Cadmium exposure enhances VE-cadherin expression in endothelial cells via suppression of ROCK signaling

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Abstract. Vascular endothelium is a target of cadmium (Cd), which is a global pollutant of the environment. However, the detailed effects and underlying mechanisms remain to be elucidated. In the present study, human umbilical vein endothelial cells (HUVECs) were treated with 0.1, 1, 5, 10, 50 μM cadmium chloride ($CdCl_2$) for 12 h. It was found that vascular endothelial (VE)-cadherin mRNA and protein expression was upregulated by Cd in HUVECs in a dose-dependent manner. Higher levels of VE-cadherin were detected at cell-to-cell junctions in HUVECs treated with 10 μM $CdCl_2$ compared with normal condition. The phosphorylation level of myosin-binding subunit of myosin phosphatase, a downstream substrate of Rho-associated protein kinase (ROCK), was reduced by 10 μM $CdCl_2$, suggesting that Cd inhibited the Rho/ROCK pathway. Activation of ROCK by narciclasine reversed the Cd-induced increase of VE-cadherin expression. By contrast, ROCK pathway inhibitor Y27632 increased VE-cadherin expression in HUVECs. Following inhibition of the ROCK pathway, Cd did not significantly alter the level of VE-cadherin. Taken together, the results suggested that Cd exposure enhanced VE-cadherin expression in endothelial cells via suppression of ROCK signaling.

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Introduction

Cadmium (Cd) is one of the global pollutants of the environment (1). Tobacco smoke, Cd-contaminated food and industrial contamination are the main sources of Cd toxicity in humans (2,3). Chronic exposure to Cd has a major effect on humans, causing damage to multiple organs including kidneys, liver, lung, pancreas and testes (3-5). In addition, studies have reported that the vascular endothelial cell (VEC) is another main target of Cd (6,7). Accumulated Cd impairs endothelial function at a variety of molecular levels, including cell adhesion molecules, metal ion transporters and protein kinase signaling pathways (7,8). As blood vessels are widely distributed in organs, functional damage of VECs further causes toxicity in the parenchymal cells of human organs (8,9). Therefore, it is of great significance to investigate the underlying mechanism of Cd toxicity on VECs.

Vascular endothelial (VE) cadherin, a principal adhesion protein, is composed of a large ectodomain with five cadherin repeats, a transmembrane domain and a cytoplasmic tail (10). Cell-to-cell adhesion is established by interactions of ectodomain of VE-cadherin (11). The cytoplasmic tail of VE-cadherin is regulated by a range of phosphatases and kinases (12). Phosphorylation of the VE-cadherin cytoplasmic tail leads to VE-cadherin dissociation from the junctions (11). VE-cadherin is produced by various stimuli to regulate vascular permeability (13-16). It has been reported that

VE-cadherin mRNA expression is reduced in chick embryo model treated with 50 μM Cd (17). In addition, low concentration of Cd induces membrane dissociation of VE-cadherin in human renal glomerular endothelial cells and human umbilical vein endothelial cells (HUVECs) (18,19). Further research is required on the effect of Cd on VE-cadherin.

Rho is a member of guanosine triphosphatase (GTPase) family (20). Rho-associated coiled-coil kinase (ROCK), including ROCK1 and ROCK2, is a serine/threonine kinase downstream of Rho GTPases (20). ROCK pathway regulates various cellular functions, including contraction, cytoskeleton organization, cell-to-cell adhesion and permeability (21). Lipopolysaccharide has been reported to activate the RhoA/ROCK signaling pathway to weaken cell junctions by reducing the expression of VE-cadherin and altering distribution of VE-cadherin (22). However, whether Cd regulates VE-cadherin by ROCK pathway is uncertain.

In the present study, the results showed that Cd increased VE-cadherin expression in HUVECs in a dose-dependent manner. It also found that 10 μM Cd inhibited ROCK pathway. Narciclasine, an activator of ROCK pathway, reversed Cd-induced VE-cadherin expression. In addition, the expression of VE-cadherin is unchanged in HUVECs treated with 10 μM narciclasine in the absence or presence of 10 μM $CdCl_2$. ROCK pathway inhibitor Y27632 increased VE-cadherin expression in HUVECs in a dose-dependent manner. With pretreatment of 20 μM Y27632, 10 μM Cd did not alter VE-cadherin expression. The present study demonstrated a role for the ROCK pathway in regulating the VE-cadherin expression induced by Cd in HUVECs.

Materials and methods

Reagents and antibodies. $CdCl_2$ was purchased from Millipore Sigma and dissolved in phosphate buffered saline (PBS). Narciclasine (MedChem Express) and Y27632 (Selleck Chemicals) were dissolved in dimethyl sulfoxide (DMSO). The primary antibody against VE-cadherin was purchased from Abcam. The primary antibodies against myosin phosphatase-targeting subunit (MYPT), phosphorylated (p)-MYPT (Ser507) and GAPDH were purchased from Cell Signaling Technology, Inc. The secondary antibody was goat anti-rabbit IgG (cat. no. 7074; Cell Signaling Technology, Inc.).

Cell Culture. HUVECs were purchased from American Type Culture Collection and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Lonza Group Ltd.), 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin at 37˚C in 5% CO_2.

Reverse transcription-quantitative (RT-q) PCR. HUVECs were seeded at a density of $5x10^5$ cells/ml in a 65-mm dish with different treatment. RNA extraction, cDNA synthesis and qPCR were performed according to the manufacturer's protocols. Total RNA from the treated HUVECs was isolated with the E.Z.N.A. Total RNA kit II (Omega Bio-Tek, Inc.). Complementary cDNA was synthesized using the RevertAid First strand cDNA Synthesis kit (Thermo Fisher Scientific, Inc.). Diluted cDNA (4.6 μl; 50 ngcDNA) and 5.4 μl of primer and supermix mixture (SYBR premix Ex Tap™ II and dH_2O; Takara Biotechnology Co., Ltd.) were used in each RT-qPCR reaction. The RT-qPCR process was performed on the CFX96 Real-Time System (Bio-Rad Laboratories, Inc.). Reaction conditions were: 95˚C for 5 min, 40 cycles of 95˚C for 10 sec and 60˚C for 32 sec. All PCR reactions were repeated three times and the mRNA levels were normalized to β-actin. Relative quantitative values were obtained from cycle threshold (Ct) and the $2^{-\Delta\Delta Cq}$ method (23). The human VE-cadherin PCR primers were 5'-CAGCCCAAAGTGTGTGAGAA-3' (sense) and 5'-CGGTCAAACTGCCCATACTT-3' (antisense). The human β-actin PCR primers were 5'-TTGCCGACAGGATGC AGAA-3' (sense) and 5'-GCCGATCCACACGGAGTACT-3' (antisense).

Western blotting. Western blotting was performed as previously described (24). Cells were homogenized with in RIPA buffer at 4˚C. Protein concentrations were measured using Pierce BCA Assay kit (Thermo Fisher Scientific, Inc.). Supernatants of cell lysates were mixed with loading buffer and heated at 95˚C for 5 min. Samples containing 25 μg protein was separated by 10% SDS-PAGE and transferred to 0.45 μm polyvinylidene difluoride (PVDF) membrane. Membranes were blocked with 5% non-fat milk for 2 hat room temperature and incubated with primary antibody against VE-cadherin (1:1,000; cat. no. ab33168; Abcam), MYPT (1:1,000; cat. no. 2634; Cell Signaling Technology, Inc.), phosphorylated (p)-MYPT (1:1,000; cat. no. 3040; Cell Signaling Technology, Inc.) and GAPDH (1:3,000, cat. no. 2118; Cell Signaling Technology, Inc.) at 4˚C overnight. After washing with TBS-T (0.5% Tween), the membranes were incubated with secondary antibodies for 2 h at room temperature and then washed with TBS-T. The secondary antibody was HRP-linked goat anti-rabbit IgG antibody (1:8,000; cat. no. 7074; Cell Signaling Technology, Inc.). Following the manufacturer's instructions, specific binding was revealed by an ECL kit (Pierce; Thermo Fisher Scientific, Inc.). Densitometry analysis was performed with ImageJ software 1.48 (National Institute Health).

Immunofluorescence. HUVECs were grown into monolayer on fibronectin-coated glass chamber slides and were then treated with 10 μM $CdCl_2$ for 12 h. Then, cells were washed with PBS and fixed with 4% paraformaldehyde for 15 min. After washing three times with PBS for 10 min, the cells were stained with a primary antibody against human VE-cadherin (cat. no. a33168; Abcam) at a dilution of 1:500 overnight at 4˚C and were incubated with the Alexa Fluor 546 donkey anti-rabbit secondary antibody (1:200; cat. no. A10040; Thermo Fisher Scientific, Inc.) for 2 h at room temperature. After washing three times with PBS for 10 min at room temperature, samples were imaged using an Olympus BX51 fluorescence microscope (Olympus Corporation) with an excitation wavelength of 546 nm (magnification, x200). The chamber slide with the monolayer was divided in 16 equal areas (4x4) and one field was randomly chosen in each area. The image mostly close to the average staining intensity was chosen as representative image.

Statistical data analysis. All the data were analyzed using GraphPad Prism 5.0 (GraphPad Software, Inc.). Data are presented as means ± SD. Statistical significance was assessed

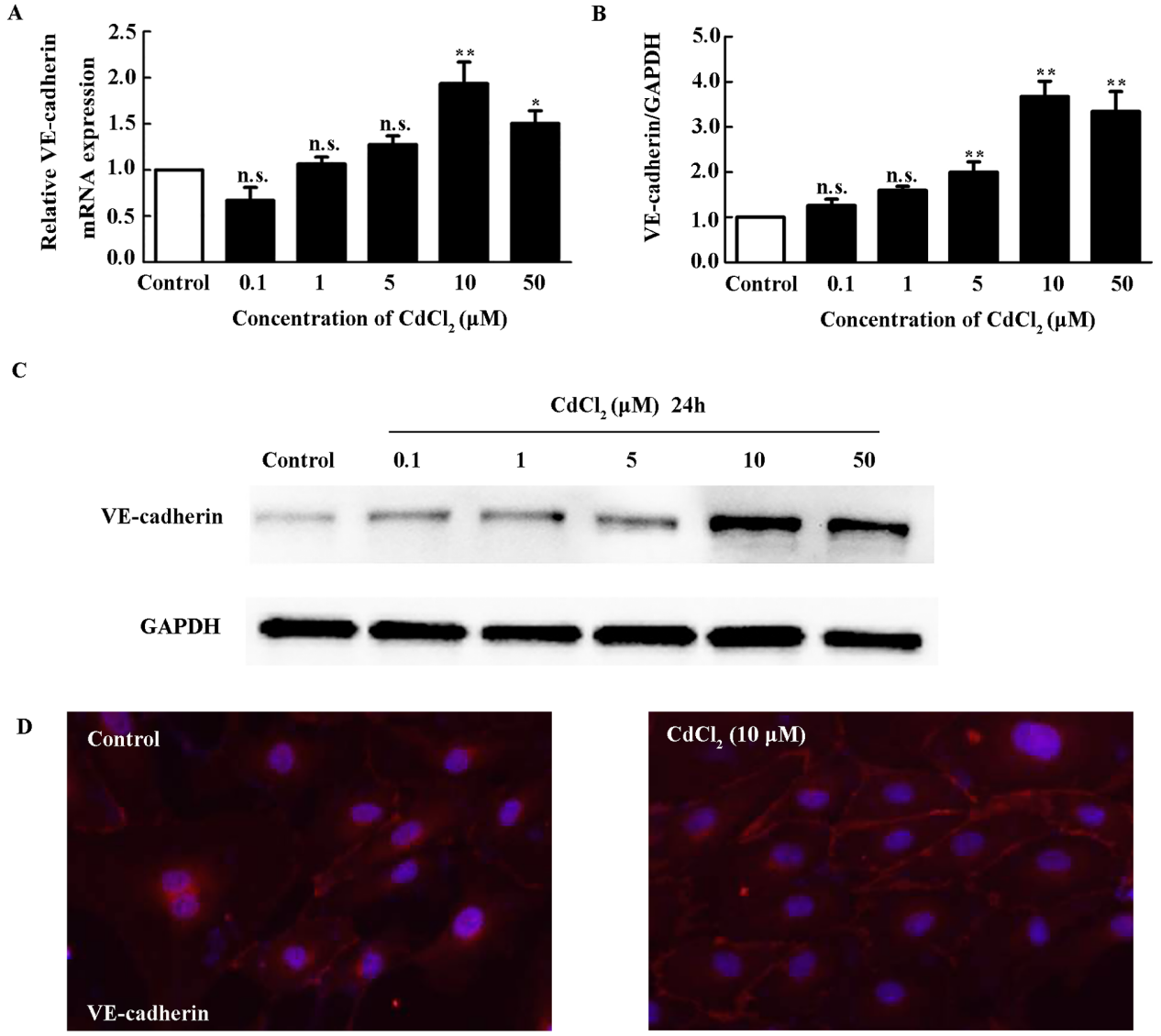

Figure 1. Effects of Cd on VE-cadherin expression in HUVECs. (A) Relative VE-cadherin mRNA expression measured by reverse transcription-quantitative PCR in HUVECs treated with different concentrations of $CdCl_2$ for 12 h (n=4). (B) Densitometry analyses of the immunoblots in (C) (n=5). (C) Immunoblots of VE-cadherin from protein samples of HUVECs exposed to different concentrations of $CdCl_2$ for 12 h. GAPDH was used as loading control. (D) Immunofluoresence staining of VE-cadherin on HUVEC monolayer, treated with 10 μM $CdCl_2$. Magnification, x200. Statistical significance was assessed using one-way analysis of variance followed by Tukey's post hoc test. *P<0.05 and **P<0.01 compared with control. HUVECs, human umbilical vein endothelial cells; n.s., non-significant; VE-cadherin, vascular endothelial cadherin.

using one-way analysis of variance followed by Tukey's post hoc test or Student's t-test. A statistical difference of P<0.05 was considered significant.

Results

High dose Cd increases VE-Cadherin expression. The present study analyzed the mRNA and protein expression of *VE-cadherin* in HUVECs treated with different concentrations of Cd for 12 h. Fig. 1A showed that relative *VE-cadherin* mRNA expression was not changed at concentrations of 0.1, 1 and 5 μM of Cd. However, it was significantly upregulated by 10 and 50 μM Cd. Western blotting showed that Cd increases VE-cadherin protein expression following treatment with Cd at concentrations of 1, 5, 10 and 50 μM (Fig. 1B and C). A previous study showed that Cd disrupts VE-cadherin mediated cell-to-cell adhesion of HUVECs (25). Immunofluorescent staining with VE-cadherin antibody on HUVECs treated with Cd was performed. As shown in Fig. 1D, VE-cadherin is mainly distributed in cytoplasm membrane under normal condition. After treatment with 10 μM Cd, the fluorescence became stronger, suggesting that higher levels of VE-cadherin were detected at cell-to-cell junctions between cells.

Cd inhibits ROCK activity in HUVECs. To examine whether Cd affected ROCK activity, the phosphorylation levels of ROCK downstream substrate, myosin-binding subunit of myosin phosphatase (MYPT), were evaluated by western blotting. HUVECs treated with 10 μM Cd showed a significant decrease in p-MYPT after 12 h (Fig. 2A and B), suggesting that Cd inhibited the Rho/ROCK pathway.

ROCK activation reduces Cd-induced increase of VE-cadherin expression. Narciclasine is an activator of

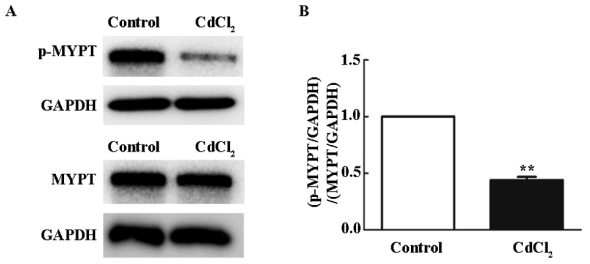

Figure 2. Effect of Cd on ROCK activity. (A) Immunoblots of p-MYPT and MYPT from protein samples of HUVECs treated with 10 μM $CdCl_2$ for 12 h. GAPDH was used as loading control. (B) Densitometry analyses of the immunoblots in (A). Statistical significance was assessed using Student's t-test. n=4; **P<0.01. ROCK, rho-associated coiled-coil kinase; p-, phosphorylated; MYPT, myosin phosphatase-targeting subunit.

the Rho/ROCK pathway (26). The present study examined whether narciclasine inhibited Cd-induced VE-cadherin expression. As shown in Fig. 3A-C, 10 μM narciclasine reduced mRNA and protein level of VE-cadherin upon Cd treatment (P<0.01). With pretreatment of 10 μM narciclasine, 10 μM Cd did not increases VE-cadherin protein in HUVECs (Fig. 3D), suggesting Cd induced VE-cadherin expression through inhibition of ROCK signaling.

Inhibition of ROCK upregulates the expression of VE-cadherin. To examine the effect of ROCK pathway on VE-cadherin, HUVECs were treated with different concentrations of ROCK inhibitors Y27632 for 12 h. Y27632 increased the expression of *VE-cadherin* mRNA at 10 and 20 μM (P<0.05; Fig. 4A). Y27632 also increased the protein levels of VE-cadherin (P<0.05; Fig. 4B and C), suggesting that ROCK pathway negatively regulated VE-cadherin expression. With pretreatment of 10 μM Y27632, Cd did not significantly alter the level of VE-cadherin in HUVECs (Fig. 4D).

Discussion

Cd exposure has been reported to cause dysfunction of VECs (8,27). Depending on the dose of exposure, Cd differentially affects vascular VECs, including permeability, apoptosis and proliferation (7,28,29). The present study demonstrated that Cd upregulated expression of VE-cadherin via inhibition of ROCK activities.

The regulation of signaling pathways in response to Cd toxicity is dependent on Cd concentration (30). A previous study demonstrated that low-dose Cd (4 μM) impairs adherens junctions by inducing VE-cadherin and β-catenin redistribution, causing hyperpermeability in HUVEC monolayers (19,31). In the present study, Cd increased VE-cadherin expression in HUVECs in a concentration-dependent manner. The effect of Cd (10 μM) on VE-cadherin was the more remarked than other concentration. In a previous study, treatment of HUVECs with Cd reduces VE-cadherin localization to cell junctions in a concentration-dependent manner (32). Similarly, in the present study, lower levels of VE-cadherin were noted at cell-to-cell junctions between cells following 10 μM Cd treatment. However, Cd (10 μM) has been reported to induce VEC hyperpermeability, suggesting that the increased expression

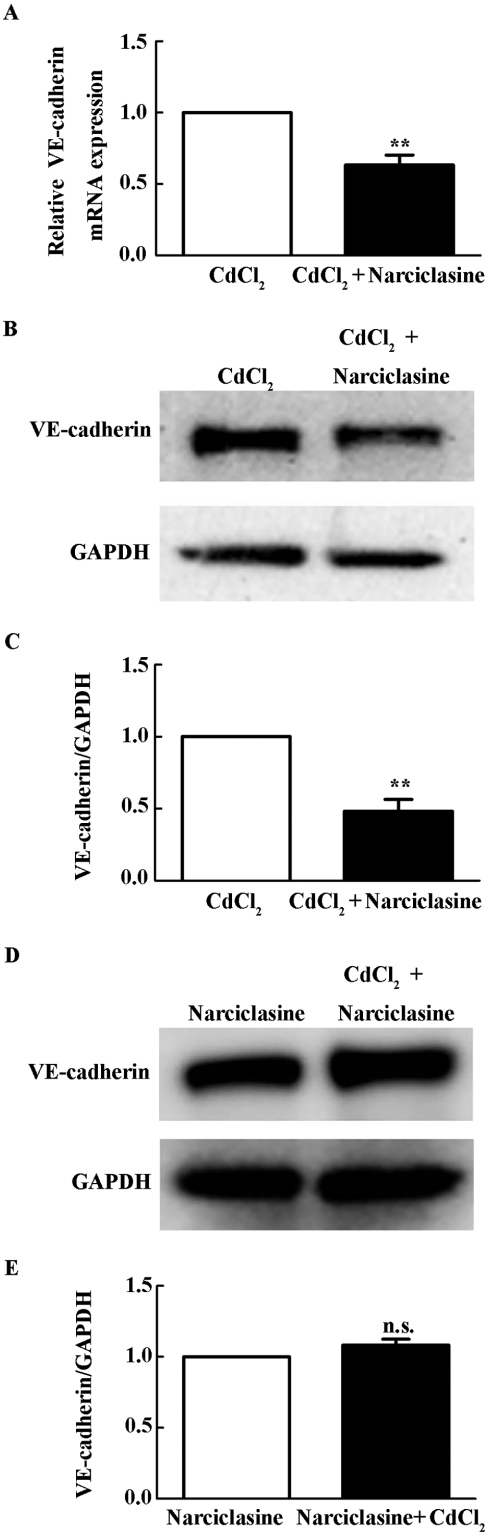

Figure 3. Effects of narciclasine on VE-cadherin expression in HUVECs. (A) Relative VE-cadherin mRNA expression measured by reverse transcription-quantitative PCR in HUVECs treated with 10 μM $CdCl_2$ for 12 h in the absence or presence of narciclasine. Statistical significance was assessed using Student's t-test; n=6; **P<0.01. (B) Immunoblots of VE-cadherin from protein samples of HUVECs treated with 10 μM $CdCl_2$ for 12 h in the absence or presence of 10 μM narciclasine. (C) Densitometry analyses of the immunoblots in (B). Statistical significance was assessed using Student's t-test; n=6; **P<0.01. (D) Western blot analysis of the expression of VE-cadherin in HUVECs treated with 10 μM narciclasinein the absence or presence of 10 μM $CdCl_2$. GAPDH was used as loading control. (E) Densitometry analyses of the immunoblots in (D). Statistical significance was assessed using Student's t-test. n=4.VE-cadherin, vascular endothelial cadherin; HUVECs, human umbilical vein endothelial cells; n.s., non-significant.

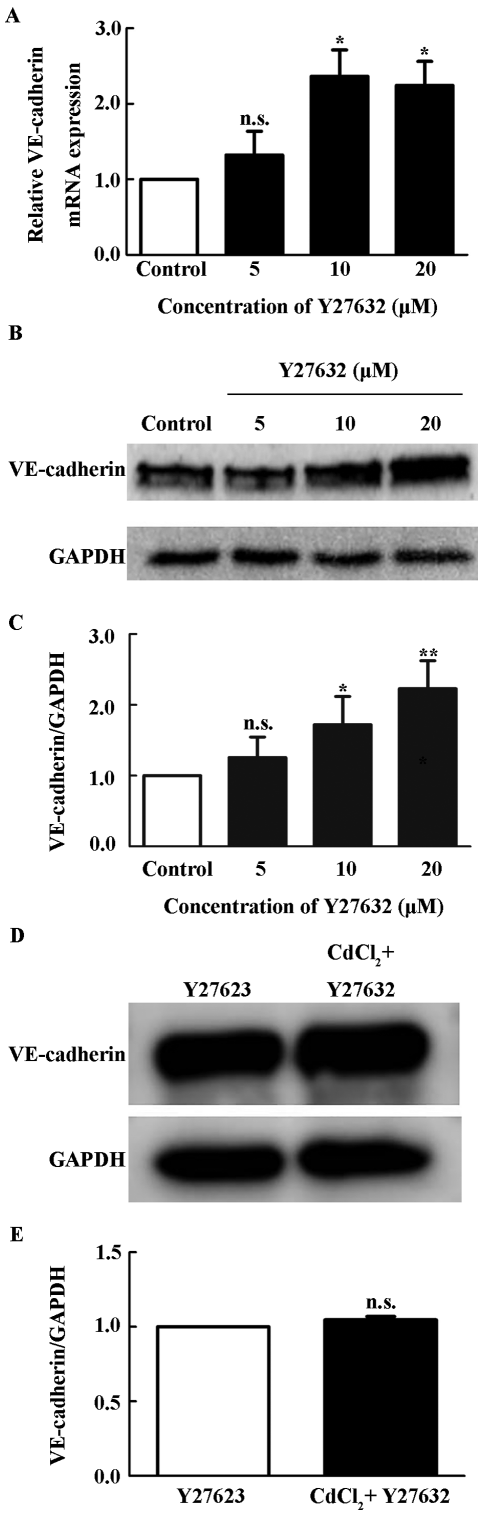

Figure 4. Effects of Y27632 on VE-cadherin expression in HUVECs. (A) Relative VE-cadherin mRNA expression measured by reverse transcription-quantitative PCR in HUVECs treated with Y27632 at different concentrations for 12 h. Statistical significance was assessed using one-way analysis of variance followed by Tukey's post hoc test; n=6; *P<0.05. (B) Immunoblots of VE-cadherin from protein samples of HUVECs exposed to different concentrations of Y27632 for 12 h. GAPDH was used as loading control. (C) Densitometry analyses of the immunoblots in (B). Statistical significance was assessed using one-way analysis of variance followed by Tukey's post hoc test; n=5; *P<0.05; **P<0.01. (D) Western blot analysis of the expression of VE-cadherin in HUVECs treated with 20 μM Y27632 for 12 h in the absence or presence of $CdCl_2$. (E) Densitometry analyses of the immunoblots in (D). Statistical significance was assessed using Student's t-test; n=4. VE-cadherin, vascular endothelial cadherin; HUVECs, human umbilical vein endothelial cells; n.s., non-significant.

of VE-cadherin induced by 10 μM Cd fails to rescue vascular hyperpermeability (33). One reason may be that apoptosis and senescence of VECs during 10 μM Cd exposure leads to enhanced vessel wall permeability to cytokines, growth factors, lipids and immune cells (34).

The present study demonstrated that 10 μM of Cd inhibited ROCK activity. The ROCK pathway increases vascular permeability by causing junction protein remodeling and endothelial barrier dysfunction (35,36). ROCK inhibits the expression of tight junction components, including occludin and claudin-1 (37,38). The present study found that ROCK also negatively regulated the expression of VE-cadherin in HUVECs, In addition, Cd did not increase the expression of VE-cadherin in the presence of ROCK inhibitor Y27632, suggesting that ROCK mediated Cd-induced VE-cadherin expression. The results of the present study are consistent with previous studies. For example, the ROCK pathway inhibitor partially limits the increased monolayer permeability in lethal toxin-treated VECs through restoration of VE-cadherin expression and membrane localization (39). Inhibition of ROCK decreases the tension across VE-cadherin adhesion and VE-cadherin dissociation rate, resulting in accumulation of VE-cadherin in adherens junctions (40). FPND, a ROCK inhibitor, protects vascular integrity through cytoskeletal rearrangement and enhancement of cell-to-cell junctions in VECs via the ROCK1 and VE-cadherin signaling pathways (41).

In conclusion, the results of the present study suggested that ROCK inhibition contributes to Cd-induced expression of VE-cadherin in endothelial cells. It increases our understanding of Cd-induced vascular dysfunction.

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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

MG and HW conceived and designed the experiments. XiaoruiL, XiaoL and RS performed the experiments. XiaoruiL, XiaoL and RS analyzed the data. XiaoruiL, MG and HW wrote the paper. XiaoruiL and MG confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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