Cellular Physiology and Biochemistry

Cell Physiol Biochem 2018;45:1717-1730
DOI: 10.1159/000487780
Published online: February 28, 2018
Accepted: January 16, 2018

Original Paper

Mdia1 is Crucial for Advanced Glycation End Product-Induced Endothelial Hyperpermeability

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Key Words
Advanced glycation end products (AGEs) • Mammalian diaphanous-related formin • Endothelial hyperpermeability • Receptor for advanced glycation end products (RAGE) • Oxidative stress

Abstract
Background/Aims: Disruption of endothelial barrier integrity in response to advanced glycation end products (AEGs) stimulation contributes to vasculopathy associated with diabetes mellitus. Mammalian diaphanous-related formin (mDia1) has been reported to bind to the cytoplasmic domain of the receptor for advanced glycation end products (RAGE), which induces a series of cellular processes. This study directly evaluated the participation of mDia1 in AGE-induced hyperpermeability and revealed the precise intracellular signal transductions of this pathological process. ***Methods:*** Human umbilical vein endothelial cells (HUVECs) were used in the *in vitro* studies. Trans-endothelial electric resistance and permeability coefficient for dextran (Pd) were measured to analyze cell permeability. Western blotting, immunofluorescence staining and flow cytometry assay were performed to investigate the underlying mechanism. Dextran flux across the mesentery in mice was monitored to investigate *in vivo* microvascular permeability. ***Results:*** we found that AGEs evoked Nox4 membrane translocation, reactive oxygen species production, phosphorylation of Src and VE-cadherin, dissociation of adherens junctions and eventual endothelial hyperpermeability through RAGE-mDia1 binding. Cells overexpressing mDia1 by recombinant adenovirus infection showed stronger cellular responses induced by AGEs. Down-regulation of mDia1 by infection with an adenovirus encoding siRNA or blockade of RAGE-mDia1 binding by transfection with RAGE mutant plasmids into HUVECs abolished these AGE-induced effects. Furthermore, knockdown of mDia1 using an adenovirus or genetical knockout of RAGE in C57 mice rescued AGE-evoked

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microvascular hyperpermeability. ***Conclusion:*** Our study revealed that mDia1 plays a critical role in AGE-induced microvascular hyperpermeability through binding to RAGE.

Introduction

The vascular endothelium lining the inner surface of blood vessels serves as the first interface for circulating blood components to interact with cells of the vascular wall and surrounding extravascular tissues. A major function of microvascular endothelia is to provide a semi-permeable barrier that controls blood-tissue exchange of fluids, nutrients and metabolic wastes. Endothelial barrier dysfunction occurs because of inflammation caused by trauma, ischemia-reperfusion injury, arteriosclerosis, and especially diabetes mellitus. Advanced glycation end products (AGEs) are a group of compounds formed irreversibly in serum and tissues as a result of non-enzymatic reactions between glucose and macromolecules [1]. The crucial roles of AGEs in the development of diabetes and various diabetic cardiovascular complications, especially vascular barrier dysfunction, have been emphasized in previous reports [2, 3]. Binding of AGEs to the receptor for advanced glycation end products (RAGE) forms a complex that affects cellular functions through complicated signal transduction pathways such as mitogen-activated protein kinases and Rho kinases [4, 5]. Our previous studies have demonstrated that oxidative stress induced by activation of NADPH oxidase is initiated via the interaction of AGEs and RAGE, followed by disorganization of adherens junctions protein VE-cadherin, resulting in endothelial hyperpermeability [6, 7]. However, RAGE does not activate NADPH oxidase directly because of the lack of kinase activity in its cytoplasmic domains, and the detailed molecular mechanisms underlying AGE-RAGE signaling pathways remain to be further elucidated.

The diaphanous-related formins family is emerging as sub-class of formins family because of their specific functional domains, the GTPase-binding domain and diaphanousauto-regulatory domain, which is widely expressed in eukaryotic cells from yeast to mammals. Mammalian diaphanous 1 (DIAPH1, also named mDia1) is one of the best-characterized members of this family. By acting as a potent actin and microtubule polymerization factor, mDia1 regulates a number of key cellular functions such as cell adhesion, movement, cytokinesis, morphogenesis, cell polarity formation, and serum response factor activation [8-10]. Recent studies have identified the formin homology (FH1) domain of mDia1 as a binding partner that interacts directly with the short cytoplasmic tail of RAGE (C-terminal RAGE, ctRAGE). Additionally, mDia1 integrates oxidative and signal transduction pathways triggered by RAGE ligands in pathological neointimal expansion [11, 12]. *In vitro* binding studies have revealed that two amino acids, Arg-5 and Gln-6 of ctRAGE, are essential for interactions with mDia1. When Arg-5 and Gln-6 are mutated to alanines, RAGE ligand-induced downstream signals are suppressed because of the loss of the binding epitope for mDia1 in vascular smooth muscle cells (SMCs) [8]. Because our previous studies confirmed that AGEs affect the functions and morphology of endothelial cells by interacting with RAGE, we presumed that mDia1 initiates the cytoplasmic signal transduction pathway of HUVEC hyperpermeability via binding to the cytoplasmic domain of RAGE after AGEs stimulation.

In this study, we focused on the role of mDia1 in mediating AGE-induced endothelial hyperpermeability and the interactions of signaling molecules underlying RAGE functions. Based on previous studies, AGE-induced cell injury is circumvented by interfering with the NADPH oxidase-related reactive oxygen species (ROS) pathways [13, 14]. Moreover, Nox4 functioned as the major catalytic component of endothelial NADPH oxidase and the main source of ROS production [15]. Our present study demonstrates that mDia1 plays a critical role in transducing the signal of AGEs upon ligation of RAGE to activate Nox4 by recruiting Nox4 to the cell membrane. Therefore, the Nox4-derived ROS elevation triggers subsequent signaling pathways including the phosphorylation of Src and VE-cadherin, and eventually results in endothelial hyperpermeability.

Materials and Methods

Cells, plasmid, antibodies and reagents

HUVECs were obtained from ScienCell. Antibodies recognizing total Src and phosphorylated Src (Y416) were obtained from Cell Signaling (Beverly, MA). Antibodies recognizing total RAGE, mDia1, VE-cadherin and p-VE-cadherin (Y658) were from Abcam (Cambridge, MA), human RAGE small interfering (si) RNA, mDia1 siRNA, Nox4 siRNA and control nonsense siRNA were from Santa Cruz Biotechnology (Santa Cruz, CA). RAGE wild-type, mutant (both Arg-5 and Gln-6 were mutated to Ala) and control plasmids were obtained from Sangon Biotech (Shanghai, China). mDia1 interfered, overexpressed and control adenovirus were obtained from Vigene Biosciences (Shangdong, China). Interfered adenovirus against mouse mDia1 was obtained from Gene (Shanghai, China). Lipofectamine 2000 and lipofectamine 3000 were from Invitrogen (Carlsbad, USA). Oxidative stress inhibitor apocynin was acquired from Merck (Germany). FITC-dextran and DCFH-DA were obtained from Sigma (Shanghai, China), Membrane and Cytosol Protein Extraction Kit was purchased from Thermo (MA, USA).

Preparation of AGE-modified bovine serum albumin

AGE-modified bovine serum albumin (AGE-BSA) was prepared based on the protocol of Schmidt AM et al. [16], with slight modification. Briefly, BSA (50 mg/mL, pH 7.4) was incubated in PBS with D-glucose (100 mmol/L) at 37°C for 8 weeks. Control albumin was incubated without glucose. Both solutions were extensively concentrated and purified at the end of the incubation period. AGE-specific fluorescence (excitation, 370 nm; emission, 440 nm) [17] was determined using ratio spectrofluorometry. AGE-BSA contained 75.20 U/mg protein of AGEs, whereas native albumin contained less than 0.9 U/mg protein of AGEs. The endotoxin content was detected using TCL kit and was found to be<0.5 EU/mL in both solutions.

Transfection of HUVECs with siRNA and plasmid

Transfection was performed based on the instructions provided by the manufacturer with slight modifications. HUVECs were cultured in DMEM/F12 without antibiotics for 24 h, when grew to 50%~70% confluence, cells were transfected with 25 nM RAGE siRNA, 25 nM Nox4 siRNA or control nonsense siRNA. The transfection of RAGE WT or Mut plasmids was conducted when cells grew to 80% ~ 90% confluences. 48 h after transfection, cells were ready for stimulus, after which relevant detections were performed.

Infection of cells with adenovirus

The interference adenovirus against human or mouse mDia1 as well as mDia1 overexpression recombinant adenovirus were constructed by specialized company, and the infection was conducted according to the provided specification. Briefly, Cells were seeded in 3.5 cm dishes to 50% ~ 60% confluences, then 10 μL $1*10^{9}$ vp/mL adenoviruses were added in the dishes, after 8 h, the adenovirus-containing DMEM was replaced by culture media and cells were incubated in the incubator for 48 h before exposed to stimulus and followed by relevant detections.

Measurement of trans-endothelial electrical resistance

Trans-endothelial electric resistance (TER) of HUVECs monolayer was measured using EVOM^2 (World Precision Instruments, USA) as reported [18]. Briefly, 100 μL of cells at 10^5/mL were seeded onto upper chamber of a trans-well with pore size of 0.4 μm. When endothelial monolayer grew confluent, TER was measured. The mean value of TER was expressed in the common unit (Ω cm^2) after subtraction of the value of a blank cell-free filter. The relative changes of TER to baseline value were calculated by the formula:

TER = TER of experimental wells / baseline TER of experimental wells - 1.

Endothelial monolayer permeability assay

Cells were grown onto transwell membrane till confluent and the tracer FITC-labeled dextran (1 mg/mL) was added to the top chamber for 45 min. Then the concentration of dextran in upper bottom chamber was determined with a HTS 7000 microplate reader. The permeability of endothelial monolayer were evaluated by the permeability coefficient of dextran (Pd) calculated as follows: Pd = [A] / t × 1 / A × V / [L], where [A] is the dextran concentration of bottom chamber, t refers to time, A indicates the area of the membrane (in cm^2), V is the volume of the bottom chamber, [L] is the luminal dextran concentration of upper chamber.

In vivo measurement of microvascular permeability

6–8-week-old male C57 mice were used to measure dextran flux across the mesenteric microvessels. Mice were administered intraperitoneally with PBS or PBS-diluted AGEs (10 mg/kg) for 7 consecutive days. For adenovirus treatment group, Ad-sh-mDia1was given IV 2 days before PBS or AGEs injection. Then mice were anesthetized with 13.3% ethyl carbamate plus 0.5% chloralose (0.65 mL/kg) followed by cannulation of jugular veins and intravenously injection of FITC-dextran at 15 mg/kg with continuous infusion at 15 mg/kg/min. Afterwards, the mesenteric microvessel was examined by an intravatal upright microscope and fluorescent image was obtained. Mesenteric transvascular flux was calculated with the following equation: $\Delta I = 1 - (I_i - I_o)/I_i$, in which ΔI indicates changes in light intensity, I_i refers to the light intensity inside the vessel, while I_o means the light intensity outside the vessel.

Isolation of mouse pulmonary microvascular endothelial cells (PMVECs)

Isolation of mouse PMVECs was performed according to the protocol of Magdalena Sobczak et al. [19]. Briefly, 6–8-week-old male C57 mice were anesthetized, and their lungs were excised, sliced, and digested in collagenase type I for 45 min, followed by filtration through 70 μm nylon filters. Then the cell suspension was centrifuged, and the cells were collected and incubated with 10 μL CD31 microbeads per 1×10^7 total cells for 15 min at 4°C. The cell suspension was then applied to a column. Labeled cells were captured in the magnetic field, while unlabeled cells passed through the column. Finally, the column was removed from the magnetic field and magnetically labeled cells were collect in a tube.

Western blotting

Cells were lysed using the lysis buffer (20 mmol/L Tris pH7.4, 2.5 mmol/L EDTA, 1% Triton X-100, 1% deoxycholic acid, 0.1% SDS, 100 mmol/L NaCl, 10 mmol/L NaF, 1mmol/L Na_3VO_4), which is supplemented with protease and phosphatase inhibitors. Protein samples were separated by SDS-PAGE gel and then transferred to polyvinylidenedifluoride (PVDF) membranes. After blocked with 5% BSA, the membranes were incubated with primary antibodies recognizing Src, p-Src, RAGE, mDia1, VE-cadherin or p-VE-cadherin (Y658) with a 1:1000 dilution and β-actin with1:2000 at 4 °C overnight, washed three times with each time for 10 min, and incubated with respective secondary antibody at room temperature for 1 h. After washed three times for 10 min each time, protein bands were visualized with chemiluminescence and densitometric analysis was operated by an imaging station.

Quantitation of cellular ROS level

The cellular ROS level in HUVECs was detected by quantitating the oxidative conversion of cell permeable DCFH-DA to fluorescent dichlorofluorescein (DCF) according to the previous reported assay [20]. DCFH-DA was prepared with a dilution in DMSO at 1:1000 before use. After treatment, HUVECs were washed, trypsinized, and incubated with 10 μmol/L DCFH-DA in a light-protected humidified chamber at 37°C. After 30 min incubation, cells were then washed twice with PBS and analyzed by BD FACSVerse™ flow cytometer (BD Biosciences). The excitation wave length for DCF was at 470 nm with an emission at 530 nm.

Fluorescent staining

HUVECs were plated in microwells and cultured to confluence. Treated with stimulation, sequentially, cells were washed in PBS three times with each time for 2 min, fixed in 4% formaldehyde for 10 min and permeabilized with 0.5% Triton X-100 at 4 °C for 15 min. Cells were washed three times, blocked in 5% BSA for 1 h, and incubated with VE-cadherin (1:200) at 4 °C overnight. After washed in PBS, cells were stained with FITC-conjugated secondary antibody (1:50). Afterwards, cells were washed three times in PBS and detected by a ZeissLSM780 laser confocal scanning microscope (Zeiss, Germany).

Statistics analysis

All data were expressed as means ± SE from at least three independent experiments. Data were statistically analyzed by one-way ANOVA with LSD or Dunnet's T3 test using SPSS 13.0 and *p* value less than 0.05 was considered significant.

Results

Involvement of mDia1 in AGE-induced endothelial hyperpermeability

Our previous study revealed that AGEs evoked dose- and time-dependent patterns of monolayer permeability of endothelial cells [21]. To evaluate the role of mDia1 in these effects, we employed siRNA targeting mDia1 and control siRNA. The effects of AGEs on monolayer permeability were explored using dextran Pd and TER. Cells were stimulated with AGEs (100 mg/L) for 8 h after siRNA transfection. HUVECs treated with AGEs showed a significant decrease in TER and an increase in Pa%. However, treatment with siRNA against mDia1 attenuated the increase in endothelial permeability (Fig. 1A, B). To confirm the role of mDia1 in increasing endothelial permeability induced by AGEs, we used an RNA interference

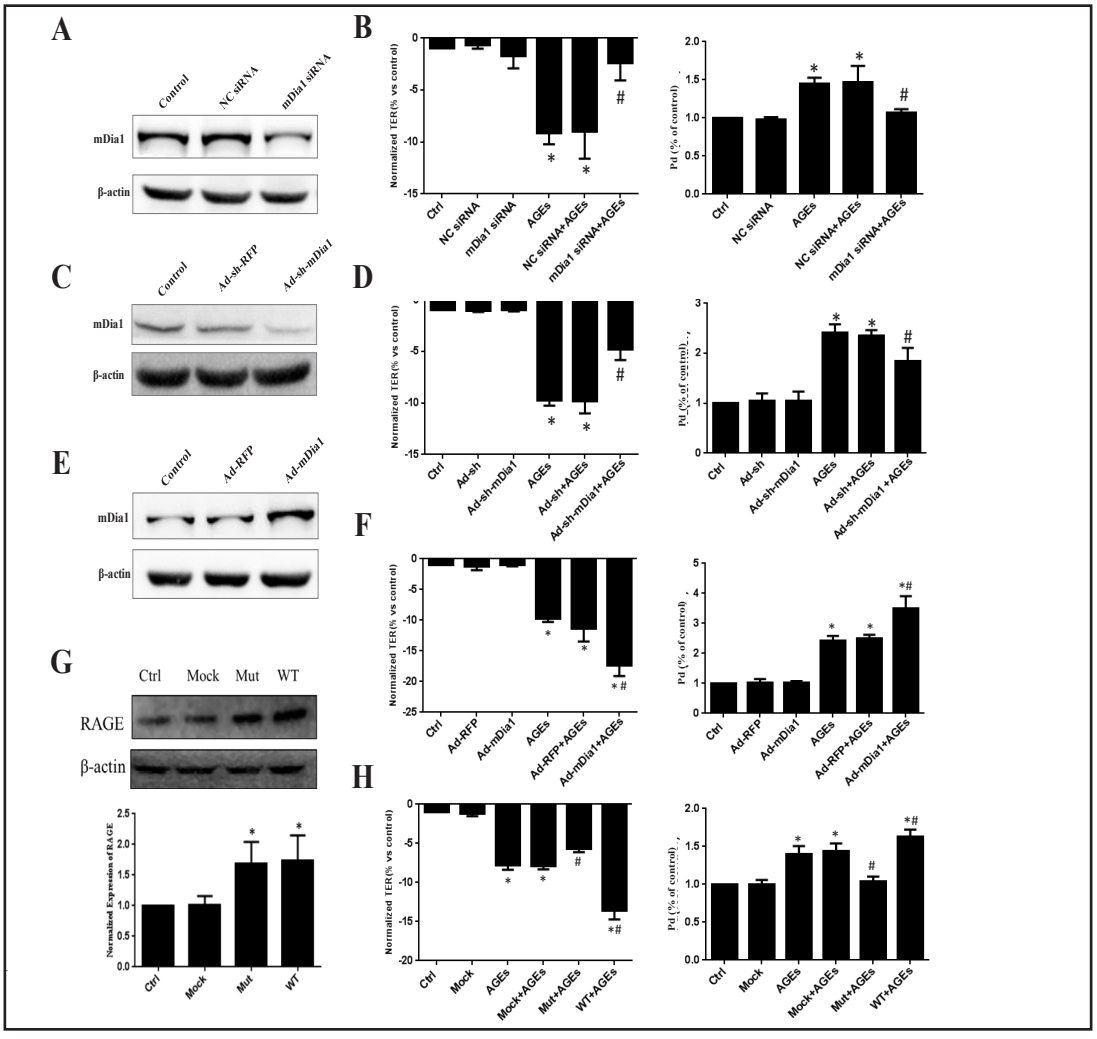

Fig. 1. Involvement of mDia1 in AGE-induced endothelial hyperpermeability. HUVECs were transfected with siRNA for 48 h to down-regulate mDia1 expression (A), followed by AGEs (100 mg/L) stimulation for 8 h. Endothelial monolayer permeability was assessed by TER and Pd (B). n=3, *P<0.05 versus control; #P<0.05 versus AGEs. A mDia1 interference adenovirus (Ad-sh-mDia1) (C) or mDia1 overexpression adenovirus (Ad-mDia1) (E) were used to down- or up-regulate mDia1 in HUVECs, respectively, followed by AGE (100 mg/L) stimulation for 8 h. Endothelial monolayer permeability was assessed by TER and Pd (D, F). n=3, *P<0.05 versus control; #P<0.05 versus AGEs. Dominant mutant or wild-type plasmids of RAGE were transfected into HUVECs (G), and then the cells were stimulated by AGEs (100 mg/L) for 8 h. TER and Pd were measured to detect endothelial permeability (H). n=3, *P<0.05 versus control; #P<0.05 versus AGEs.

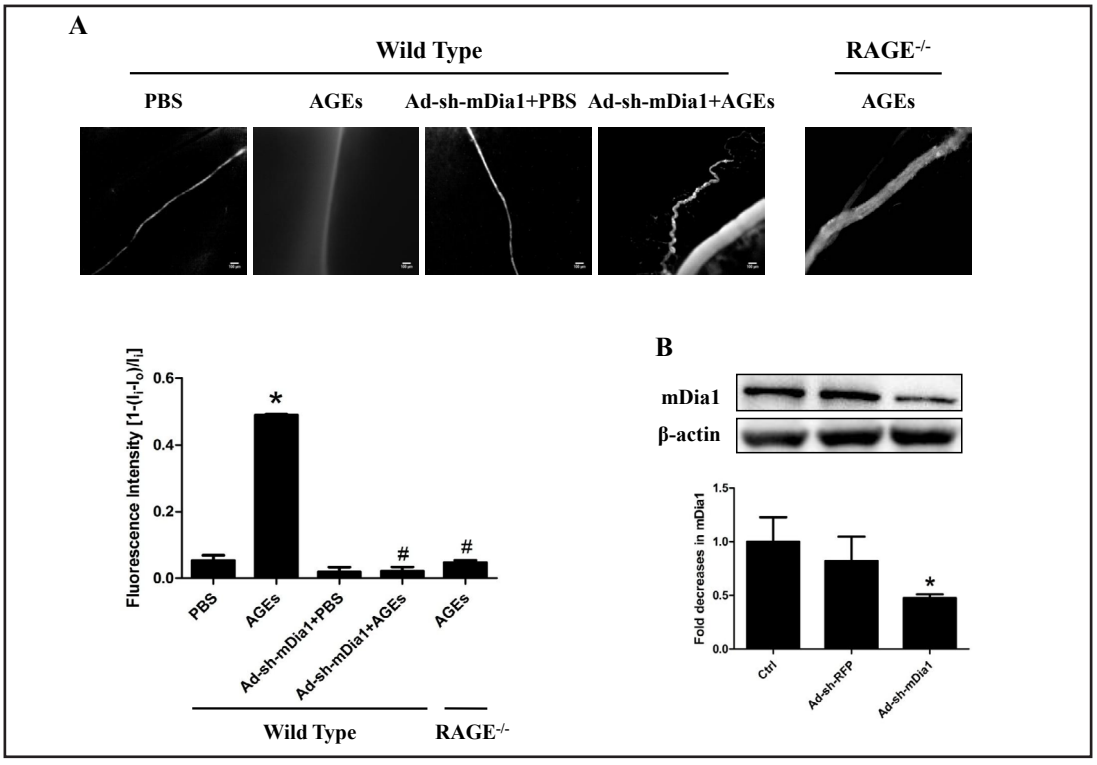

Fig. 2. Knockdown of mDia1 or RAGE prevents AGE-induced vascular barrier dysfunction in vivo. (A, B) An interference adenovirus against mouse mDia1 was intravenously administered to WT mice that were intraperitoneally injected with PBS or AGEs at 10 mg/kg after 2 days for 7 consecutive days. For the RAGE$^{-/-}$ group, RAGE$^{-/-}$ mice were administered with AGEs for 7 consecutive days. FITC-dextran flux across mesenteric microvessels was measured by an intravital upright microscope. n=3–4, *P<0.05 versus control; $^{\#}$P<0.05 versus AGEs. (C) An interference adenovirus against mouse mDia1 was constructed and applied to mouse PMVECs. Then, total mDia1 proteins were detected. n=3, *P<0.05 versus control.

adenovirus against human mDia1 (Ad-sh-mDia1) and an overexpression adenovirus (Ad-mDia1). We found that Ad-sh-mDia1 abolished AGE-evoked monolayer hyperpermeability (Fig. 1C, D). whereas overexpression of mDia1 triggered endothelial hyperpermeability and further enhanced this effect in the presence of AGEs (Fig. 1E, F). These data indicated that mDia1 was strongly linked to AGE-induced disruption of the endothelial barrier.

To clarify the role of RAGE- mDia1 binding in AGE-induced endothelial barrier disruption, RAGE mutant (Mut) plasmids were constructed by converting Arg-5 and Gln-6 to alanines in the cytosolic segment of RAGE, which failed to bind to mDia1. To test whether the plasmids were successfully transfected and expressed in HUVECs, we detected the protein expression of RAGE by western blotting. Transfection of mutant and wide-type (WT) plasmids resulted in consistent overexpression of RAGE, whereas the control plasmid treatment maintained similar expression compared with the control group (Fig. 1G). Cells were pre-transfected with WT, Mut or control (Mock) plasmids using lipofectamine for 48 h before treatment with 100 mg/L AGEs for 8 h. The results indicated that RAGE mutant remarkably supressed the AGE-mediated barrier dysfunction in contrast to the enhanced effect by the WT plasmid. There was no significant change compared with the control plasmid group (Fig. 1H). These data confirmed the specific binding sites of RAGE and mDia1in the process of AGE-mediated hyperpermeability.

To further assess the pivotal role of mDia1 in AGE-evoked microvascular hyperpermeability, an interference adenovirus against mouse mDia1 was constructed and tested in mouse PMVECs (Fig. 2C). Then, the mDia1 interference adenovirus was administered to mice before AGEs injection, and dextran flux across the mouse mesenteric microvessels was

monitored. The results showed that, compared with the PBS group, mice injected with AGEs presented severe dextran leakage, whereas knockdown of mDia1 significantly attenuated AGE-induced dextran extraction (Fig. 2A, B). RAGE$^{-/-}$ mice were administered with AGEs for 7 consecutive days, and permeability of mesenteric microvessels was measured. The results showed that knockout of RAGE significantly attenuated AGE-induced dextran leakage (Fig. 2A, B), indicating that RAGE is required for AGE-induced endothelial barrier perturbation.

mDia1 and RAGE binding leads to activation of Nox4 and increased generation of ROS

We have demonstrated that the NADPH oxidase inhibitor, apocynin suppresses AGE-induced endothelial hyperpermeability [6], indicating the involvement of oxidative stress in this pathological process. However, the link between RAGE and oxidative stress induced by AGEs has remained unclear. In this study, we first assessed ROS generation evoked by AGEs by using flow cytometry. Cells were analyzed immediately or at 10, 15, 20, 30 min after application of AGEs at 100 mg/L. ROS generation was significantly increased at 10 min, rapidly reach a peak at 15 min, and was maintained to 30 min (Fig. 3A). When RAGE or mDia1 were down-regulated by their siRNAs, both down-regulations led to notable diminishment of ROS generation, compared with the group with AGEs stimulation alone (Fig. 3B and C).

To further test the hypothesis that mDia1 might be involved in AGE-induced oxidative stress, HUVECs were pretreated with Ad-sh-mDia1 or Ad-mDia1 for 48 h before exposure to 100 mg/L AGEs for 15 min. HUVECs pretreated with control adenoviruses (Ad-sh-RFP and Ad-RFP) for 48 h before AGEs stimulation showed a significant increase in the ROS generation,

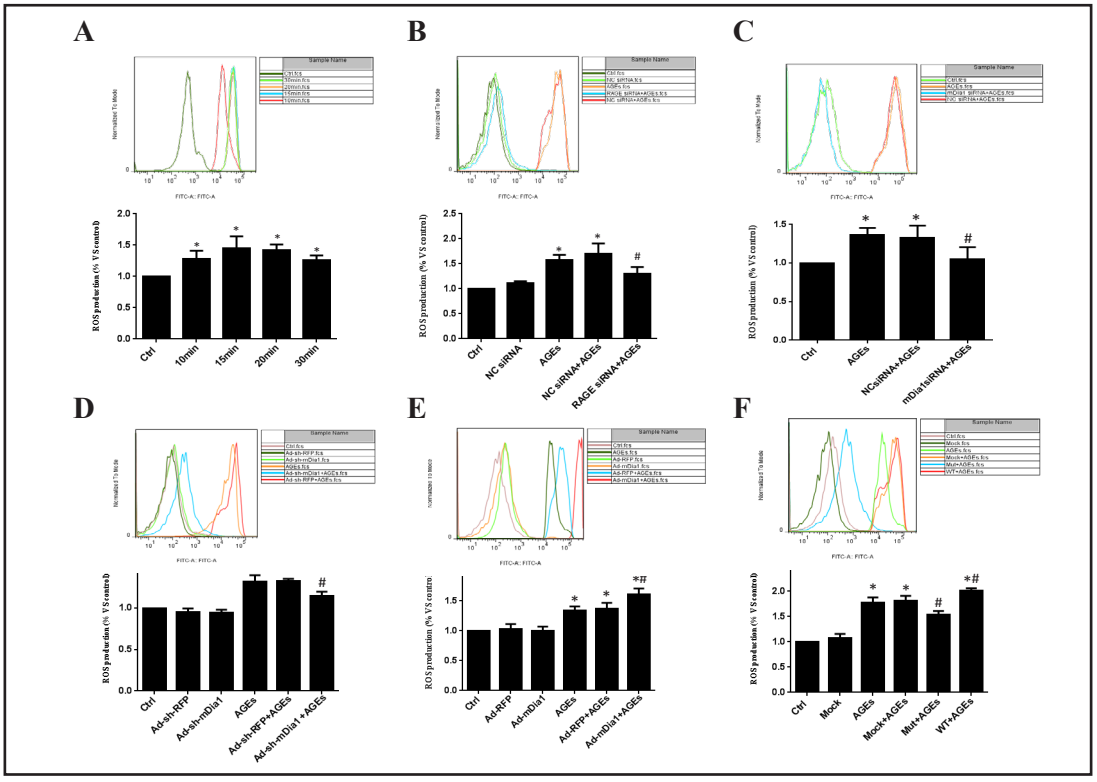

Fig. 3. AGE-mediated up-regulation of ROS requires RAGE-mDia1 binding. (A) HUVECs were treated with 100 mg/L AGEs for 10, 15, 20 or 30 min, ROS generation was then detected by flow cytometry. (B) Cells were transfected with RAGE siRNA for 48 h before AGEs stimulation for 15 min. ROS generation was then detected by flow cytometry. (C, D) Ad-sh-mDia1 was used to knockdown mDia1 in HUVECs, followed by AGEs application for 15 min, ROS generation was then detected by flow cytometry. (E) HUVECs were infected with an adenovirus to overexpress mDia1 before AGEs treatment for 15 min, ROS generation was then measured. (F) Dominant Mut or WT plasmids of RAGE were transfected at 48 h before AGEs treatment. ROS generation was then measured. n=3, *P<0.05 versus control; #P<0.05 versus AGEs.

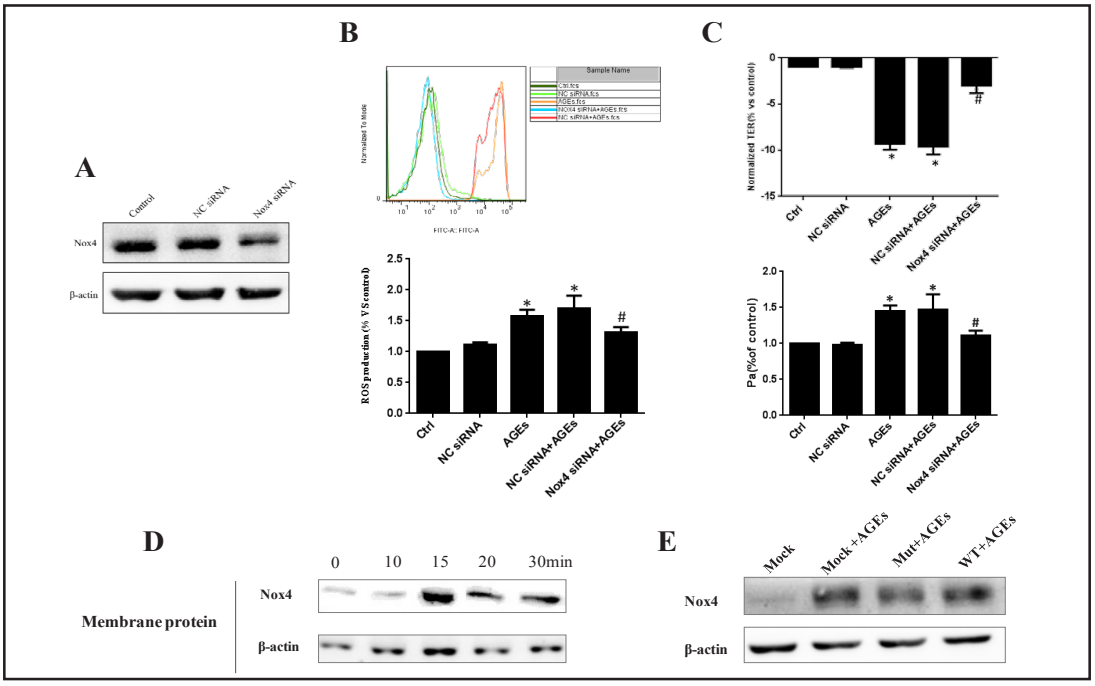

Fig. 4. RAGE-mDia1 binding is required for AGE-mediated activation of Nox4. HUVECs were transfected with siRNA for 48 h to down-regulate Nox4 expression (A), followed by AGEs stimulation, ROS generation was then detected by flow cytometry (B) and endothelial monolayer permeability was assessed by TER and Pd (C). n=3, *P<0.05 versus control; #P<0.05 versus AGEs. (D) HUVECs were treated with 100 mg/L AGEs for 10, 15, 20 or 30 min, membrane proteins were then extracted and Nox4 was detected through specific antibody. (E) Dominant Mut or WT plasmids of RAGE were transfected into HUVECs, then cells were stimulated by AGEs for 15 min. Membrane proteins were extracted and Nox4 was detected.

and this increase was even remarkably enhanced by Ad-mDia1 treatment. However it was decreased by Ad-sh-mDia1 treatment (Fig. 3D and E). These data indicated that mDia1 is essential for the AGE-induced increase in oxidative stress of HUVECs. Moreover, WT and Mut plasmids of RAGE were used to determine whether mDia1 played its role by conjugation with RAGE. Cells pre-transfected with Mut plasmid showed decreases of ROS production, whereas the WT plasmid group showed elevation of ROS (Fig. 3F). These results implied that the interaction of RAGE and mDia1 mediates the AGE-induced ROS production.

Considering that oxidative stress is involved in AGE-induced endothelial hyperpermeability [6], we next examined the role of Nox4 in this pathological process by knockdown of Nox4 in HUVECs by siRNA. As a result, both ROS generation and endothelial permeability showed significant decreases after AGEs stimulation (Fig. 4A–C). The mechanism of AGE-induced cellular oxidative stress was then explored by measuring the expression of Nox4 membrane enrichment (translocation), because Nox4 translocates to membrane to produce ROS. Membrane proteins were extracted from HUVECs after stimulation with AGEs for indicated times, and then analyzed by western blotting. As shown in Fig. 4D, Nox4 expression in the membrane peaked at 15 min after stimulation, suggesting that the AGE-induced Nox4 membrane enrichment increased the level of ROS. Subsequently, we suppressed the conjugation between mDia1 and RAGE by transfection of RAGE Mut plasmid, which effectively attenuated AGE-induced Nox4 membrane enrichment (Fig. 4E). These results indicate that Nox4 membrane enrichment induced by AGEs is partially dependent on the binding of mDia1 to RAGE.

AGE-induced Src phosphorylation requires the interaction of RAGE with mDia1

Src is activated by Nox4-derived ROS elevation to mediate subsequent downstream signaling [22]. In this study, we observed that the phosphorylation of SrcY419 in endothelial

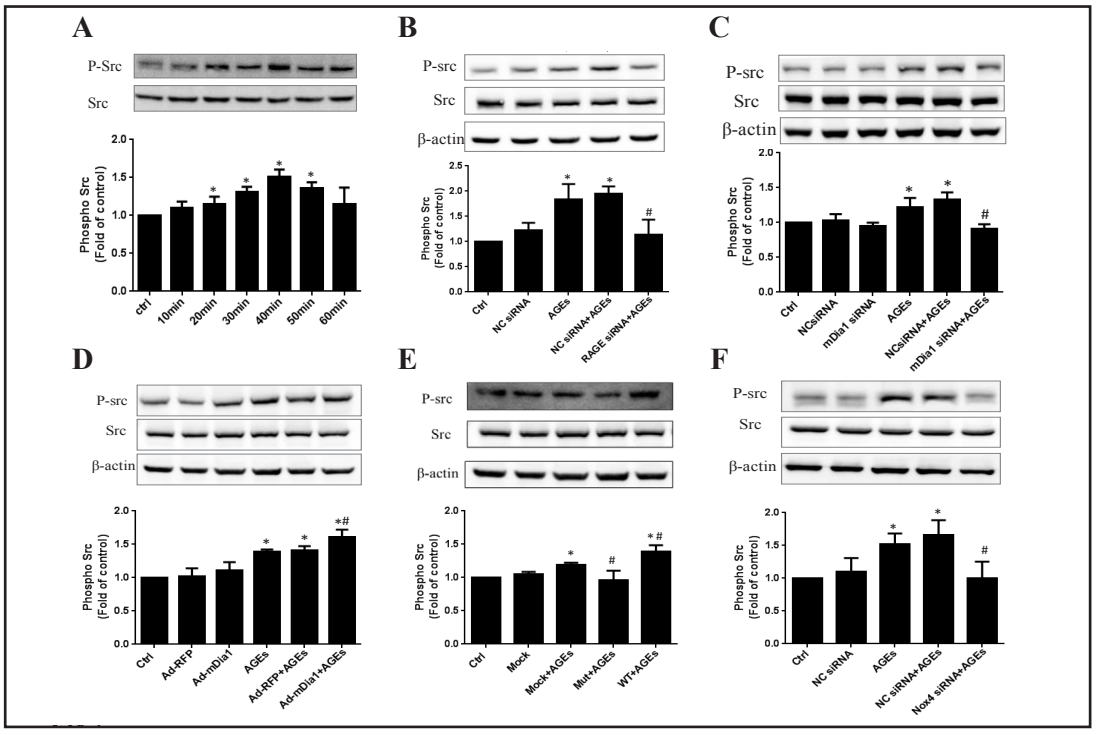

Fig. 5. AGE-induced Src phosphorylation requires the interaction of RAGE and mDia1. (A) HUVECs were incubated with 100 mg/L AGEs for 10, 20, 30, 40, 50 or 60 min, phosphorylation of Src was then measured. Cells were transfected with RAGE siRNA (B), mDia1 siRNA (C) and Nox4 siRNA (F), respectively, and stimulated by AGEs for 40 min. the phosphorylation of Src was then measured. (D) HUVECs were infected with human Ad-sh-mDia1 at 48 h before AGEs treatment, the phosphorylation of Src was then measured. (E) Dominant Mut or WT plasmids of RAGE were applied to HUVECs before AGEs stimulation, the phosphorylation of Src was then measured. n=3, *P<0.05 versus control; #P<0.05 versus AGEs.

cells was increased in a time-dependent manner after 100 mg/L AGEs treatment (Fig. 5A). However, this effect was abolished by the treatment with RAGE siRNA, mDia1 siRNA, Ad-sh-mDia1 or the RAGE Mut plasmid (Fig. 5B, C and E), and enhanced by Ad-mDia1 and RAGE WT plasmid treatments (Fig. 5D and E) compared with AGEs treatment alone. These findings suggest that binding of mDia1 to RAGE plays an important role in the mediation of AGE-induced Src phosphorylation.

Because the essential role of oxidative stress in AGE-induced endothelial cell responses was confirmed in our previous study, we further investigated whether oxidative stress was involved in the AGE-induced Src phosphorylation. HUVECs were pretreated with Nox4 siRNA for 48 h before stimulation with AGEs at 100 mg/L. We found that Nox4 siRNA attenuated the Src phosphorylation evoked by AGEs (Fig. 5F).

AGE-induced VE-cadherin Y658 phosphorylation and disruption require the interaction of RAGE with mDia1

Considering that VE-cadherin acts downstream of Src and causes the disruption of endothelial barrier in response to permeability-increasing agents such as bradykinin and histamine [23], we investigated whether RAGE and mDia1 are involved in activation of VE-cadherin under AGEs treatment. We found that treatment of HUVECs with 100 mg/L AGEsresulted in a notable increase in VE-cadherin Y658 phosphorylation in a time-dependent manner (Fig .6A). Pretreatment with mDia1 siRNA and the RAGE Mut plasmid significantly diminished VE-cadherin phosphorylation, whereas RAGE WT plasmid treatment suppressed this effect (Fig. 6B and C), implying an indispensable role of mDia1 and RAGE in AGE-induced VE-cadherin phosphorylation.

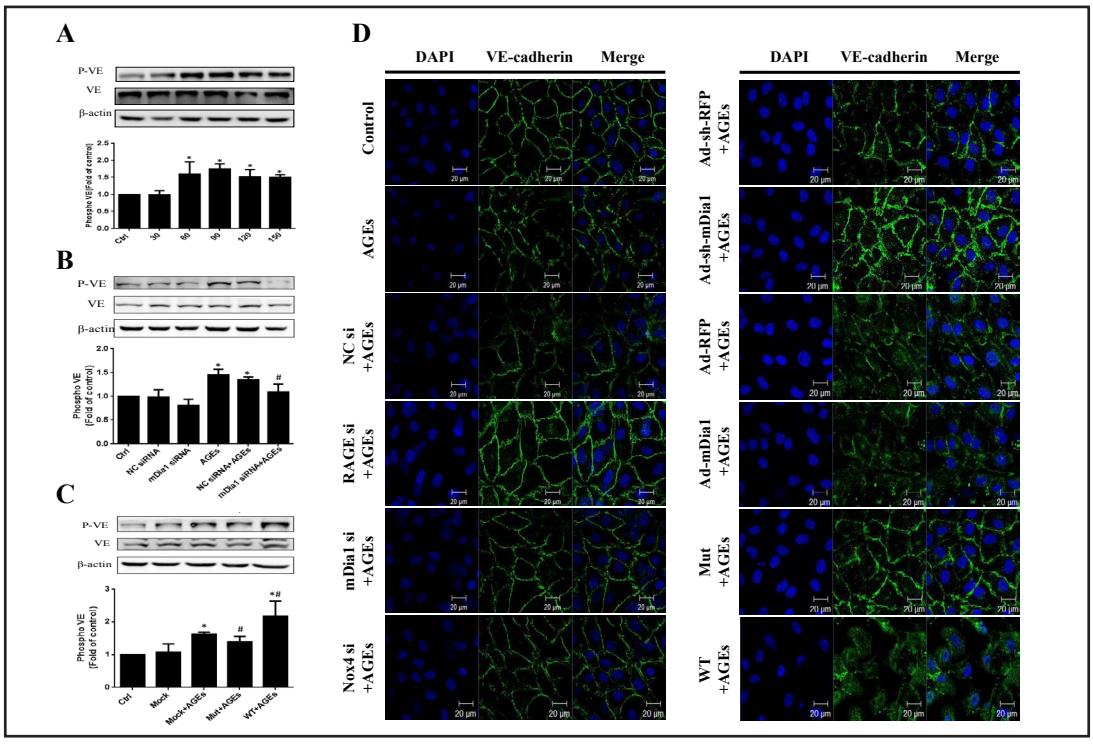

Fig. 6. RAGE-mDia1 binding is required for AGE-induced phosphorylation of VE-cadherin and disruption of adherens junctions. (A) HUVECs were incubated with 100 mg/L AGEs for 30, 60, 90, 120 or 150 min, and then phosphorylation of VE-cadherin was measured. (B) HUVECs were transfected with mDia1 siRNA at 48 h before AGEs treatment for 90 min. Phosphorylation of VE-cadherin was then measured. (C) Dominant Mut or WT plasmids of RAGE were transfected into HUVECs before AGEs stimulation. Phosphorylation of VE-cadherin was then measured. n=3, *P<0.05 versus control; #P<0.05 versus AGEs. (D) HUVECs were transfected with RAGE siRNA, mDia1 siRNA, NOX4 siRNA, the dominant Mut plasmid of RAGE, or the WT plasmid of RAGE or infected with Ad-sh-mDia1 or Ad-mDia1 48 h before AGEs treatment for 8 h. Immunofluorescence was then performed and VE-cadherin dissociation was observed by confocal laser microscope.

Furthermore, dissociation of VE-cadherin was estimated by confocal laser microscopy. We confirmed that AGE-induced dissociation of VE-cadherin could be prevented by RAGE, mDia1, and Nox4 siRNAs, or Ad-sh-mDia1 treatment, but promoted by Ad-mDia1 or RAGE WT plasmid treatments (Fig. 6D). These data suggest that mDia1, RAGE, and Nox4 all play roles in AGE-induced VE-cadherin dissociation.

Discussion

Diabetic microvascular diseases remains as one of the most common complications in patients with diabetes mellitus, including retinopathy, neuropathy, nephropathy, and limb ischemia [24]. Microvascular barrier dysfunction and endothelial hyperpermeability leading to tissue edema and organ dysfunction are critical in the pathogenesis of these diseases. Although several studies have reported that AGEs contribute to elevated vascular endothelial monolayer permeability and microvascular permeability [2, 21, 25, 26], the mechanisms involved in these processes are complex and remain to be determined. Our study first confirmed the participation of mDia1 in endothelial hyperpermeability in response to AGEs. We demonstrated that AGEs elicited a series of potent signal transductions via RAGE-mDia1 binding. Either Down-regulation of mDia1 expression by siRNA or Ad-sh-mDia1 or genetic knockout of RAGE or inhibition of RAGE-mDia1 binding using the RAGE

Mut plasmid attenuated AGE-induced cellular responses and thus rescued microvascular hyperpermeability evoked by AGEs. mDia1 acts as a key signaling node and triggers cellular oxidative stress by recruiting Nox4 to the cell membrane, facilating its activation. The elevated ROS generation derived by activated Nox4 therefore evoked phosphorylation of Src which in turn facilitating the phosphorylation of VE-cadherin and eventually led to endothelial hyperpermeability (Fig. 7).

ctRAGE has been shown to be a pivotal element required for RAGE ligand-stimulated cellular responses in multiple studies [27-29]. In addition, RAGE-dependent signal transduction requires the interaction between the ctRAGE and mDia1 that has been identified as a binding partner of the cytoplasmic domain. Further studies revealed that Arg-5 and Gln-6 of ctRAGE are essential for its interaction with mDia1. When both are mutated to alanines, the loss of binding sites for mDia1 abolishes RAGE ligand-induced downstream signaling pathways such as Rho GTPases Rac1 and Cdc42, and AKT [8, 12]. In the present study, we found either down-regulation of RAGE and mDia1 or transfection with RAGE Mut plasmid in HUVECs rescued AGE-induced cellular reactions. Thus, we demonstrated that AGE-stimulated endothelial dysfunctions require RAGE-mDia1 binding.

It is well established that oxidative stress is a major process involved in AGE-RAGE-mediated pathological effects. AGEs are capable of inducing cytosolic ROS production that is largely attributed to the activation of the NADPH oxidase system [15, 30]. The role of mDia1 in oxidative stress has been studied in SMCs and a murine model of arterial injury induced by endothelial denudation of the femoral artery. Oxidative stress induced by the injury was markedly attenuated in RAGE and mDia1 null mice compared with WT control mice. Deletion of mDia1 *in vivo* reduced NADPH oxidase in the injured vessels, and exposure of SMCs retrieved from the aorta of mice to RAGE ligand S100B enhanced ROS production, which was not found in SMCs retrieved from RAGE or mDia1 null mice [11]. Our previous study showed that the AGE-induced hyperpermeability response in HUVECs was abated by the NADPH oxidase inhibitor apocynin [6]. Here, deletion of mDia1 in endothelial cells mimiced the protective effect of RAGE and Nox4 deficiency, as shown by the reduction in ROS production and the diminishment of HUVECs hyperpermeability, in contrast to the opposite effects in mDia1-overexpressing cells. Activation of NADPH oxidase is an ordered multistep process including protein phosphorylation, GTPase activation and translocation of certain cytosolic proteins to the plasma membrane [31]. Furthermore, in mDia1-RAGE association-deficient cells, membrane translocation of Nox4 was inhibited, suggesting that RAGE-induced NADPH oxidase activation requires the cooperation of mDia1.

As a homologue of gp91phox/Nox2, Nox4 is abundantly expressed in endothelial cells and functionally active with p22phox as a superoxide-producing enzyme, making it an important membrane component of endothelial NADPH oxidase [15]. Another study and our present data revealed recruitment of Nox4 to the membrane and subsequent Src activation evoked by the translocation [22]. Moreover, inhibition of oxidative stress attenuated AGE-induced Src phosphorylation. Nox4-derived ROS in cellular oxidative stress is responsible for Src oxidation [32], It has been previously shown that Nox4 recruitment to the plasma membrane scaffold SHPS-1 allows localized ROS generation to mediate sustained Src oxidation and activation [22]. The Src kinase family regulates diverse processes such as cellular differentiation, cell adhesion, migration, and oncogenesis. They are also potent effectors of the formin family of proteins [33]. We confirmed for the first time the crucial role of mDia1 and the mDia1-RAGE association in AGE-derived Src phosphorylation. Recent reports have identified mDia1 as a key molecule that is essential for Src kinase membrane translocation [11, 34]. It was shown that Src and mDia1 bind directly via their SH3 and FH1 domains, respectively, suggesting that mDia1 and Src move together through processive actin polymerization by mDia1 [34]. Here, our results are in complete agreement with these reports and illustrate this relationship under AGE-RAGE ligation in HUVECs. However, the precise mechanism by which the mDia1-RAGE interaction induces Nox4 recruitment remains a subject for future investigation.

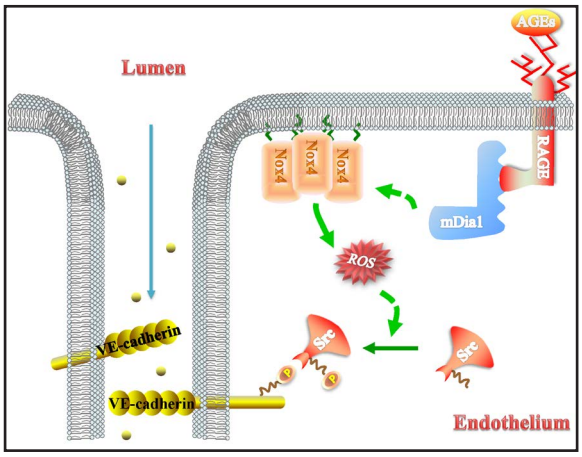

Fig. 7. Schematic of the mediation of AGE-induced endothelial hyperpermeability by mDia1. Binding of AGEs to RAGE facilitates its combination with mDia1, which in turn contributes to recruitment and membrane translocation of Nox4. The excessive production of ROS derived from activated Nox4 leads to phosphorylation of Src, and subsequently causes phosphorylation of VE-cadherin at Y658, which facilitates disruption of adherence junctions and eventually leads to hyperpermeability.

Various proteins, including NADPH oxidase, vascular endothelial growth factor (VEGF), matrix metalloproteinases, and VE-cadherin have been reported to be implicated in the elevated endothelial permeability in response to AGEs stimulation in different contexts. Src as a downstream molecule of VEGF signaling participates in mediating VEGF-induced vascular permeability [18, 35]. Phosphorylation of Src contributes to activation of VE-cadherin, which has been identified as a critical step in the induction of permeability by growth factors and proinflammatory cytokines [23, 36]. VE-cadherin functions as a mediation protein at endothelial adherens junctions, and the internalization and cleavage of VE-cadherin can cause adherens junctions to be dismantled [37-39]. VE-cadherin phosphorylation at Y658 is considered to be a prerequisite for rapid and fully reversible opening of cell-cell junctions [37]. Thus, in the present study, we further determined whether inhibition of mDial or mDia1-RAGE binding could block phosphorylation and dissociation of VE-cadherin. The obtained results strongly verified our speculation. Moreover, we revealed a novel intracellular signaling pathway by which mDia1 acts in AGE-evoked endothelial hyperpermeability.

Conclusion

Our study shows for the first time that mDia1 exerts a critical effect on AGE-induced endothelial hyperpermeability in HUVECs. mDia1 acts as a key signaling node and functions by binding to RAGE to trigger a series of downstream pathways in response to AGEs, including oxidative stress, and activation of Src and VE-cadherin, leading to endothelial hyperpermeability. Because deletion of mDia1 or blockade of RAGE-mDia1prevented the increase in endothelial permeability, mDia1 may be an appropriate target for the prevention and treatment of AGE-associated endothelial hyperpermeability in diabetic microvascular diseases.

Abbreviations

AGEs (Advanced glycation end products); ctRAGE (C-terminal RAGE, Cytoplasmic tail of RAGE); mDia1 (Mammalian diaphanous 1); Pd (Permeability coefficient for dextran); RAGE (Receptor for Advanced glycation end products); ROS (Reactive Oxygen Species); TER (Trans-endothelial electric resistance).

Acknowledgements

All animal experiments were approved by the Animal Care Committee of the Southern Medical University of China and strictly followed the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health.

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

This work was supported by National Natural Science Foundation of China (81670076, 81370226); Guangdong Natural Science Foundation (2017A030313462); The Team-project of Natural Science Foundation of Guangdong, China (S2013030013217).

QH and XG conceived the study, arranged the collaboration, edited and compiled the final version for submission. XZ and JW initiated the manuscript and participated in its design. JX, QX and WW performed laboratory and studied design. All authors reviewed and approved the final manuscript.

We thank Biying Zhou for providing technical support in mice experiment.

Disclosure Statement

The authors declare that they have no competing interests.

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