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Original Article

Tongxinluo protects against hypoxia-induced breakdown of the endothelial barrier through inducing tight junction protein expression

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Abstract: The hypoxia can cause abnormal expression of tight junction proteins, leading to disruption of tight junctions (TJs) and increasing permeability of the endothelial barrier. Tongxinluo (TXL) can improve endothelial cell function and protect the brain against blood-brain barrier disruption. However, it remains unclear whether there is a direct relationship between protective effect of TXL on endothelial functions and TXL-induced tight junction protein expression. The aim of present study was to investigate the mechanism of TXL actions whereby TXL protects against hypoxia-induced tight junction disruption. We found that hypoxia disrupted, while TXL treatment protected the TJs in the microvasculature of the mouse brain, and that TXL promoted the expressions of TJ proteins VE-cadherin, β-catenin and ZO-1 in human cardiac microvascular endothelial cells (HCMECs) under hypoxia conditions. Mechanistic studies suggested that upregulation of TJ protein expressions is attributable to KLF4 phosphorylation at sites Ser444 and Ser415 induced by TXL. Furthermore, our study shows that Akt signaling plays a key role in TXL-induced KLF4 phosphorylation. In conclusion, the results of our study reveal a novel mechanism whereby TXL protects against hypoxia-induced tight junction disruption through promoting KLF4 phosphorylation and inducing tight junction protein expression.

Keywords: Tight junctions, endothelial cell, hypoxia, KLF4, phosphorylation, tongxinluo

Introduction

Vascular endothelium forms a continuous inner lining of the blood vessels, and permits the movement of molecules between circulating blood and interstitial space [1]. Under normal conditions, the endothelium functions as a semipermeable barrier. The vascular endothelial integrity is critical for maintenance of vessel wall homeostasis under physiological and pathophysiological states. Several lines of evidence have shown that the barrier function of the endothelial monolayer depends mainly on cell-to-cell connections, including tight junctions (TJs) and adherens junctions (AJs), and that TJs of endothelium, which is constituted by many transmembrane proteins, such as β-catenin, ZO-1, VE-cadherin and claudin, play a pivotal role in modulating its barrier permeability [2, 3]. Disruption of these junctions perturbs endothelial barrier function and leads to an increase in endothelial permeability, which allows the movement of leukocytes from the blood stream to infiltratein to the interstitial space and initiates inflammatory cascade [3, 4]. Abnormal expression of tight junction proteins can result in endothelial barrier dysfunction and causes various cardiovascular diseases.

Tissue ischemia and hypoxia caused by many factors, such as atherosclerotic lesion and microvascular obstruction can activate proteases, resulting in degradation of endothelial tight junction proteins and increased permeability of the endothelial barrier [5, 6]. The endothelial cell response to hypoxia involves hypoxia-mediated suppression of endothelial barrier function and increase in vascular leakage [7]. Although many of the factors involved in maintaining endothelial barrier have been identified, protective mechanism against hypoxia-induced

tight junction disruption remains poorly understood. Krüppel-like factors (KLFs) are increasingly appreciated as key regulators of endothelial cell biology [8]. It has been known that KLF4 regulates key endothelial targets that modulate the inflammatory and coagulant state of the endothelium [9], and that KLF4 plays a critical role in the regulation of endothelial functions [10]. However, it remains unclear whether and how KLF4 modulates TJs of the endothelium.

Tongxinluo (TXL) is a traditional Chinese medicine that is extracted, concentrated and standardized from a mixture of 12 medicinal constituents, such as ginseng, *Radix paeoniae*rubra, borneol, and spiny jujuba seed [11], and is approved in 1996 by the State Food and Drug Administration of China for treatment of angina pectoris and ischemic stroke. The previous studies have shown that TXL can improve endothelial cell function [12], protect the brain against blood-brain barrier disruption [13], and reduce inflammation and apoptosis [14, 15]. However, it remains largely unclear whether there is a direct relationship between protective effect of TXL on endothelial functions and TXL-induced KLF4 phosphorylation and tight junction protein expression. In this study, we investigated whether and how TXL protected against hypoxia-induced tight junction disruption by regulating KLF4 expression and phosphorylation.

Material and methods

Preparation of TXL

TXL ultrafine powder was provided by the Yiling Pharmaceutic (Shijiazhuang, China). The herbal drug was authenticated and standardized to marker compounds according to the Chinese Pharmacopoeia 2005 [16]. The components of the TXL powder have been described previously [15]. TXL powder was weighed and dissolved in the phosphate-buffered saline. The ultrasound was used to promote the melting for about 1 h. The drug was then centrifuged at 1000 × g for 10 min, and the supernatant was put into the microfilter (0.22 μm) to eliminate bacteria, which was then aliquoted and stored at -20°C before use.

Animal models

Adult male C57BL/6J mice with a mean weight of 25 g were provided by the Experimental Animal Center of Hebei Medical University. All study procedures were approved by the local Animal Care and Use Committee of Hebei Medical University. Mice were randomly divided into one of the following 4 groups: control group, TXL-pretreated group, hypoxia group, and TXL-pretreated plushypoxia group (6 mice per group). The last 2 groups were placed in a hypobaric chamber (barometric pressure [PB] = 404 mm Hg, oxygen pressure [PO2] = 84 mm Hg) to simulate an altitude of 5000 m (6 h/d, 28 days). TXL (7.5 mg/10 g) was intragastrically administered 3 days before hypoxictreatment in the hypobaric chamber and continued for 28 days, until hypoxic treatment was finished. Mice were anesthetized with a pentobarbital injection, andthen brains were removed, frozen immediately in -40°C, and used to perform immunofluorescence staining and to isolate proteins.

Cell culture and treatment

Human cardiac microvascular endothelial cells (HCMECs) were cultured in endothelial cell medium with 10% fetal bovine serum and maintained in 5% CO_2 at 37°C in ahumidified atmosphere. All studies used cells from passages 3-6. For hypoxic treatment, cells were exposed to 95% nitrogen and 5% CO_2 at 37°C for 4 h in an incubator (Serico CB, Binder GmBH, Tultingen, Germany). Control cells were incubatedfor 4 h in 5% CO_2 and 21% O_2. Cells were transfected with either wild-type KLF4 or KLF4 with a single Ser-Ala point mutation at position 415, 444, or 470 as previously described [17], The different groups of cells were treated with or without TXL (600 μg/mL) for 24 h. For inhibitor studies, cells were pretreated for 1 h with Akt inhibitor LY294002 (Promega, Madison, WI) before the Cells were treated with TXL. Cells were harvested and lysed with lysis buffer containing 1% NP-40, 150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 10% glycerin, 1 mM Na_3VO_4, 1 mM PMSF (phenylmethylsulfonyl fluoride) and 1 mM DTT.

Immunofluorescence staining

Immunofluorescence staining of brain sections (20 μm in thickness) was performedas previously described [18]. In brief, the sections were pre-incubated with 10% normal goatserum (KPL, USA) and then incubated with primary antibodies anti-β-catenin (1:50, Abgent,

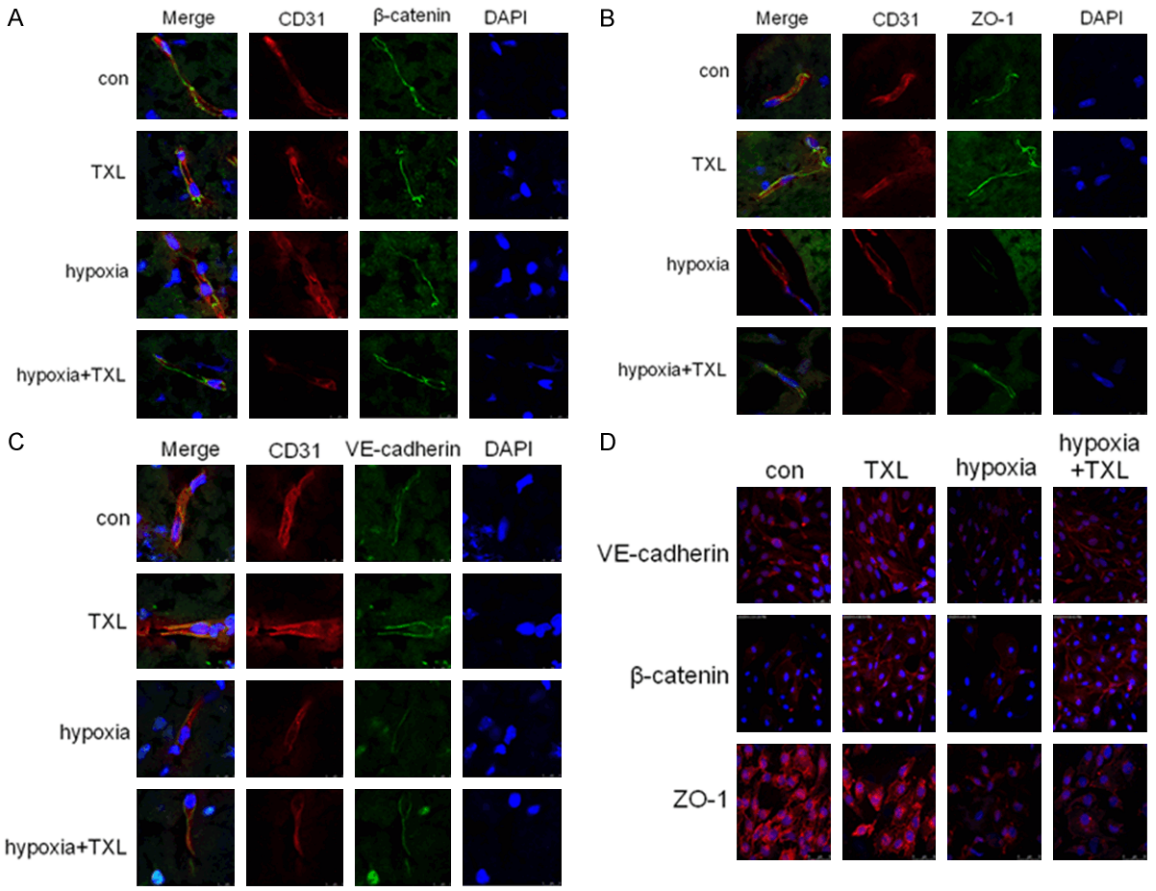

Figure 1. Hypoxia disrupted, while TXL treatment protected the TJs. Confocal images show CD31/β-catenin (A), CD31/ZO-1 (B) and CD31/VE-cadherin (C) double staining in control, TXL-, hypoxia-, and hypoxia+TXL-treated mice. Microvessels in the cortex of control and TXL-treated mice showed a continuous labeling of β-catenin, ZO-1 or VE-cadherin along the vessel. Tight junction proteins β-catenin, ZO-1 or VE-cadherin showed a discontinuous, less regular distribution in microvessels of hypoxia-treated mice. Scale bar, 10 μm. (D) Immunocytochemistry of β-catenin, ZO-1 or VE-cadherinin control, TXL-, hypoxia-, and hypoxia+TXL-treated HCMECs. β-catenin, ZO-1 and VE-cadherin were located mostly in the cytoplasmic membrane in control and TXL-treated cells, hypoxia reduced their distribution in the membrane. Scale bar, 50 μm.

USA), anti-ZO-1 (1:50, Proteintech, USA), anti-VE-cadherin (1:50, Abcam, USA), or anti-CD31 (1:50, Abcam, USA). Secondary antibodies were fluorescein-labeledantibody to rabbit IgG (1:1000, KPL, USA) and rhodamine-labeled antibody to mouse IgG (1:1000, KPL, USA). Images were captured by confocal microscopy (DM6000 CFS, Leica, Germany) and processed by LAS AF software. For cultured cells, the slides were incubated overnight with antibodies against β-catenin, VE-cadherin, or ZO-1 and subsequently incubated with the appropriate secondary antibodies. Sections were visualized with 3,3'-diaminobenzidine (DAB) and counterstained using hematoxylin. Brown and yellow coloring indicated positive stains.

Western blot analysis

Proteins isolated fromthe cultured HCMECs and brain tissues were separated by sodium dodecyl sulfate-polyacrylamidegel electrophoresis (SDS-PAGE), and transferred onto polyvinylidene difluoride membrane (Millipore). Membranes were blocked with 5% milk in Tris-HCl tween buffer solution for 2 h at 37°C and incubated overnight at 4°C with specific VE-cadherin (1:1000), beta-catenin (1:1000), ZO-1 (1:1000) and KLF4 (1:1000) antibodies. After incubation with appropriate secondary antibody, the membranes were developed with the Chemiluminescence Plus Western blot analysis kit (Millipore). For immunoblot analysis of phos-

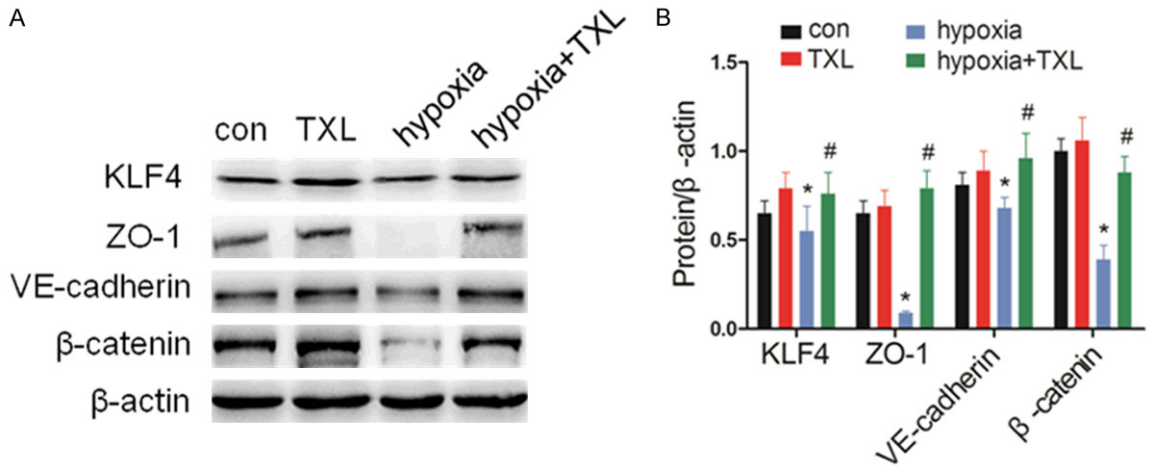

Figure 2. TXL promoted the expressions of VE-cadherin, β-catenin and ZO-1 in HCMECs. (A) HCMECs were cultured in endothelial cell medium under normoxia or hypoxia conditions and were treated with or without TXL (600 μg/mL) for 24 h. The expression of KLF4, VE-cadherin, β-catenin and ZO-1 was determined by Western blotting with their respective antibodies. (B) Densitometric scanning of the results shown in (A). Values are the mean ± SD from 3 independent experiments. *P<0.05, compared with control group; #P<0.05, compared with hypoxia group.

pho-KLF4, cell extracts were immunoprecipitated with anti-phosphoserine antibody and analyzed by Western blot for KLF4.

Statistics

All the experiments were repeated three times, and data were analyzed using the software SPSS 13.0 and expressed as means ± SD. Statistical comparison between different treatments was done by one-way ANOVA. *P* values below 0.05 were considered as a statistically significant difference.

Results

Hypoxia disrupted, while TXL treatment protected the TJs in the microvasculature of the mouse brain

To evaluate the effects of chronic hypoxia and TXL on TJs, we performed CD31/β-catenin, CD31/ZO-1 and CD31/VE-cadherin double staining. β-catenin, ZO-1 and VE-cadherin were continuously presented in the endothelial cell layer of mouse brain microvessels maintained in normoxia (**Figure 1A-C**. Microvessel walls showed significant disruption with discontinuous staining along the margins microvessel endothelial cell layer in chronic hypoxia-treated mice. In contrast, the microvessel walls of the TXL-treated group showed much smoother and more continuous labeling compared with those of mice maintained in normoxia, regardless of the presence or absence of hypoxia (**Figure 1A-C**). The fluorescence intensity of β-catenin, ZO-1 and VE-cadherin staining was increased in the hypoxia-treated mice after treatment with TXL. The TJs is a membrane-associated complex at theapico-lateral margin of epithelial cells. To gain additional support for the effects of hypoxia and TXL on TJs, we examined the levels of β-catenin, ZO-1 and VE-cadherin in the TJs of cultured HCMECs. As shown in **Figure 1D**, treatment with hypoxia caused a significant dissociation of β-catenin, ZO-1 and VE-cadherin from the TJs in cultured HCMECs, whereas such dissociation was attenuated in the TXL-treated cells, whose monolayer displayed intact and continuous TJs, and β-catenin, ZO-1 and VE-cadherin were located mostly in the TJs.

TXL promoted the expressions of TJ proteins VE-cadherin, β-catenin and ZO-1 in HCMECs under hypoxia conditions

Because the TJs is composed of multiple transmembrane proteins, including β-catenin, ZO-1 and VE-cadherin, we sought to know whether the protective effect of TXL on the TJs was related to the upregulation of β-catenin, ZO-1 and VE-cadherin expression by TXL. To do this, cultured HCMECs were pre-incubated with TXL,

and then cultured in a sealed canister for hypoxia. Western blot analysis shows that the expression of β-catenin, ZO-1 and VE-cadherin, especially ZO-1, was substantially decreased upon exposure to hypoxia (**Figure 2A**, lane 3 versus lane 1). Treatment of HCMECs with TXL significantly promoted the expression of all these three proteins (**Figure 2A**, lane 4 versus lane 3), suggesting that TXL protects against hypoxia-induced tight junction disruption through upregulating expression of β-catenin, ZO-1 and VE-cadherin. Notably, we also found that the zinc finger transcription factor Krüppel-like factor 4 (KLF4), which controls basic endothelial functions, was upregulated in TXL-treated HCMECs under hypoxia conditions. This result suggests that KLF4 mediates TXL-induced expression of β-catenin, ZO-1 and VE-cadherin in hypoxia-treated HCMECs.

TXL induced the expressions of β-catenin, VE-cadherin and ZO-1 via stimulating KLF4 phosphorylation

Because KLF4 activity can be regulated not only by *de novo* synthesis but also through posttranslational modifications including phosphorylation [17], we sought to determine whether KLF4-mediated expression of β-catenin, ZO-1 and VE-cadherin induced by TXL was responsible for KLF4 phosphorylation. Thus, we used antibody against phosphoserine to immunoprecipitate the lysates of hypoxia-induced HCMECs treated or not with TXL, and the immunoprecipitates pulled down with anti-phosphoserine antibody were detected by anti-KLF4 antibody. The results show a modestly higher level of KLF4 phosphorylation in hypoxia-treated HCMECs after treatment with TXL compared with hypoxia alone (**Figure 3A**, lane 4 versus lane 3), which was accompanied by an increase in all three proteins examined in this study (**Figure 3A**, lane 4 versus lane 3), indicating that TXL increases the expressions of VE-cadherin, β-catenin and ZO-1 via promoting KLF4 phosphorylation.

To further demonstrate that the phosphorylation of KLF4 is critical for the expression of three tight junction proteins, we mutated three phosphorylation sites (S470A, S444A and S415A), which are located within the domain of KLF4 interaction with other proteins, and used these phosphorylation-deficient mutants of KLF4 to transfect HCMECs and examined the effect of their mutations on expression of β-catenin, ZO-1 and VE-cadherin. As shown in **Figure 3B**, when the Ser444 and Ser415 were mutated toalanine, the expressions of β-catenin and ZO-1 were significantly reduced (**Figure 3B**, lanes 4 and 5 versus lanes 2 and 3), however, the mutation of phosphorylation site (Ser470) did not affect the expressions of β-catenin, ZO-1 and VE-cadherin (**Figure 3B**, lane 3 versus lanes 4 and 5). These results indicated that the phosphorylations of Ser444 and Ser415are important for β-catenin and ZO-1 expression. Further, when HCMECs transfected with phosphorylation-deficient mutants of KLF4 were exposed to hypoxia, compared with TXL-untreated cells, TXL treatment increased the expressions of VE-cadherin, β-catenin and ZO-1 in GFP-KLF4- and GFP-S470A-transfected cells (**Figure 3C**, lanes 2 and 3 versus lane 1). Notably, the mutations of Ser444 and Ser415 to alanine abrogated the upregulation of all three proteinsby TXL treatment (**Figure 3C**, lanes 4 and 5 versus lanes 2 and 3), again suggesting that the phosphorylations of Ser444 and Ser415 are required for TXL-induced expression of three tight junction proteins.

TXL induced KLF4 phosphorylation via AKT signaling

Because we have found that the activation of Akt signaling is responsible for KLF4 phosphorylation [17], we sought to examine whether hypoxia and TXL treatment might also affect Akt signaling. The results shown in **Figure 4A** demonstrate an obvious increase in the levels of p-Akt in cerebral tissue of hypoxia-treated mice after treatment with TXL compared with mice treated with hypoxia alone (**Figure 4A**, lanes 7 and 8 versus lanes 5 and 6), without significant change of Akt expression. Surprisingly, TXL treatment also activated Akt signaling to some extent in cerebral tissue of mice maintained under normoxia (**Figure 4A**, lanes 3 and 4 versus lanes 1 and 2). Next, we used the cultured HCMECs to assess the effect of TXL on the hypoxia-repressed Akt activation. The results showed that TXL treatment resulted inchanges similar to those seen in mouse brain tissue (**Figure 4B**). Further, we treated HCMECs with Akt inhibitor LY294002, and then examined the effect of TXL on KLF4 phosphorylation. Compared with HCMECs treated with TXL alone,

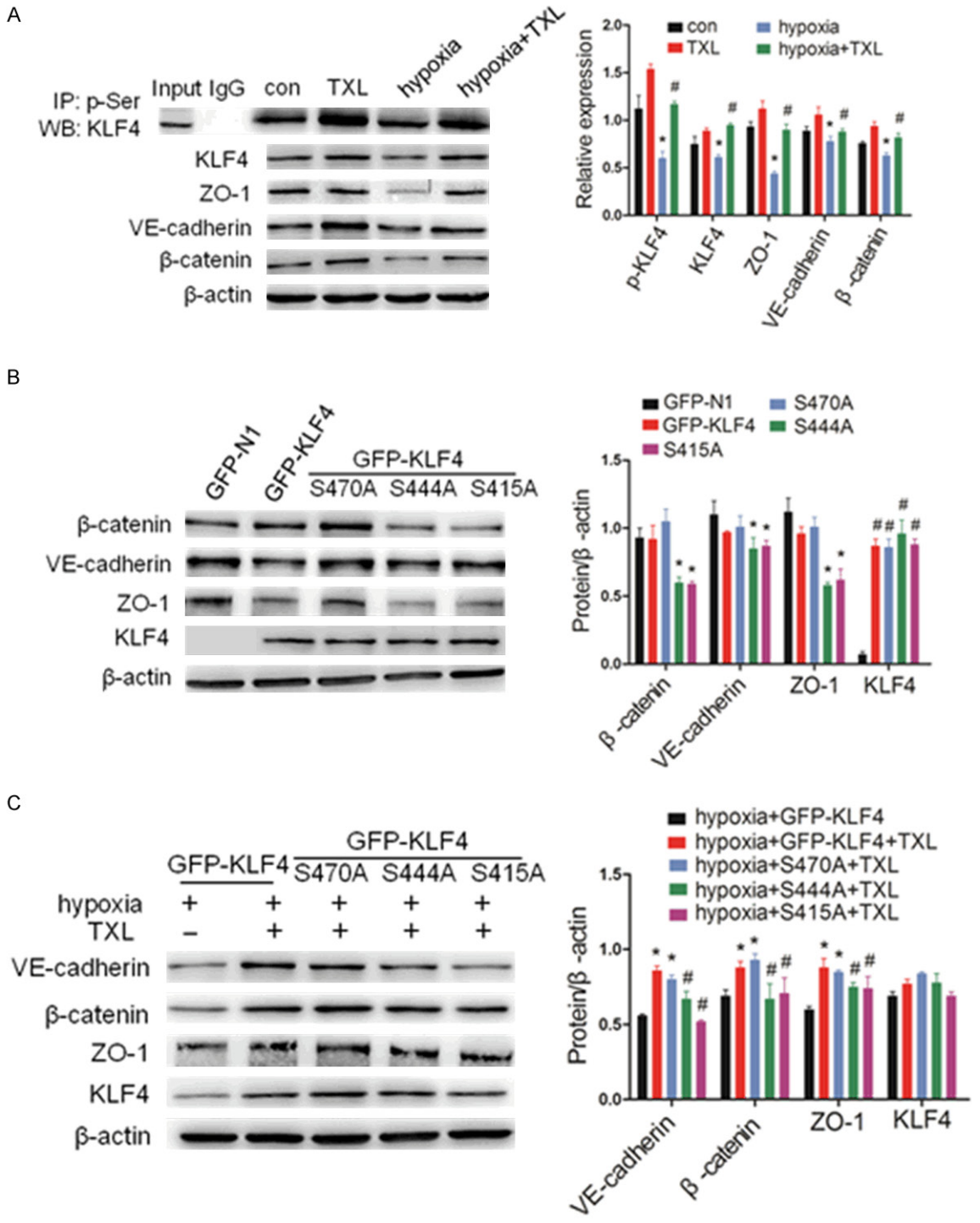

Figure 3. TXL induced the expressions of β-catenin, VE-cadherin and ZO-1 via stimulating KLF4 phosphorylation. A. HCMECs were cultured under normoxia or hypoxia conditions and were treated with or without TXL (600 μg/mL) for 24 h. Phospho-KLF4 was detected by immunoprecipitation with an anti-phosphoserine antibody and immunoblotting with the anti-KLF4 antibody. KLF4, VE-cadherin, β-catenin and ZO-1 were determined by Western blotting with their respective antibodies (left panel); densitometric scanning (right panel). Values are the means ± SD from three independent experiments. *P<0.05, compared with control group; #P<0.05, compared with hypoxia group. B. HCMECs were transfected with adenovirus to overexpress KLF4 and different phosphorylation-deficient mutants of KLF4. Western blotting was used to detect VE-cadherin, β-catenin, ZO-1 and KLF4 (left panel). Densitometric scanning (right panel). Values are the means ± SD from three independent experiments. *P<0.05, compared with GFP-KLF4 group; #P<0.05, compared with GFP-N1 group. C. HCMEC stransfected with different adenovirus as in B were

treated with or without TXL under hypoxia conditions, and VE-cadherin, β-catenin, ZO-1 and KLF4 were detected by Western blotting (left panel). Densitometric scanning (right panel). Values are the means ± SD from three independent experiments. *P<0.05, compared with TXL-untreated group; #P<0.05, compared with GFP-KLF4+TXL group.

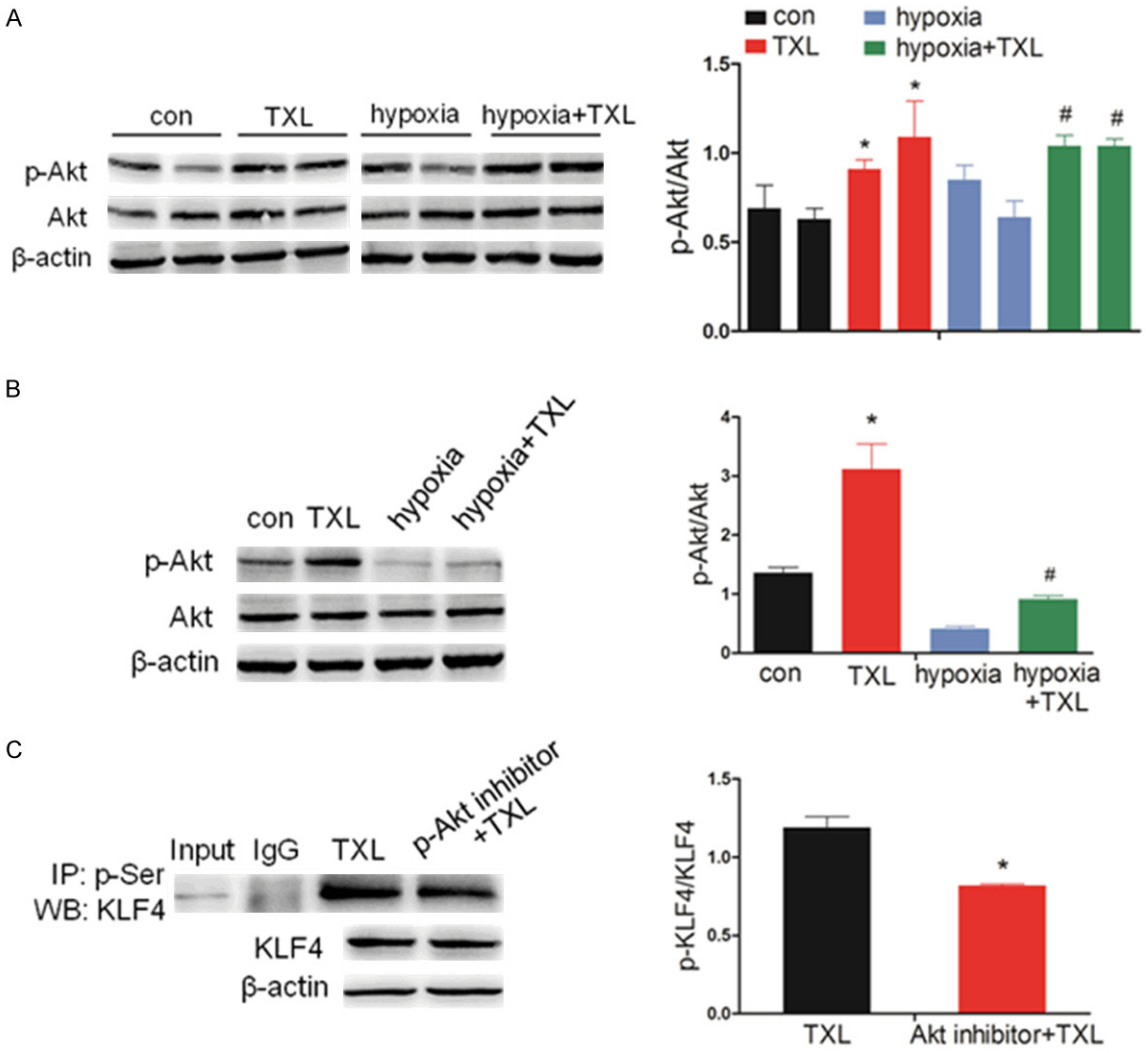

Figure 4. TXL induced KLF4 phosphorylation via AKT signaling. A. Phospho-Akt and total Akt in mouse brain tissues treated with or without hypoxia and TXL were determined by Western blotting (left panel). Densitometric scanning (right panel). Values are the means ± SD from three independent experiments. *P<0.05, compared with control group; #P<0.05, compared with hypoxia group. B. HCMECs were cultured under normoxia or hypoxia conditions and were treated with or without TXL (600 μg/mL) for 24 h. Phospho-Akt and total Akt were determined by Western blotting with their respective antibodies (left panel); densitometric scanning (right panel). Values are the means ± SD from three independent experiments. *P<0.05, compared with control group; #P<0.05, compared with hypoxia group. C. HCMECs were pretreated for 1 h with Akt inhibitor LY294002 and then treated with TXL (600 μg/mL) for 24 h. Phospho-KLF4 and total KLF4 were detected as described in **Figure 3A** (left panel); densitometric scanning (right panel). Values are the means ± SD from three independent experiments. *P<0.05, compared with TXL group.

pretreating cells with LY294002 reduced significantly KLF4 phosphorylation induced by TXL (**Figure 4C**, lane 2 versus lane 1). These findings suggest that TXL induces KLF4 phosphorylation byactivating Akt signaling.

Discussion

In the endothelium, junctional complexes comprise TJs, adherens junctions, and gap junctions [2]. TJs is essential structural components

of the endothelial barrier [2, 3]. It close the clefts between the neighboring endothelial cells and regulate the paracellular permeability of the endothelium to ions and macromolecules [2]. It is well known that endothelial TJs is composed of both transmembrane (occludin, claudins, etc.) and intracellular (ZO-1, ZO-2 and ZO-3) molecules [2, 19, 20]. Endothelial cells express celltype-specific transmembrane and intracellular proteins, suchas VE-cadherin at AJs [21] and claudin-5 at TJs [22]. Considerable evidence indicates that abnormal expression of tight junction proteins can disrupt the TJs affecting the barrier permeability of the endothelium. It has been demonstrated that several inflammatory cytokines can promote the expression of tight junction proteins. For example, interferon-λ reduces ZO-1 expression, causes redistribution of occludin and ZO-2 [23], and enhances permeability of the T84 epithelial cell line [24]. The combination of interferon-λ and tumor necrosis factor-α increases permeability of microvascular endothelial cells [25] and reduces the junctional distribution of JAM-Ain umbilical vein endothelial cells [26]. In contrast, other noninflammatory cytokines, such as epidermal growth factor, exert a positive effect on TJs [27].

In this study, we found that chronic hypoxia disrupted the TJs in the microvasculature through repressing the expression of β-catenin, ZO-1 and VE-cadherin, and increased the dissociation of β-catenin, ZO-1 and VE-cadherin from the TJs in cultured HCMECs. TXL treatment protected against hypoxia-induced tight junction disruption by inducing the expression of β-catenin, ZO-1 and VE-cadherin. It has been reported that KLF4 plays a critical role in the regulation of endothelial functions [10]. Our study shows that KLF4 was responsible for the expression of tight junction proteins induced by TXL treatment. TXL not only promoted KLF4 expression but also induced KLF4 phosphorylation in hypoxia-treated HCMECs. Analysis of phosphorylation-deficient mutants of KLF4 indicates that KLF4 mediated TXL-promoted expression of the tight junction proteins via the phosphorylations of at its sites Ser444 and Ser415. To define the signaling pathways leading to TXL-induced KLF4 phosphorylation, we examined the effects of Akt inhibitor on KLF4 phosphorylation induced by TXL. The results show that Akt inhibitor LY294002 blocked TXL-induced KLF4 phosphorylation and thereby abolished the upregulation of tight junction proteins by TXL, suggesting that Akt signaling plays a key role in TXL-induced KLF4 phosphorylation.

Our previous study has shown that TXL inhibited the vascular inflammatory response by decreasing inflammatory cytokine production and macrophage infiltration, and that TXL promoted phosphorylation in TNF-α-stimulated marrow-derived macrophages, and knockdown of Akt1 abrogated the TXL-induced suppression of miR-155 [14]. These findings suggest that TXL exerts its anti-inflammatory effects by suppressing miR-155 expression mediated by Akt1 and blocking the feedback loop between miR-155 and TNF-α. Previous studies also reported that TXL protected against palmitic acid-induced endothelial damage by initiating AMP-activated protein kinase-mediated activation of the thioredoxin antioxidant system [28]. TXL improved endothelial function in overfatigued rats through activating the JNK/c-Jun/heme oxygenase-1 pathway [29]. Moreover, TXL promoted endothelium-dependent vasodilation by upregulating eNOS expression mediated by the phosphatidylinositol 3-kinase/Akt signaling pathway [12]. Therefore, TXL-mediated vasoprotective effects may be via multiple different pathways. Protection against hypoxia-induced tight junction disruption is one of the mechanisms underlying the vasoprotective effects of TXL.

Acknowledgements

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Disclosure of conflict of interest

None.

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