution of ZO-1 in endothelial cells. Together, these results suggested that Ang II inhibited the protein expression of ZO-1 in vascular endothelial cells by downregulating VE-cadherin, thus destroying the tight junctions between endothelial cells, which may also be the mechanism by which Ang II is involved in the development of cardiovascular diseases.

Introduction

Angiotensin II (Ang II), an important effector molecule of the renin-angiotensin system, regulates the expression of various cytokines and maintains homeostasis of the cardiovascular system under physiological conditions (1). Under pathological conditions, Ang II is involved in the occurrence and development of cardiovascular diseases, including intimal hyperplasia, cardiac hypertrophy and cardiac remodeling, by activating multiple signaling pathways (2,3). Previous studies have shown that Ang II is involved in the development of various cardiovascular diseases by disrupting microvessel permeability (4). However, the exact mechanism by which Ang II affects microvascular permeability remains to be elucidated.

The integrity of the vascular endothelial cell barrier is the basis for maintaining normal vascular permeability. Multiple tight junctions, which are often found around the cells, form a continuous band to link adjacent vascular endothelial cells together, thus filling the cell gap and forming a natural physical barrier. The connected endothelial cells can maintain homeostasis and selective permeability (5). Zonula occludens-1 (ZO-1) is a 220-kDa protein of the membrane-associated guanylate kinase homologs gene family, which interacts directly with the transmembrane protein occludin, ZO-2, and AF-6, the target of the oncogene, ras (6). As a key component of junctional complexes that regulate tight junction formation, the expression and distribution of ZO-1 in endothelial cells is decisive in the process of formation of tight junctions between endothelial cells (7). Therefore, the present study investigated the effect of Ang II on the expression and distribution of ZO-1 in endothelial cells.

The dynamics of vascular endothelial (VE)-cadherin at the plasma membrane are considered to be essential in modulating endothelial adhesion strength and junction plasticity between endothelial cells (8). Previous studies have shown that downregulation of the VE-cadherin extracellular domain leads to reduced cell-cell adhesion strength (9). In addition, studies have found that VE-cadherin is involved in the formation of tight junctions between endothelial cells, not only as a major component of junctional complexes but also as a key regulator of the expression and distribution of other components (10,11).

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However, whether VE-cadherin is involved in regulating the expression of ZO-1 remains to be elucidated.

The present study investigated the effect of Ang II on the expression and distribution of ZO-1 in endothelial cells and aimed to elucidate the role of VE-cadherin in endothelial cells in order to investigate the possible mechanism underlying the effect of Ang II.

Materials and methods

Animals. Male specific pathogen-free Sprague-Dawley (SD) rats (50 days old, body weight 150–180 g) were obtained from the Animal Center Laboratory of Zhejiang Province Institute of Medicine (Zhejiang, China). Mice were housed in a sterile environment at 25°C with a 12 h light/dark cycle, 40% humidity and food and water *ad libitum*.

Reagents. M199 medium, PBS, 0.25% trypsin-EDTA, penicillin, and streptomycin were purchased from Jinuo Biotech Company (Hangzhou, China). Ang II was purchased from Sigma; EMD Millipore (Billerica, MA, USA), dimethyl sulfoxide was from MP Biomedicals; Thermo Fisher Scientific, Inc. (Waltham, MA, USA), fetal bovine serum (FBS) was from Gibco; Thermo Fisher Scientific, Inc., and DAPI was from Roche Diagnostics (Indianapolis, IN, USA). Antibodies against ZO-1 (cat. no. ab150266), VE-cadherin (cat. no. ab33168) and β -actin (cat. no. ab8226) were purchased from Abcam (Cambridge, MA, USA), and horseradish peroxidase-conjugated goat anti-mouse and goat anti-rabbit IgG antibodies were purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA, USA). The other reagents for the immunoblot assay were purchased from Beyotime Institute of Biotechnology (Jiangsu, China). The Rneasy mini-kit was purchased from Qiagen GmbH (Hilden, Germany). All primer sequences were synthesized by Guangzhou RiboBio Co., Ltd. (Guangzhou, China). The human VE-cadherin gene was constructed into the pcDNA3.1+HA vector by GeneChem Co., Ltd. (Shanghai, China). All other chemicals were commercially available and of reagent grade.

Cell culture, transfection and treatment groups. The primary culture of vascular endothelial cells was established by isolating the cells from the thoracic and abdominal aortas, which were resected from 2–3-week-old male SD rats as described in our previous study (12). The present study was approved by the Ethical Committee of Shaoxing People's Hospital (no. 2016C33227). The endothelial cells were cultured in M199 medium supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.), 1% 100 U/ml penicillin and 1% 100 mg/ml streptomycin sulfates. The cells were incubated in humidified incubators with 5% CO₂ at 37°C.

The rat VE-cadherin gene was constructed into the pcDNA3.1+HA vector and the empty vector served as the negative control. For transfection, following culture of the cells to 70–80% confluence, the pcDNA3.1+HA-VE-cadherin and pcDNA3.1+HA empty vectors were transfected using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol.

In the present study, $\sim 10^5$ cells/well were plated in a six-well plate at 37°C for 24 h. Ang II (1 μ mol/l) was used to establish

the cell model. In the valsartan group, the cells were treated with Ang II (1 μ mol/l) and valsartan (10 μ mol/l) for 24 h at 37°C; in the VE-cadherin group, the cells were transfected with VE-cadherin overexpression vector and subsequently treated with Ang II (1 μ mol/l) for 24 h at 37°C.

Total mRNA isolation and reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. Following treatment, total RNA from the treated cells was extracted using the Rneasy mini-kit. First-strand complementary DNA (cDNA) was synthesized using the Thermo Script RT-PCR kit (Invitrogen; Thermo Fisher Scientific, Inc.) with a 10- μ l reaction mixture containing 2 μ l PrimeScript RT Enzyme Mix I, 2 μ l RT Primer Mix, 4 μ l RNase Free distilled H₂O and 2 μ l total RNA, according to the manufacturer's protocol. The synthesized cDNA (2 μ l) was then used for RT-qPCR analysis. The forward and reverse primer sequences used were as follows: ZO-1, forward 5'-CCCTCAAGGAGC CATTC-3' and reverse 5'-CAGTTTGCTCCAACGAGA-3'; VE-cadherin, forward 5'-AAGCGTGAGTCGCAA-3' and reverse 5'-CTCTCAGGTTTTTCGC-3'; GAPDH, forward 5'-GAGTCAACGGATTTGGTCGT-3' and reverse 5'-TTG ATTTTGGAGGGATCTCG-3'. The reaction conditions were as follows: Step 1, 95°C for 30 sec; step 2, 40 cycles of 95°C for 5 sec and 60°C for 34 sec; step 3, 95°C for 15 sec, 60°C for 60 sec, and 95°C for 15 sec. The final densities of ZO-1 and VE-cadherin were determined relative to the corresponding density of GAPDH from the same RNA sample with the 2^{- $\Delta\Delta C_q$} method (13).

Western blot analysis. Following incubation with the corresponding intervention factors for 24 h, cellular protein was obtained following lysis using radioimmunoprecipitation assay lysis buffer for western blot analysis. The protein concentrations of the supernatant were determined using a bicinchoninic acid protein assay. Proteins (30 μ g/lane) were loaded and separated by SDS-PAGE (10%) and transferred onto polyvinylidene fluoride membranes. Subsequently, the membranes were blocked with blocking buffer for 30 min at room temperature and then incubated with rabbit anti-ZO-1 and anti-VE-cadherin 1 monoclonal antibodies (1:1,000 dilution) and rabbit anti- β -actin monoclonal antibody (1:10,000 dilution) overnight at 4°C. Following incubation, TBS-T was used to wash the membranes (three times for 10 min), following which the membranes were incubated with goat anti-rabbit IgG-HRP (1:10,000 dilution) for 1 h at room temperature. The standard chemical luminescence method was used to detect the antigen. The bands were scanned on a gel imaging and analysis system and analyzed using Quantity One 4.4 (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Immunohistochemical analysis. Following treatment, the cells were washed with PBS, fixed in 4% paraformaldehyde, and permeabilized using 0.1% Triton X-100. Subsequently, the cells were blocked with 10% goat serum (Beyotime Institute of Biotechnology) in PBST and incubated with ZO-1 antibodies (1:300) in PBST for 1 h at 37°C. After 1 h, the cells were washed in PBS with 0.1% Tween-20 and then incubated with anti-mouse fluorescein isothiocyanate (FITC)-conjugated secondary antibody (1:500) for 1 h at 37°C,

followed again by washing. Coverslips were then processed for immunofluorescence microscopy.

Statistical analysis. Statistical analyses were performed using GraphPad Prism software (version 6.0; GraphPad Software, Inc., La Jolla, CA, USA). One-way analysis of variance, followed by Tukey's post hoc analysis was performed to compare multiple experimental groups. Student's t-test was used for comparisons between two different groups. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Ang II inhibits the expression of ZO-1 in endothelial cells. Western blot and RT-qPCR analyses were performed to assess the effect of Ang II on the expression of ZO-1 in endothelial cells. As shown in Fig. 1A and B, Ang II significantly inhibited the protein and mRNA expression of ZO-1 in endothelial cells ($P < 0.05$). In addition, the immunohistochemical analysis revealed that Ang II also disturbed the distribution of ZO-1 protein around the cells (Fig. 1C). Valsartan reversed this effect of Ang II ($P < 0.05$).

Ang II decreases the expression of VE-cadherin in endothelial cells. Following incubation of the endothelial cells with Ang II for 24 h, western blot and RT-qPCR analyses showed that the expression of VE-cadherin was significantly decreased by Ang II ($P < 0.01$; Fig. 2A and B). Considering the importance of VE-cadherin in the formation of tight junctions between endothelial cells, these results led to a focus on VE-cadherin in the subsequent experiments.

Overexpression of VE-cadherin reverses the effect of Ang II on the expression of ZO-1 in endothelial cells. To further verify the role of VE-cadherin in the Ang II-induced inhibited expression of ZO-1 in endothelial cells, pcDNA3.1+HA-VE-cadherin was transfected into the endothelial cells to induce the overexpression of VE-cadherin. Fluorescence analysis showed that the plasmid had been successfully transfected into the endothelial cells (Fig. 3A). The results of the RT-qPCR analysis revealed that the mRNA levels of VE-cadherin were significantly higher in the cells transfected with pcDNA3.1+HA-VE-cadherin, compared with those in the cells transfected with the empty vector ($P < 0.01$; Fig. 3B).

Following successful transfection of the pcDNA3.1+HA-VE-cadherin or empty plasmid, the cells were incubated with Ang II. In the group overexpressing VE-cadherin, VE-cadherin not only suppressed the Ang II-induced inhibition of ZO-1 ($P < 0.01$; Fig. 4A) but also reversed the Ang II-induced disordered distribution of ZO-1 around the cells ($P < 0.01$; Fig. 4B). These results suggested that Ang II suppressed the expression of ZO-1 in the endothelial cells by downregulating VE-cadherin.

Discussion

The effect of Ang II on the function of vascular endothelial cells has attracted increasing attention from cardiologists. Previous studies have shown that Ang II can promote cellular functions by stimulating reactive oxygen species production, promoting thrombosis (14), inhibiting nitric oxide production,

and promoting vascular endothelial cell apoptosis (15). Our previous studies also showed that Ang II can affect the normal function of endothelial cells by inhibiting nitric oxide synthase or inducing inflammatory reactions in cells (12,16). Studies have also revealed another mechanism by which Ang II is involved in the development of various cardiovascular diseases, wherein it acts by disrupting capillary permeability and the tight junctions between endothelial cells (4,17). However, few reports have been published on this topic.

Tight junctions form a continuous apicolateral paracellular barrier between epithelial cells, and this barrier enables the selective and regulated movement of solutes between apical and basolateral compartments. Its disturbance can eventually result in several cardiovascular diseases (18,19). To date, numerous proteins involved in the formation of tight junctions, including the transmembrane claudin family of proteins, occludin, and the ZO scaffolding family of proteins, have been identified (20,21). ZO proteins consist of three isoforms, ZO-1, ZO-2 and ZO-3, all of which form a heteromeric complex. All the ZO proteins contain the fusion protein (PDZ), SRC homology 3 domain and glucose kinase domains, through which the occludin, claudin and actin are linked (22). Therefore, these structures connect the tight junction with the cytoskeleton to finally form a stable ligation system between cells. ZO-1 is an important marker of tight junction integrity, which is disrupted in several intestinal diseases and invasive cancer types (23); ZO-1 has also been shown to be downregulated in poorly differentiated invasive breast cancer cell lines (24). Studies have also shown that absence of ZO-1 protein prevents the formation of tight junctions between cells (25). In addition, Su *et al* (7) demonstrated that the downregulation of ZO-1 protein reduced the number of tight junctions, eventually resulting in dysfunction of the blood-testosterone barrier. Furthermore, Guo *et al* (26) reported that via the miR-181d-5p-mediated downregulation of ZO-1, the long non-coding RNA, nuclear paraspeckle assembly transcript 1, regulated the permeability of the blood-tumor barrier. Wei *et al* (27) also found that it was possible for the function of the intestinal barrier to be regulated by modulating the expression of ZO-1 through the protein kinase C ϵ -dependent pathway. In the present study, it was found that Ang II reduced the protein expression of ZO-1 in endothelial cells and also caused a disturbance in the distribution of ZO-1 protein, which is typically distributed around the cell membrane. This result suggested that Ang II inhibited the formation of tight junctions between endothelial cells, which may be the mechanism by which Ang II decreases capillary permeability, eventually resulting in the development of cardiovascular diseases.

The cadherin family is a group of calcium-dependent type I transmembrane glycoproteins that mediate islet cell adhesion, and are involved in the formation and maintenance of normal cell-cell connections and polarity, differentiation of stem cells, and invasion and metastasis of tumor cells (28). VE-cadherin, which is specific to endothelial cells, belongs to the class of classical cadherins, is mainly distributed in endothelial cells and mediates endothelial cell-cell connections (29). Several studies have shown that VE-cadherin not only mediates intercellular junctions, but also transduces signals between cells (30-32). Taddei *et al* (33) reported that the VE-cadherin-mediated upregulation of claudin-5 increased the formation of tight junctions. In addition, studies have shown that VE-cadherin

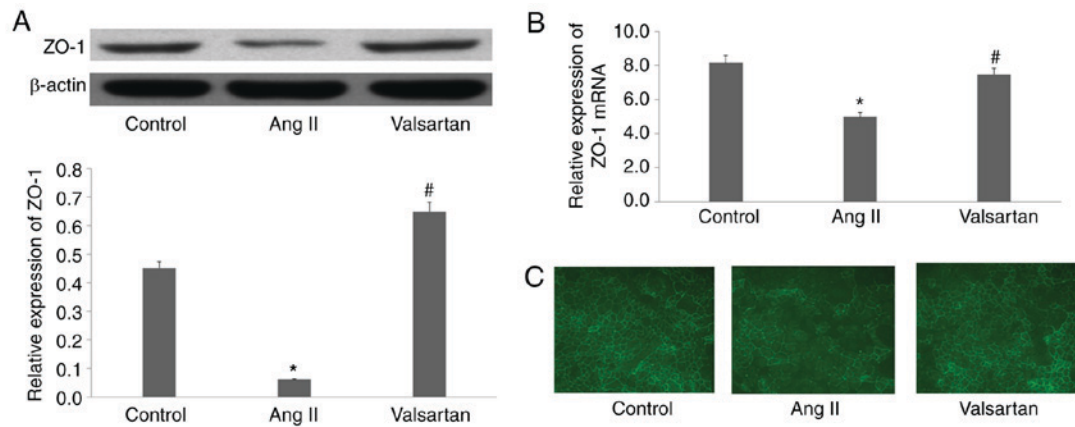


Figure 1. Effect of Ang II on expression and distribution of ZO-1 in endothelial cells. (A) Relative protein expression of ZO-1. (B) Relative mRNA expression of ZO-1. (C) Distribution of ZO-1 in endothelial cells. Magnification, x200. * $P < 0.01$, vs. Control; # $P < 0.01$, vs. Ang II. ZO-1, zonula occludens-1; Ang II, angiotensin II.

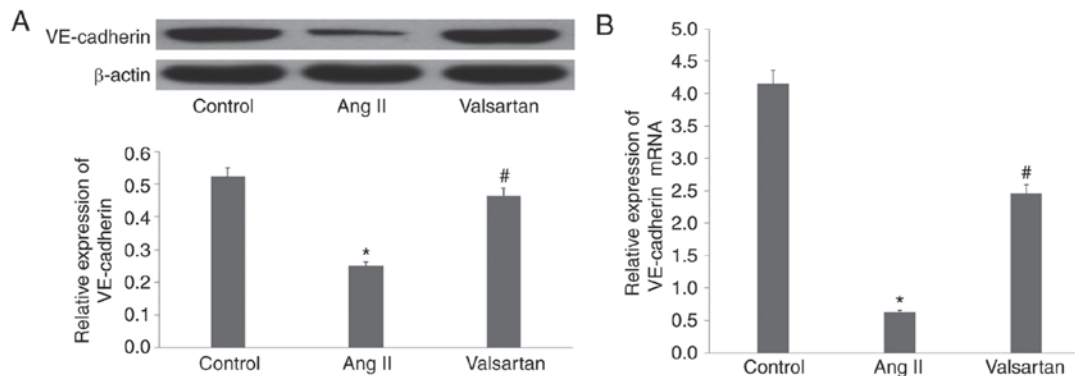


Figure 2. Ang II inhibits the expression of VE-cadherin in endothelial cells. (A) Relative protein expression of VE-cadherin. (B) Relative mRNA expression of VE-cadherin. * $P < 0.01$, vs. Control; # $P < 0.01$, vs. Ang II. VE-cadherin, vascular endothelial cadherin; Ang II, angiotensin II.

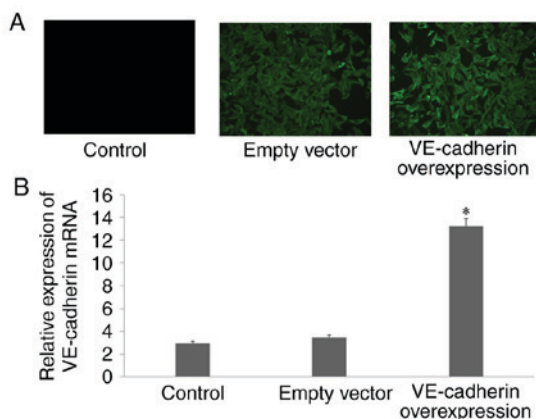


Figure 3. Successful transfection of VE-cadherin overexpression plasmid. (A) Vectors were successfully transfected into the endothelial cells. Magnification, x100. (B) Relative mRNA expression of VE-cadherin. * $P < 0.01$, vs. empty vector. VE-cadherin, vascular endothelial cadherin; Ang II, angiotensin II.

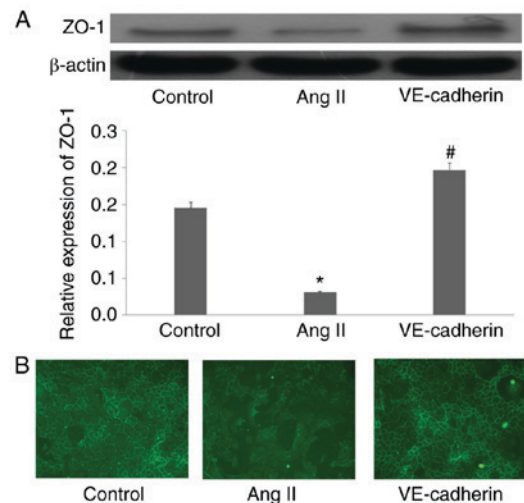


Figure 4. Overexpression of VE-cadherin reverses the effect of Ang II. (A) Relative protein expression of ZO-1. (B) Distribution of ZO-1 in endothelial cells. Magnification, x200. * $P < 0.01$, vs. Control; # $P < 0.01$, vs. Ang II. ZO-1, zonula occludens-1; Ang II, angiotensin II.

and vascular endothelial growth factor receptor 2 eventually affected endothelial cell plasticity in the course of angiogenesis (34,35). VE-cadherin can also stabilize cell-cell contacts and organize the endothelial barrier through an original outside-in signaling mechanism involving calcium signaling

and microtubule dynamics (36). In the present study, it was found that Ang II inhibited the expression of VE-cadherin in endothelial cells and downregulated the expression of

ZO-1. Therefore, considering the signal-transducing role of VE-cadherin, it was hypothesized that Ang II inhibited the expression of ZO-1 by downregulating VE-cadherin. To further validate this hypothesis, a VE-cadherin-overexpression plasmid was constructed, and it was found that Ang II did not inhibit the protein expression of ZO-1 in endothelial cells transfected with the VE-cadherin-overexpression plasmid. This result suggested that VE-cadherin was involved in the Ang II-induced downregulation of ZO-1.

The present study had a number of limitations. The effect of the overexpression of VE-cadherin was examined, however, no knockdown of VE-cadherin was performed to confirm the role of VE-cadherin in the Ang II-reduced expression of ZO-1. In addition, the experiments were performed in cells only, with no experiments performed in animals. These experiments are to be included in future investigations.

In conclusion, the present study found that Ang II reduced the expression of ZO-1 and caused a disturbance in the distribution of ZO-1 in endothelial cells. It was then showed that Ang II decreased the expression of VE-cadherin and that the overexpression of VE-cadherin reversed the inhibitory effect of Ang II on ZO-1. Taken together, these results suggested that Ang II inhibited the protein expression of ZO-1 in vascular endothelial cells by downregulating the expression of VE-cadherin. This may be the molecular mechanism by which Ang II decreases the formation of tight junctions between cells, eventually resulting in the development of cardiovascular diseases.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

LL, PZ, HL, JC and FP performed the cell experiments. LM performed the statistical analysis. HG designed the study.

Ethics approval and consent to participate

The present study was approved by the Ethical Committee of Shaoxing People's Hospital (grant no. 2016RCA027).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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