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Hypoxia induces FoxO3a-mediated dysfunction of blood–brain barrier

Soo-Wang Hyun [a,b,1], Yi-Sook Jung [a,c,*,2]

[a] College of Pharmacy, Ajou University, Suwon 443-749, Republic of Korea
[b] Graduate Program of Molecular Medicine, Ajou University School of Medicine, Suwon 443-749, Republic of Korea
[c] Research Institute of Pharmaceutical Sciences and Technology, Ajou University, Suwon 443-749, Republic of Korea

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ABSTRACT

The Forkhead box O 3a (FoxO3a), a transcription factor, is known to be involve in change of endothelial permeability. During hypoxia, blood–brain barrier (BBB) permeability is increased through degradation of vascular endothelium cadherin (VE-cadherin) and clsudin-5. The hypoxia also increased mRNA levels of matrix metalloproteinase (MMP)-3/9 and promoted translocation of FoxO3a into nucleus in endothelial cells. However, little is known about the role of FoxO3a in hypoxia-induced BBB hyperpermeability. Here, we examined whether FoxO3a regulates hypoxia-induced BBB permeability through induction of MMPs. The transfection of siFoxO3a suppressed hypoxia-induced BBB hyperpermeability. The transfection of siFoxO3a also inhibited hypoxia-induced degradation of VE-cadherin and claudin-5. In addition, the transfection of siFoxO3a reduced hypoxia-induced increase of MMP-3 mRNA levels. However, transfection of siFoxO3a did not inhibits transcription of MMP-9 induced by hypoxia. Taken together, our findings suggest that FoxO3a is involved in hypoxia-induced degradation of VE-cadherin and claudin-5 through induction of MMPs indirectly.

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1. Introduction

The blood–brain barrier (BBB) is structural and functional barrier which impedes and regulates the influx of many compounds and pathogens from blood circulation to brain tissue [1]. As primary barrier of the BBB, brain microvascular endothelial cells is sealed by tight and adherens junctions [12], which form the junction complex between adjacent endothelial cells [16] and play a crucial role in the maintenance of endothelial barriers [14]. The BBB is disrupted under pathological conditions such as cerebral ischemia, leading to vasogenic edema and secondary brain damage [5]. Matrix metalloproteinases (MMPs) is the one of the main causes in brain ischemia-induced BBB disruption [18,22,24]. Among MMPs, especially, MMP-9 is involved in BBB dysfunction after brain ischemia [26]. Recent studies suggest that activation of MMP-9 caused severe BBB dysfunction, and this effect is associated with degrading the tight junctions, substantially contributing to brain edema [26]. MMP-9 has also been shown to degrade claudin-5, occludin, and ZO-1 in cultured brain endothelial cells [3].

Forkhead box O (FoxO) is the O type subfamily of the forkhead transcription factor superfamily and includes FoxO1, 3a, 4 and 6 in mammalian species [25]. FoxO proteins regulates transcriptionally activates or inhibits a series of downstream targets, thereby involved in diverse cellular functions including regulation of cell permeability [2,4,25,27]. It is known that FoxO1/β-catenin caused claudin-5 repression in IL-1β-mediated barrier dysfunction [2]. FoxO1 also increased caveolae-mediated transcytosis across the BBB [23]. In addition, FoxO4 regulated intestinal permeability through tight junction proteins, including ZO-1 and claudin-1 [27]. However, little is known that FoxO3a protein has a role in regulation of brain endothelial cell permeability. Lee and colleagues reported that the functional activation of MMP-9 via FoxO3a-mediated MMP-3 activation is involved in regulating EC survival and degrading extracellular matrix [8]. Emerging data indicated that FoxO3a may play an important role during injuries that involve cerebral ischemia and oxidative stresses [4,13]. In the present study, we investigated the roles of FoxO3a in regulation of BBB permeability after hypoxia.

2. Materials and methods

2.1. Cell culture

The bEnd.3 cells was purchased from the ATCC (Manassas, VA) and grown in Dulbecco's Modified Eagle Medium (DMEM)

* Corresponding author at: College of Pharmacy, Ajou University, Suwon 443-749, Republic of Korea. Fax: +82 31 219 3435.
E-mail address: yisjung@ajou.ac.kr (Y.-S. Jung).

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(Gibco-BRL, Grand Island, NY) with 10% fetal bovine serum. For challenge of hypoxia, confluent bEnd.3 cells were incubated in an anaerobic chamber (Forma Scientific, Marietta, OH) at 37 °C under an atmosphere composed of 5% CO_2, 10% H_2 and 85% N_2 in glucose free DMEM that had been saturated with N_2 gas for 30 min.

2.2. Endothelial cell monolayer permeability assay

bEnd.3 cells were grown on the inside of gelatin-coated transwell insert (0.4 μm, Corning Costar Co., NY) and exposed to hypoxic condition for 4 h. Next, 165 μg/ml of Evans blue-0.1% albumin (Sigma–Aldrich Co., St. Louis, MO) and NaF (Sigma–Aldrich Co., St. Louis, MO) were added to the upper chamber 1 h before measurement. The intensity of the diffused Evans blue-0.1% albumin and NaF in the lower chamber was measured at 650 nm and 485/535 nm, respectively. Results are expressed as ratios of Evans blue-0.1% albumin and NaF concentration in the lower chamber to the total concentration of Evans blue-0.1% albumin concentration and NaF added to the upper chamber at the start of the experiment, respectively. Transendothelial electrical resistance (TEER) across the membrane was measured with a Millicell ERS-2 Voltohmmeter (Millipore Co., Billerica, MA). The gelatin-coated transwell inserts were placed in 24 well plates containing culture medium and then used to measure background resistance. The resistance measurements of these blank filters were then subtracted from those of filters with cells. The values are shown as $\Omega \times cm^2$ based on culture inserts.

2.3. Reverse transcription polymerase chain reaction (RT-PCR)

RT-PCR was performed by modification of the procedures described previously [10]. Briefly, total RNA was isolated for RT. After RT reaction, cDNA was amplified using specific primers for MMP-3 and-9. All sample detected and analyzed using GelDoc™ (Bio-Rad, Hercules, CA).

2.4. Western blot analysis

Western blot analysis was performed by modification of the procedures described previously [6]. To obtain the protein, briefly, the cells were lysed and centrifuged at 14,000 rpm for 15 min and the supernatant was collected. Proteins were separated by SDS–PAGE and reacted with antibodies specific for VE-cadherin (Invitrogen, Carlsbad, CA) and claudin-5 (Invitrogen, Carlsbad, CA). All sample detected and analyzed using LAS4000 mini (Fuji Photo Film, Tokyo, Japan).

2.5. Immunocytochemistry for translocation of FoxO3a

Immunocytochemistry was performed by modification of the procedures described previously [9]. Briefly, samples were fixed in methanol for 10 min at room temperature and then soaked in 3% bovine serum albumin (BSA) blocking solution at room temperature. Samples were incubated with anti-FoxO3a antibody (Cell Signaling Technology, Inc., Danvers, MA) overnight and then with secondary antibody labeled with Alexa 488 (Invitrogen, Carlsbad, CA). Finally, samples were incubated with hoechst 33258. All samples were observed under a Nicon C2 confocal microscope (Nicon, Tokyo, Japan).

2.6. Small interfering RNA (siRNA) transfection

Transfection of siFoxO3a was performed by modification of the procedures described previously [7]. For gene silencing in vitro, the FoxO3a siRNAs and a control siRNA were from Santa Cruz Biotechnology, Inc. (Dallas, TX).

2.7. Statistical analysis

All data are expressed as the means ± SEMs. Statistical comparisons were conducted by Student's *t*-test and/or one-way analysis of variance (ANOVA). *p* values of less than 0.01 or 0.05 were considered significant.

3. Results

3.1. Hypoxia promoted FoxO3a nuclear location

The accumulation of FoxO3a in nucleus was measured to determine whether hypoxia induce translocation of FoxO3a into nucleus in brain endothelial cells. The cells were exposed to hypoxic condition for 15 min, 30 min and 60 min. As shown in Fig. 1A, the accumulation of FoxO3a in nucleus was detectable at 60 min of hypoxia.

3.2. FoxO3a regulates hypoxia-induced BBB hyperpermeability

To investigate the role of FoxO3a in hypoxia-induced BBB hyperpermeability, we transfected siFoxO3a in brain endothelial cells. Western blotting results revealed that protein of FoxO3a in brain endothelial cells was reduced after transiently transfected with FoxO3a siRNA (Fig. 1E). The BBB permeability was measured using TEER (Fig. 1B), Evans blue (Fig. 1C) and NaF (Fig. 1D). TEER values significantly decreased after 4 h of hypoxia compared with normoxia (data not shown). The inhibition of FoxO3a did not alter TEER values under normoxia, whereas significantly prevented hypoxia-induced changes in TEER. The concentration of Evans blue and NaF in lower chamber also significantly increased at 4 h of hypoxia compared with normoxia (data not shown). The concentration of Evans blue and NaF in lower chamber was greater in hypoxia group than in the control group, and this increase was significantly attenuated by the knockdown of FoxO3a with siRNA.

3.3. FoxO3a affects the degradation of VE-cadherin and claudin-5 in hypoxia model

To investigate the role of FoxO3a in hypoxia-induced degradation of *VE-cadherin and claudin-5*, we transfected siFoxO3a in brain endothelial cells. The disruption of VE-cadherin and claudin-5 in the hypoxia was quantified using Western blotting at 2, 4 and 6 h of hypoxia. As shown in Fig. 2A, VE-cadherin and claudin-5 significantly reduced at 4 h of hypoxia. As shown in Fig. 2, the level of VE-cadherin (B) and claudin-5 (C) was lower in hypoxia group than in the control group, and this decrease was significantly prevented by the knockdown of FoxO3a.

3.4. FoxO3a modulates expression of MMP-3 and MMP-9 in hypoxia model

To investigate the role of FoxO3a in hypoxia-induced expression of MMP-3 and MMP-9, we transfected siFoxO3a in brain endothelial cells. The expression of MMP-3 and MMP-9 were quantified using RT-PCR at 1, 2 and 4 h of hypoxia. The expression of MMP-3 and MMP-9 in the hypoxia remarkably increased at 2 h of hypoxia (Fig. 3A). The expression of MMP-3 also was greater in hypoxia group than in the control group, and this increase was significantly prevented by the knockdown of FoxO3a. During hypoxia, however, no differences in mRNA level of MMP-9 were found between vehicle and siFoxO3a groups (Fig. 3B).

4. Discussion

This study demonstrates that FoxO3a involves in hypoxia-induced BBB dysfunction through degradation of VE-cadherin and claudin-5 indirectly.

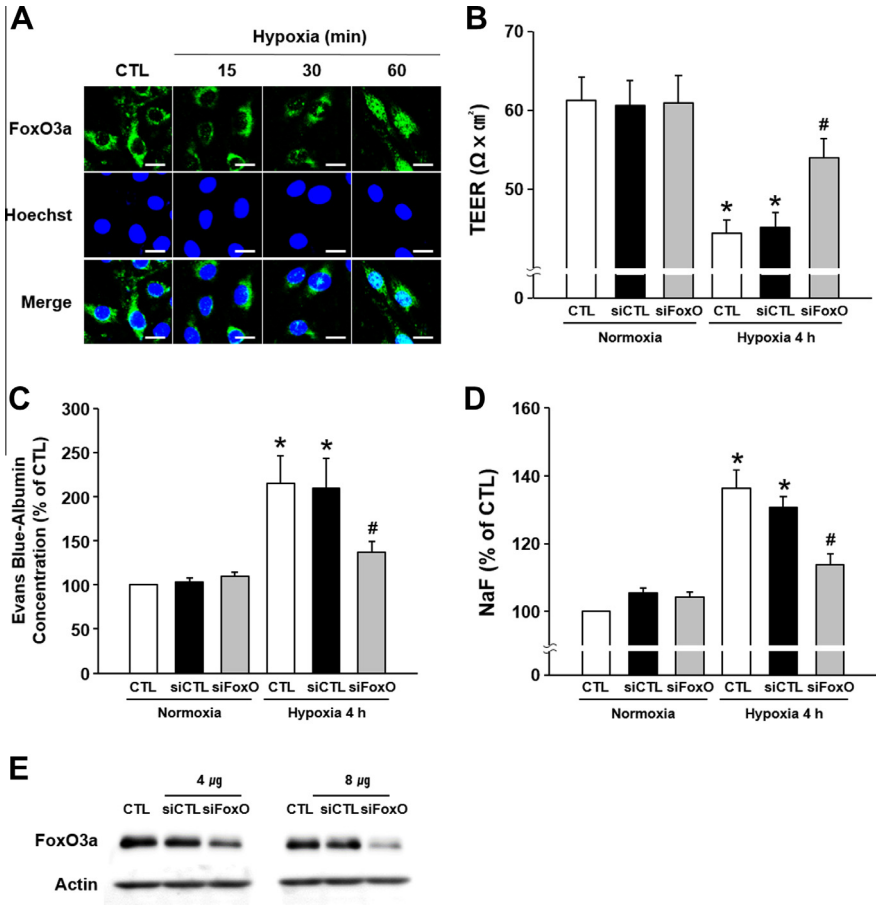

Fig. 1. Hypoxia increased BBB permeability, promoting FoxO3a nuclear location. (A) The immunofluorescent staining of FoxO3a (green) and hoechst 33258 (blue) in brain endothelial cells. The immunofluorscent staining was detected at control (CTL), 15, 30 and 60 min of hypoxia. (Scale bars: 10 μm) (B) Quantitative analysis of TEER in brain endothelial cell monolayer transfected with FoxO3a siRNA or control siRNA (siCTL) at 4 h after hypoxia. Data shown are mean ± SEM. ($n = 4$ and more). $^{*}p < 0.01$ vs. normoxia control (CTL), $^{\#}p < 0.01$ vs. hypoxia control. Quantitative analysis of Evans blue contents (C) and NaF (D) in brain endothelial cell monolayer transfected with FoxO3a siRNA or control siRNA at 4 h after hypoxia. Data shown are mean ± SEM. ($n = 4$ and more). $^{*}p < 0.01$ vs. normoxia CTL, $^{\#}p < 0.01$ vs. hypoxia CTL. (E) Brain endothelial cells was transfected with FoxO3a siRNA (siFoxO3a, 4 or 8 μg) or control siRNA, as described in Section 2. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

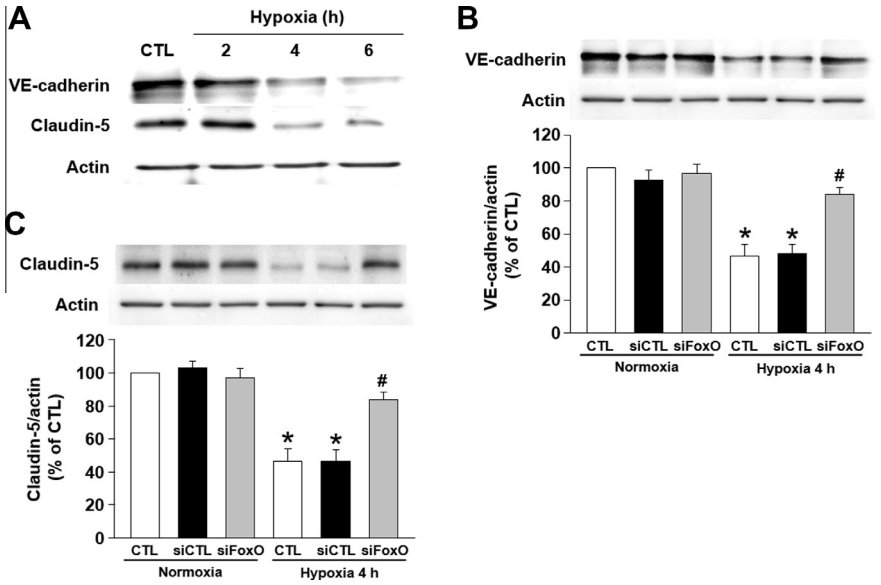

Fig. 2. FoxO3a silencing suppresses hypoxia-induced degradation of VE-cadherin and claudin-5. (A) Quantitative analysis of degradation of VE-cadherin and claudin-5 in brain endothelial cells. The degradation of VE-cadherin and claudin-5 were measured at control (CTL), 2, 4 and 6 h of hypoxia. Quantitative analysis of degradation of VE-cadherin (B) and claudin-5 (C) in brain endothelial cells transfected with FoxO3a siRNA or control siRNA at 4 h after hypoxia. Data shown are mean ± SEM. ($n = 4$ and more). $^{*}p < 0.01$ vs. normoxia CTL, $^{\#}p < 0.01$ vs. hypoxia CTL.

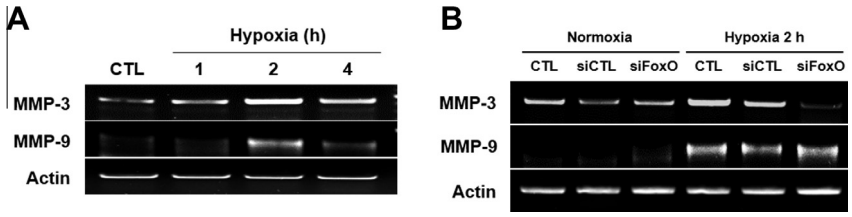

Fig. 3. FoxO3a silencing suppresses hypoxia-induced an increase of MMP-3. (A) The expression of an increase of MMP-3/9 mRNA levels in brain endothelial cells. The mRNA levels of MMP-3/9 were measured at control (CTL), 1, 2 and 4 h of hypoxia. (B) The expression of an increase of MMP-3/9 mRNA levels in brain endothelial cells transfected with FoxO3a siRNA or control siRNA at 2 h after hypoxia.

BBB is a diffusion barrier, composing of endothelial cells, astrocytes, pericytes and extracellular-matrix (ECM). One of the primary functions of the BBB is the strict regulation of paracellular permeability [19]. The first-line of the defense between blood circulation and brain tissue is the endothelium [19]. The endothelial cells are connected by junction complex, in which tight junction (TJ) and adherens junction (AJ) play a critical role in maintaining the integrity of the BBB [17]. TJ and AJ proteins among adjacent cells form the basic structure of BBB and regulate paracellular permeability [11]. At both TJ and AJ proteins, adhesion is mediated by transmembrane proteins that are linked inside the cells to the actin cytoskeleton [21]. The adhesion is also mediated by a member of cadherins and claudin family at AJ and TJ, respectively [21]. The transmembrane adhesion proteins such as VE-cadherin at AJ and claudin-5 at TJ were expressed in brain endothelial cells [15]. Claudins are principal barrier-forming proteins and consist of at least 24 members, with each showing a specific organ and tissue distribution [14]. Especially, brain endothelial cells mainly possess the claudin-5 and possibly some other claudins [20]. Importantly, claudin-5 deletion resulted in a size-selective increase in permeability [20]. The claudin-5-deficient mice also present an altered BBB with higher permeability [12]. VE-cadherin is the major transmembrane protein of the endothelial adherens junction. VE-cadherin is an important determinant of microvascular integrity and together with catenin forms a complex that links to the actin microfilament network of the cell skeleton. VE-cadherin and catenin complex also may regulate vascular permeability and affect BBB function [20]. In addition, overexpression of FoxO3a causes degradation of AJ such as VE-cadherin and β-catenin through activation of MMP-9 in endothelial cells [8]. In our results from this study, hypoxia-induced disruption of TJ and AJ proteins attenuated by transfection of siFosO3a, thereby we suggest that FoxO3a related to hypoxia-induced disruption of TJ and AJ proteins, in part. In addition, Lee and colleagues reported that enzymatic activities of MMP-9 increased after FoxO3a activation in endothelial cells but their regulation involves indirect mechanism, MMP-3 activation [8]. The mRNA and protein amount of MMP-9 were not changed by FoxO3a activation [8]. FoxO3a-induced MMP-3 activation contributes to increased enzymatic activity of MMP-9 [8]. The consensus binding site for the forkhead transcription factors did not exist in the promoter sequences of MMP-9 [8]. Hypoxia-induced an increase of mRNA level of MMP-3 but not MMP-9 significantly changed after siFoxO3a transfection in this study. MMPs mediate BBB disruption and vasogenic edema after cerebral ischemia by degrading the extracellular matrix, basal lamina proteins and TJ around the BBB [18]. Among MMPs, especially MMP-9 disrupts the BBB by degrading the TJ [5]. Therefore, we suggest that FoxO3a regulates indirectly the activity of MMP-9 via MMP-3, and then increases the BBB permeability during hypoxia.

Taken together, the results from this study suggest that FoxO3a may be involved indirectly in significant BBB dysfunction after hypoxia, and these are associated with degradation of VE-cadherin and claudin-5 though MMPs.

Acknowledgments

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