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Fc-saxatilin inhibits VEGF-induced permeability by regulating claudin-5 expression in human brain microvascular endothelial cells

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ABSTRACT

The disruption of the blood–brain barrier influences the degree of brain damage and prognosis in cerebral ischemia or other brain diseases accompanied by inflammation. Vascular endothelial growth factor (VEGF) released during brain ischemia or inflammation has been implicated in the breakdown of the blood–brain barrier by increasing endothelial permeability. Saxatilin, a disintegrin-containing RGD motif, has been reported to disaggregate platelets via interactions with platelet integrins and to have a thrombolysis effect. Additionally, the Fc–saxatilin fusion protein reduces vascular leakage in cerebral ischemia in mice. In this study, we show that Fc–saxatilin prevents VEGF-induced permeability in human brain microvascular endothelial cells (HBMECs). The activation of Src and Fak, downstream signaling proteins of VEGF in the induction of endothelial permeability, was inhibited by Fc–saxatilin in HBMECs. The downregulation of a tight junction protein, claudin-5, at the protein and mRNA levels by VEGF was recovered by Fc–saxatilin. Our findings suggest that Fc–saxatilin attenuates VEGF-induced endothelial permeability via the regulation of downstream signaling, and this may contribute to its protective effect against vascular leakage in the ischemic brain.

1. Introduction

Saxatilin is an RGD (Arg-Gly-Asp) motif-containing disintegrin isolated from Korean snake (*Gloydius saxatilis*) venom (Hong et al., 2002). Due to its high affinity to the platelet integrin αIIb β3 (glycoprotein IIb/IIIa), saxatilin has been reported to have inhibitory effects on platelet activation and aggregation in vitro (Jang et al., 2007). Saxatilin also accelerates thrombolysis in a mouse model of carotid artery thrombosis (Kwon et al., 2013). Additionally, the Fc–saxatilin fusion protein has a protective effect against ischemia-induced vascular injury by interacting with integrin $\alpha_v\beta_3$ on endothelial cells (Choi et al., 2017).

The disruption of the blood–brain barrier (BBB) in focal cerebral ischemia is one of the most critical events contributing to cerebral ischemia/reperfusion injury. Vascular endothelial growth factor (VEGF) is involved in BBB leakage in the ischemic brain. VEGF is a mitogenic growth factor associated with angiogenesis and a potent inducer of endothelial permeability. The expression of VEGF is up-regulated in the brains of both transient and permanent middle cerebral artery occlusion models (Hayashi et al., 1997; Kovacs et al., 1996). While VEGF contributes to vascular leakage and lesions at an early stage of the ischemic brain, it ameliorates tissue damage by inducing angiogenesis in the penumbra region at a later stage (Zhang et al., 2000). Several studies have demonstrated that the antagonism of VEGF in the acute stage of stroke reduces tissue injuries by suppressing vascular permeability in the ischemic brain (van Bruggen et al., 1999), implicating VEGF in the pathogenesis of stroke.

The promotion of permeability by VEGF is initiated by binding to VEGF receptors and is mediated by several intermediate molecules, including Src and Fak. Src is phosphorylated in response to VEGF. Activated Src in turn regulates the expression or stability of junctional proteins. A deficiency of Src or the blockade of Src phosphorylation results in abnormal vascular permeability in response to VEGF (Eliceiri et al., 1999). Fak, a kinase activated by integrins, also mediates VEGF-induced vascular permeability (Chen et al., 2012). The genetic or pharmacological inhibition of Fak activity in endothelial cells blocks VEGF-induced permeability mediated by VEGF receptors and Src activation in vivo (Chen et al., 2012).

Claudin-5 is a tight junction protein and a major structural component of the BBB. This tight junction protein mediates the selective permeability of the BBB, and claudin-5 knockout mice show selective BBB opening (Nitta et al., 2003). Its expression is dynamically regulated by VEGF at the level of transcription in human brain endothelial cells

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(Argaw et al., 2009). The direct injection of VEGF in the mouse cerebral cortex downregulates claudin-5 and induces the breakdown of the BBB (Argaw et al., 2009). Interestingly, the inhibition of Src phosphorylation by a Src inhibitor, PP2, preserves claudin-5 expression and attenuates BBB disruption in mouse ischemic brains (Bai et al., 2014).

VEGF signaling is associated with integrin signaling initiated by interactions with the extracellular matrix. We thus investigated the effect of Fc–saxatilin, a disintegrin, on VEGF-induced permeability in brain endothelial cells. We evaluated whether the activation of Src and Fak kinases by VEGF is affected by Fc–saxatilin. Lastly, we explored the effect of Fc–saxatilin on VEGF-mediated claudin-5 disruption.

2. Materials and methods

2.1. Cell culture and reagents

Human brain microvascular endothelial cells (HBMECs) were purchased from Neuromics (HEC02; Minneapolis, MN, USA). HBMECs were maintained in ENDO-growth medium (MED001; Neuromics) or endothelial growth medium (EGM)-2 (Lonza, Walkersville, MD, USA) supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin at 37 °C in humidified incubators with 5% CO_2 and 95% room air. HBMECs and HUVECs were used between passages three and seven. HBMECs or HUVECs were treated by Fc-saxatilin diluted in PBS. VEGF was purchased from R&D systems biotechnology (Minneapolis, MN, USA). Human Immunoglobulin G4 (IgG4) Fc region was fused to N-terminal of Saxatilin (pYK603) and the fusion protein was expressed in CHO cells. Fc-Saxatilin was purified by protein A affinity column (Mabselect Sure resion, GE, Chicago, IL, USA) and anion exchange chromatography (AIEX; HiTrap Capto Q ImpRes, GE, Chicago, IL, USA).

2.2. Assay of endothelial cell monolayer permeability

The effect of hypoxia on endothelial monolayer permeability to fluorescein isothiocyanate-conjugated 70-kDa dextran (FITC-dextran; Sigma-Aldrich Chemicals, St. Louis, MO, USA) was assessed using Transwell permeable membranes (24-well cell culture inserts) with a 0.4-μm pore size (Costar, Corning, NY, USA). Cells (10^5) were seeded on gelatin-coated Transwell filters and allowed to grow for 5 days. The cells were incubated with Fc–saxatilin (50 ng/ml, 100 ng/ml or 300 ng/ml) or PBS only for 10 min before exposure to VEGF (100 ng/ml). PBS only treated cells served as control. The treated cells were then incubated for 24 h. After 24 h, FITC-dextran 70 (70 kDa, 1 mg/ml; Sigma) was added to the upper compartment. 25 μl medium were removed from every lower chamber of the transwell at 15 min later and diluted 1:20 with the medium. The diluted samples were transferred into a black 96-well plate (Costar, Corning, NY, USA) as triplicates and the fluorescence intensity of FITC-dextran was measured using a fluorescence plate reader (BioTek Inc., Winooski, VT, USA).

2.3. Western blot

Cells cultured in 60-mm plates were washed with ice-cold phosphate-buffered saline (PBS) and lysed in 200 μl of RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 1% sodium deoxycholate, 0.1% SDS, and 2 mM EDTA) containing proteinase inhibitors and phosphatase inhibitors (GenDepot, Kathy, TX, USA). The lysates were centrifuged at 16,000 × *g* (Hanil Biomed Inc., Gwangju, Republic of Korea) for 15 min. The cell lysates were analyzed by SDS-PAGE on 8% or 10% gels and transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore, Burlington, MA, USA). Immunoblotting was performed with anti-claudin-5 (Invitrogen Corp. Camarillo, CA, USA), anti-pFak, anti -Fak, anti-pSrc (Cell Signaling Technology Inc., Danvers, MA, USA), anti-Src, and anti-β-actin antibodies (Abs) (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

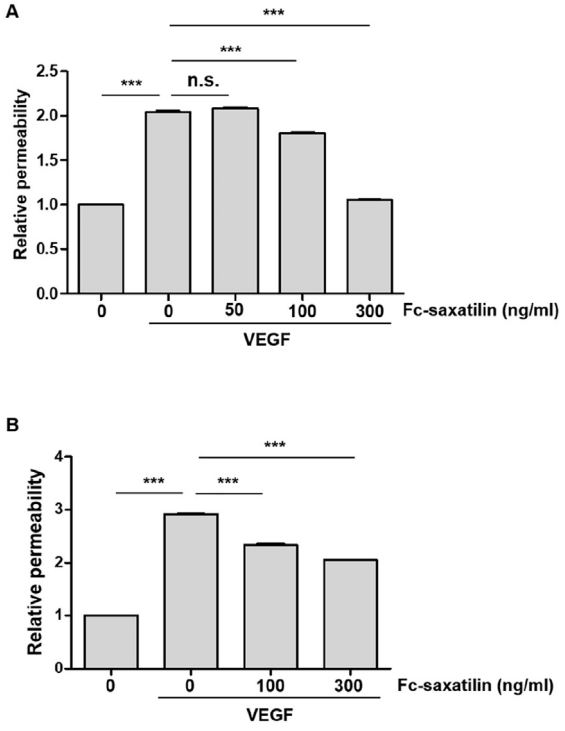

Fig. 1. Fc–saxatilin inhibits VEGF-induced endothelial permeability. A) The transwell cultured with human brain microvascular endothelial cells (HBMECs) were pretreated with vehicle (PBS, 0) only or Fc-saxatilin (50, 100, or 300 ng/ml) in the basal medium (0.5% FBS) for 10 min and then exposed to VEGF (100 ng/ml) for 24 h. Cells treated with only PBS served as a control. B) Human umblical vein endothelial cells (HUVECs) cultured in transwell were pretreated with PBS or Fc-saxatilin (100 or 300 ng/ml) for 10 min. Cells were then treated with VEGF (100 ng/ml) and incubated for 24 h. A Transwell permeability assay was performed as described in materials and methods, and the diffused fluorescent tracer was measured and normalized by the control. All experiments were assayed in triplicate and repeated three separate times with similar results. A representative of three experiments is shown ($n = 3$); $^{***}P < 0.0001$ compared to PBS only- or VEGF only-treated cells.

2.4. Immunostaining of claudin-5

Cells grown on gelatin-coated glass coverslips were washed once with Dulbecco's phosphate-buffered saline (DPBS) and fixed with 4% paraformaldehyde in PBS for 15 min at room temperature. The cells were then washed and permeabilized with 0.1% Triton X-100 in PBS for 12 min at 4 °C. Next, the cells were incubated with 3% BSA in PBS for 30 min at room temperature and with an anti-claudin-5 Ab (Invitrogen, Carlsbad, CA, USA) in PBS containing 3% BSA overnight at 4 °C. The cells were then washed three times for 5 min with DPBS and incubated with a 1:200 dilution of a FITC-conjugated secondary Ab in PBS containing 3% BSA for 1 h at room temperature. Cells were washed with DPBS once and then nuclei were stained with DAPI (1:5000). After three washes for 5 min with DPBS, the coverslips were drained and mounted in a mounting solution (Dako, Santa Clara, CA, USA).

2.5. Quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR)

Total RNA was isolated from HBMECs using the RNeasy Kit (Qiagen, Hilden, Germany). cDNA (20 μl) was synthesized from total RNA (1 μg) using the AccuPower RocketScript Cycle RT PreMix (Bioneer Corp., Daejeon, Republic of Korea). The abundance of transcripts in each cDNA sample was measured by real-time PCR with specific primers. The reactions contained PowerSYBR Green PCK Master Mix (Applied Biosystems, Foster City, CA, USA), 10 pmol both forward and reverse

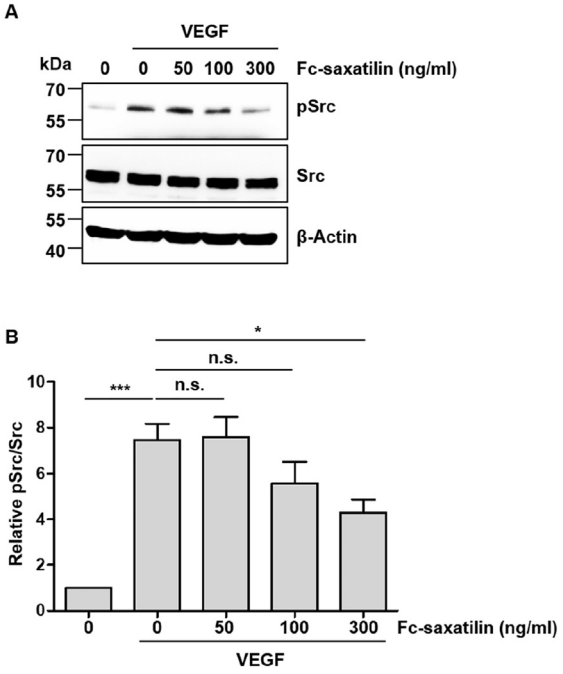

Fig. 2. Fc–saxatilin attenuates the phosphorylation of Src induced by VEGF in HBMECs. A) HBMECs were treated with pretreated with vehicle (PSB, 0) or Fc-saxatilin (50, 100, or 300 ng/ml) in the basal medium (0.5% FBS) for 10 min and treated with VEGF (100 ng/ml) for 1 h. Control cells were incubated in the medium without Fc-saxatilin and VEGF. Cell lysates were subjected to an immunoblot analysis with antibodies against either phospho-Src, Src, or actin, as indicated. Representative data from three separate experiments are shown. B) Bands in the immunoblots were quantified using ImageJ and normalized to actin ($n = 3$); $^{***}P < 0.0001$ compared to vehicle only-treated control, $^{*}P < 0.05$ compared to VEGF-treated control.

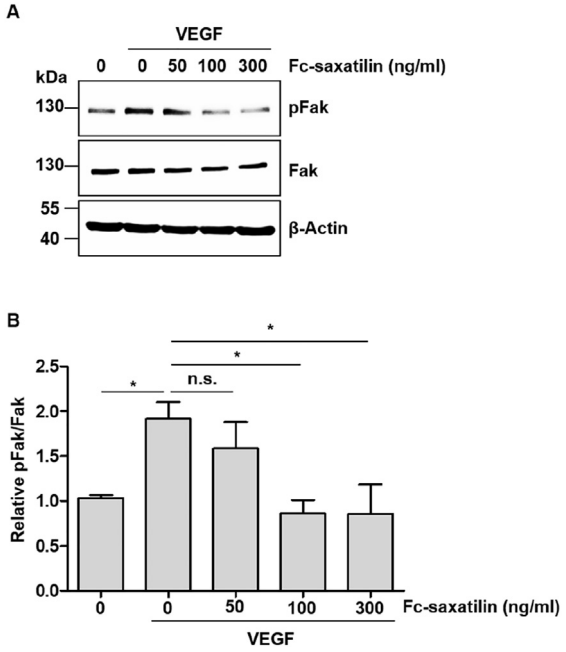

Fig. 3. Fc–saxatilin attenuates the phosphorylation of Fak induced by VEGF. A) HBMECs were treated with pretreated with vehicle (PBS, 0) or Fc-saxatilin (50, 100, or 300 ng/ml) in the basal medium (0.5% FBS) for 10 min and treated with VEGF (100 ng/ml) for 1 h. Control cells were incubated in the control medium without Fc-saxatilin and VEGF. Cell lysates were subjected to an immunoblot analysis with antibodies against either phospho-Fak, Fak, or actin, as indicated. Representative data from three separate experiments are shown. B) Bands in the immunoblots were quantified using ImageJ and normalized to actin (n = 3); $^{*}P < 0.05$ compared to the vehicle only-treated control or VEGF-treated control.

primers, and 1 μl of cDNA corresponding to 1 μg of total RNA. The reaction conditions included 50 cycles of PCR amplification (95 °C for 10 s, 55 °C for 15 s, and 72 °C for 20 s) using a StepOne Plus (Applied Biosystems) detection system. All results were normalized to *GAPDH* mRNA levels. The following primers were used: human claudin-5 forward, 5′-GTCCGCGAGTTTTACGACC-3′ and reverse 5′-GCTGAGTACTT CACGGGGAA-3′; human *GAPDH* forward, 5′-ACCCAGAAGACTGTGGA TGG-3′ and reverse, 5′-TCTAGACGGCAGGTCAGGTC-3′.

2.6. Statistical analysis

Data are expressed as means ± standard error of the mean (SEM). Statistical analyses were performed using GraphPad Prism 5.0 (GraphPad, La Jolla, CA, USA). Differences between two-groups were evaluated using two-tailed Student's *t*-test. For all statistical analysis, a *P*-value of < 0.05 was considered statistically significant.

3. Results

3.1. Fc–saxatilin prevents VEGF-induced permeability in human brain endothelial cells

To determine the effect of Fc–saxatilin on VEGF-induced endothelial permeability in the brain, we treated HBMECs with VEGF in the presence or absence of Fc–saxatilin. Intercellular permeability was measured by the leakage of FITC-dextran through the monolayer of HBMECs after treatment. VEGF significantly increased the passage of FITC-dextran across the endothelial monolayer, indicating its permeability-promoting effect (Fig. 1). Fc–saxatilin efficiently blocked the increase in endothelial permeability by VEGF in a dose-dependent manner (Fig. 1).

3.2. Fc–saxatilin inhibits VEGF downstream signaling in HBMECs

VEGF regulates vascular permeability via downstream signaling mediated by Src kinase. We thus assessed the effect of Fc–saxatilin on Src activation. Src phosphorylation increased in response to VEGF in HBMECs, consistent with the enhanced permeability (Fig. 2). Fc–saxatilin inhibited the phosphorylation of Src induced by VEGF in a dose-dependent manner. Another downstream signaling protein that could be activated by VEGF or integrin is focal adhesion kinase (Fak). The genetic or pharmacological inhibition of Fak in endothelial cells blocks VEGF-induced endothelial permeability and Src activation in vitro and in vivo (Chen et al., 2012). We evaluated the activation of Fak by detecting Fak phosphorylation in brain endothelial cells. VEGF induced Fak phosphorylation significantly (Fig. 3). This induction was blocked by Fc–saxatilin (Fig. 3). Taken together, these results suggest that Fc–saxatilin inhibits the downstream signaling important for VEGF-induced vascular permeability.

3.3. Fc-saxatilin reverses the claudin-5 reduction induced by VEGF in HBMECs

To determine whether the protective effect of Fc–saxatilin on VEGF-induced endothelial permeability is associated with changes in claudin-5, we analyzed cell lysates after the treatment of HBMECs with VEGF in the presence or absence of Fc–saxatilin. Claudin-5 was detected by western blotting as a band at around 22 kDa and was strongly down-regulated by VEGF (Fig. 4A and B). The reduction in the claudin-5 protein level was partially recovered in Fc–saxatilin-treated HBMECs (Fig. 4A and B). Another tight junction protein, occludin, was not significantly altered by VEGF in the absence or presence of Fc–saxatilin (Fig. 4A). Immunofluorescence staining of HBMECs with a claudin-5-

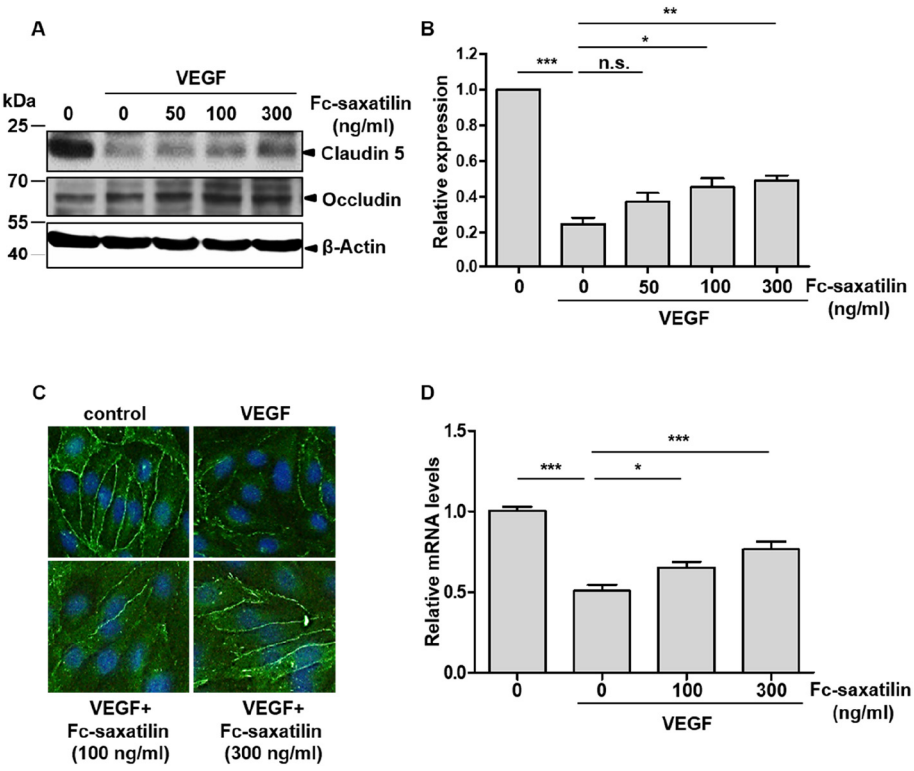

Fig. 4. Fc–saxatilin attenuates the reduction in claudin-5 expression induced by VEGF. A) HBMECs were treated with pretreated with vehicle (PBS, 0) or Fc-saxatilin (50, 100, or 300 ng/ml) in the basal medium (0.5% FBS) for 10 min and treated with VEGF (100 ng/ml) for 24 h. Control cells were incubated in the basal medium without Fc-saxatilin and VEGF. Cell lysates were subjected to an immunoblot analysis with antibodies against either claudin-5, occludin, or actin, as indicated. Representative data from three separate experiments are shown. B) Claudin-5 bands in the immunoblots from (A) were quantified using ImageJ and were normalized to actin ($n = 3$); $^{***}P < 0.0001$ compared to vehicle only-treated control; $^{*}P < 0.05$, $^{**}P < 0.01$ compared to VEGF-treated control. C) HBMECs were treated with VEGF (100 ng/ml) in the presence or absence of Fc–saxatilin (100 or 300 ng/ml) for 24 h. Cells were stained with an antibody (Ab) against claudin-5 (Green) and DAPI (blue; nucleus) and images were obtained under a fluorescence microscope (Olympus, X400). Claudin-5 is shown as a continuous line in the cell membrane (arrows). In VEGF-treated cells, the intensity of claudin-5 is severely reduced (Fc–saxatilin 0), and the intensity is recovered in the Fc–saxatilin groups (100 and 300 ng/ml). D) Total RNA was extracted HBMECs treated with VEGF (100 ng/ml) in the presence or absence of Fc–saxatilin. Relative levels of claudin-5 mRNA were measured by semi-quantitative RT-PCR ($n = 4$); $^{*}P < 0.05$, $^{**}P < 0.01$, or $^{***}P < 0.0001$ compared to vehicle only-treated control or VEGF-treated control. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

specific antibody showed the clear localization of claudin-5 to the plasma membrane in areas of cell–cell contact. The expression of claudin-5 at the membrane was disrupted by VEGF (Fig. 4C). The disruption of claudin-5 expression was recovered at the membrane region of cell–cell contact by Fc–saxatilin (Fig. 4C). To determine whether this change in claudin-5 expression was due to transcriptional regulation, we performed real-time PCR. Claudin-5 mRNA was down-regulated by VEGF and this downregulation was also inhibited by Fc–saxatilin (Fig. 4D). These results suggest that the regulation of claudin-5 expression might contribute to the recovery of VEGF-induced endothelial permeability by Fc–saxatilin.

4. Discussion

We showed that Fc–saxatilin prevents VEGF-induced permeability by blocking the VEGF-induced reduction of claudin-5 in HBMECs. The signaling mediators linking VEGF to vascular permeability were also inhibited by Fc–saxatilin. We previously showed that Fc–saxatilin blocks vascular leakage during ischemic stroke in mice. Our results provide an additional mechanism underlying the protective effect of Fc–saxatilin on the vascular barrier in the ischemic brain.

Disintegrins are family of small molecular weight proteins first identified from viper venom. These proteins contain RGD (Arg-Gly-Asp) or KGD (Lys-Gly-Asp) sequence motif that binds specifically to integrin receptors and act as integrin antagonists. Since integrins contribute to diverse physiological and pathological process by mediating cell-cell or cell-extracellular matrix interaction, disintegrins have been demonstrated to inhibit these processes such as platelets aggregation which is essential in thrombosis and haemostasis. In addition, due to the specific interaction with integrins, disintegrins could also interfere cell survival, proliferation, migration, invasion which are important processes in tumor development. Disintegrins thus have been exploited for the development of therapeutics for the prevention of thrombosis and cancer.

Fc–saxatilin, a disintegrin fusion protein binds to endothelial integrin $\alpha_v\beta_3$ with a high affinity and blocks signaling by interfering with the interaction between integrins and their extracellular matrix ligands (Choi et al., 2017). Integrin signaling exhibits cross-talk with VEGF signaling in cells (Byzova et al., 2000; Mahabeleshwar et al., 2007). In particular, integrin $\alpha_v\beta_3$ enhances VEGF signaling in endothelial cells attached to its ligand, vitronectin, and promotes angiogenic programming (Soldi et al., 1999). Integrin $\alpha_v\beta_3$ is up-regulated in the microvessels of ischemic brains with increased VEGF (Abumiya et al., 1999). The blockade of these integrins with RGD-containing peptides leads to decreased vascular leakage following middle cerebral artery occlusion in vivo (Shimamura et al., 2006). Consistent with these studies, the VEGF-induced increase in the permeability of HBMECs was reduced to the level in control cells by another integrin blocker, Fc–saxatilin (Fig. 1).

Src is a member of the non-receptor tyrosine kinase family. Src is a well-known downstream molecule linking VEGF signaling to vascular permeability (Eliceiri et al., 1999). Activated Src is involved in increasing vascular permeability by the modification of the actin cytoskeleton and VE-cadherin mediated cell-to-cell junctions (Hu et al., 2008; Mucha et al., 2003; Sun et al., 2012). The mechanism underlying the direct regulation of claudin-5 by Src has not been clearly defined. However, the phosphorylation of Src and downregulated expression of claudin-5 are well-correlated with VEGF-induced BBB breakdown in transient focal cerebral ischemia of rats (Bai et al., 2014). The activation of Src by VEGF leads to the phosphorylation and degradation of VE-cadherin, an adherent junction protein (Dejana et al., 2008; Weis et al., 2004). Interestingly, VE-cadherin phosphorylation has been linked to a reduction in the expression of claudin-5 in endothelial cells (Taddei et al., 2008). These findings suggest that VEGF may indirectly

regulate claudin-5 expression by controlling adherent junctions via Src. The effect of Fc–saxatilin on claudin-5 expression in HBMECs could be attributed to the inhibition of VEGF-Src-claudin-5 aixs.

Owing to the essential role of Src in VEGF-induced vascular permeability, its roles in the disruption of the BBB and brain damage in stroke have been investigated in several studies. In animal models of ischemic stroke, a genetic deficiency in Src reduces vascular permeability as well as neuronal damage after stroke (Paul et al., 2001). In this model, VEGF expression was correlated with vascular permeability. The Src kinase inhibitors PP1 or PP2 consistently reduce the infarct size of the ischemic brain and vascular permeability (Bai et al., 2014; Paul et al., 2001; Zan et al., 2014). The results of the present study suggest that Fc–saxatilin plays a role in reducing ischemia-induced vascular leakage by inhibiting VEGF-induced Src activation.

Fak mediates integrin signaling and is involved in the formation of a focal adhesion complex of endothelial cells on the matrix and cell migration. Fak phosphorylation is increased in response to VEGF stimuli via an undefined mechanism (Abedi and Zachary, 1997). Considering that Fc–saxatilin is a disintegrin with a high affinity to specific integrins and that Fak is a direct downstream protein activated by integrin ligation, it could be hypothesized that the blockade of integrins by Fc–saxatilin influences VEGF-induced Fak signaling in endothelial cells. Several studies have demonstrated the cross-talk between integrins and VEGF signaling regulates angiogenesis (Brakenhielm, 2007; Mahabeleshwar et al., 2007). Additionally, endothelial-specific *Fak* knockout mice have reduced VEGF-induced permeability and related downstream signaling (Chen et al., 2012). In line with this, dominant-negative Fak mutants exhibit the suppression of VEGF-stimulated permeability in vitro and in coronary vessels ex vivo (Wu et al., 2003). Our results reveal that Fc–saxatilin blocks VEGF-induced Fak activity and permeability, supporting the role of crosstalk between integrins and VEGF signaling in brain endothelial cells. Taken together, this study provides evidence for a possible mechanism in which Fc–saxatilin mitigates vascular leakage in the ischemic mouse brain by regulating integrin–Fak signaling.

In conclusion, our results showed that Fc–saxatilin blocks brain endothelial permeability induced by VEGF. Our findings indicated that Fc–saxatilin is a potential candidate for the prevention of vascular leakage in ischemic brains because VEGF is an important factor for the disruption of the BBB in pathological conditions, including stroke.

Acknowledgments

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Declaration of competing interest

The authors declare that they have no conflicts of interest.

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