Minoxidil sulfate induced the increase in blood–brain tumor barrier permeability through ROS/RhoA/PI3K/PKB signaling pathway

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Abstract
Adenosine 5'-triphosphate-sensitive potassium channel (KATP channel) activator, minoxidil sulfate (MS), can selectively increase the permeability of the blood–tumor barrier (BTB); however, the mechanism by which this occurs is still under investigation. Using a rat glioma (C6) model, we first examined the expression levels of occludin and claudin-5 at different time points after intracarotid infusion of MS (30 μg/kg/min) by western blotting. Compared to MS treatment for 0 min group, the protein expression levels of occludin and claudin-5 in brain tumor tissue of rats showed no changes within 1 h and began to decrease significantly after 2 h of MS infusion. Based on these findings, we then used an in vitro BTB model and selective inhibitors of diverse signaling pathways to investigate whether reactive oxygen species (ROS)/RhoA/PI3K/PKB pathway play a key role in the process of the increase of BTB permeability induced by MS. The inhibitor of ROS or RhoA or PI3K or PKB significantly attenuated the expression of tight junction (TJ) protein and the increase of the BTB permeability after 2 h of MS treatment. In addition, the significant increases in RhoA activity and PKB phosphorylation after MS administration were observed, which were partly inhibited by N-2-mercaptopropionyl glycine (MPG) or C3 exoenzyme or LY294002 pretreatment. The present study indicates that the activation of signaling cascades involving ROS/RhoA/PI3K/PKB in BTB was required for the increase of BTB permeability induced by MS. Taken together, all of these results suggested that MS might increase BTB permeability in a time-dependent manner by down-regulating TJ protein expression and this effect could be related to ROS/RhoA/PI3K/ PKB signal pathway.

1. Introduction
Brain glioma is one of the most common brain tumors in the central nervous system (CNS) and has the second highest mortality rate among brain tumors, which make it an important area of study (Drappatz et al., 2007; Hayashi et al., 2006). The blood–brain barrier (BBB) mainly consists of microvascular endothelial cells and overlying astrocytic foot processes, which regulates the entry of blood-borne molecules into brain, and preserves ionic homeostasis within the brain microenvironment (Svetlana et al., 2008). Tight junctions (TJ) are a critical constituent of BBB and it consists of dense TJ proteins connecting adjacent endothelial cells (Wolburg and Lippoldt, 2002). The existence of BBB may impede antitumor drugs entering the tumor tissue. Thus selective opening of the BBB is a crucial event needed to allow the entry of anti-cancer agents into the brain, which is a prerequisite for chemotherapy of brain tumors (Komarova and Malik, 2010). Ningaraj et al. demonstrated that minoxidil sulfate (MS), a KATP channel activator, could selectively increase the blood–brain tumor barrier (BBT) permeability without affecting the normal BBB (Ningaraj et al., 2003). However, the mechanisms responsible for this MS-induced effect are still unclear. Understanding the mechanism might prospectively contribute to an improved action of antitumor therapy.
permeability of BTB induced by MS is correlated with the transcellular pathway, which is supported by the accelerated formation of transport vesicles in both brain tumor capillary endothelium and tumor cells (Ningaraj et al., 2003). However, recent studies have demonstrated that accelerated formation of transport vesicles preceded the opening of TJ in the endothelium of lesion vessels (Nag et al., 2007; Zhong et al., 2008). Moreover, it is demonstrated that reactive oxygen species (ROS) could time-dependently regulate the expression and distribution of TJ protein and then induce the increase in the permeability of BBB (Schreibelt et al., 2007; Pun et al., 2009). ROS specifically activate the Protein kinase B (PKB) signaling pathway via RhoA/PI3K in brain microvascular endothelial cells. And the activation of the PKB pathway causes dissociation and subsequent disappearance of the TJ proteins (Schreibelt et al., 2007; Gu et al., 2012). In our previous study, we demonstrated that the ROS scavenger (MPG) greatly attenuated the increase of BTB permeability that was induced by MS (Gu et al., 2011). Therefore, we hypothesize that MS could also improve the paracellular transport pathway by regulating the expression of TJ proteins and this process is mediated by ROS/RhoA/PI3K/PKB signaling pathway.

To test the idea, we utilized in vivo and in vitro BTB model and evaluated the expression of TJ proteins, the changes of BTB permeability, ROS, RhoA activation and PKB phosphorylation in different time point groups.

2. Methods

2.1. Experiments in vivo

2.1.1. Animals implanted with rat brain glioma cells (C6 cells)

Adult male Wistar rats (body weight 180–220 g) were purchased from the Center for Experimental Animals of Shenyang Pharmaceutical University. All animal experiments were conducted in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals as well as the policies of our university authority. In brief, rats were anesthetized with 10% chloral hydrate (3.5 ml/kg, i.p.) and then they received an intracerebral injection of 1 × 10^6 C6 cells in 10 μl of medium using a Hamilton syringe. The coordinates were 1 mm anterior bregma, 3 mm lateral to the midline and 5.5 mm deep to the caudate nucleus. C6 cells were maintained in a monolayer culture in DMEM medium with 10% (vol/vol) fetal calf serum. Eighteen days after tumor implantation, the rats were prepared for the following experiments.

2.1.2. Treatment with minoxidil sulfate

Through a ventral neck midline incision, the common and external carotid arteries were isolated, ipsilateral to the tumor. Both the distal end of the external carotid artery and the proximal end of the common carotid artery were ligated. MS (Sigma–Aldrich) was pumped into the brain via the proximal end of the common carotid artery at a dosage of 30 mg/kg/min, following the previous studies (Ningaraj et al., 2003). We selected 0 min, 15 min, 30 min, 1 h, 2 h and 4 h after the start of MS infusion as the time points for the following investigations (Evans blue leakage and Western blots). Six rats from each group were used in the following experiments respectively.

2.1.3. Measurement of BTB permeability by Evans blue leakage

BTB permeability was quantitatively evaluated by extravasation of Evans blue (EB) as a marker of albumin extravasation. Briefly, 2% EB in saline (2 ml/kg) was injected intravenously (Fig. 1). Two hours after EB injection, rats in different time point groups were deeply anesthetized with chloral hydrate and transcardially perfused until colorless perfusion fluid was obtained from the right atrium. Brain tumor tissue were weighed and immersed in formaldehyde (1 ml/100 mg) at 60 °C for 24 h. The supernatant was obtained, and its optical density was determined with a spectrophotometer (at 620 nm). The quantitative calculation of the dye content in the brain was based on the external standards dissolved in the same solvent.

2.1.4. Fluorescence assays of Evans blue

Two hours after EB injection, rats for different time point groups were fixed by transcardiac perfusion with saline, followed by perfusion with 4% paraformaldehyde. The brain tumor tissues in different groups were cut in 10 μm section at −25 °C using frozen-section machine. The sections were stained with hematoxylin and eosin to confirm the presence of tumor. And then the images were analyzed using immunofluorescence microscopy (IX71, Olympus).

2.1.5. Western blot analysis

The effect of MS on occludin and claudin-5 was analyzed via Western blotting. Brain tumor tissue from different time point groups was removed, respectively. Protein homogenates of tumor samples were prepared by rapid homogenization in 10 volumes of lysis buffer. Samples were centrifuged at 17,000 g for 1 h. The protein concentration of soluble materials was determined by the BCA protein assay kit (Bicin-chonic Acid, Kangwei Biotechnology, Inc.). The Protein lysates (20 μg per lane for each sample) were fractioned and concentrated on 12% and 4% SDS-polyacrylamide gels, followed by transferring to nitrocellulose membranes (Santa Cruz Biotechnology, Inc.). The membranes were blocked in blocking buffer (5% non-fat dairy milk dissolved in Tween-Tris-buffered saline, TTBS) for 2 h at room temperature. The blots were then incubated with rabbit polyclonal antibody anti-occludin (Invitrogen, Life technology), anti-claudin-5 (Invitrogen, Life technology) and anti-β-actin (dilution 1:8000; Santa Cruz Biotechnology, Inc.) overnight at 4 °C. The occludin, claudin-5 bands and β-actin on these immunoblots were visualized using enhanced chemiluminescence (ECL kit, Santa Cruz Biotechnology, Inc.). And β-actin represents a control procedure. The occludin, claudin-5 and β-actin protein bands were scanned using Chemi Imager 5500 V2.03 software, and IDVs were calculated by Fluor Chen 2.0 software and normalized with that of β-actin.

2.2. Experiments in vitro

2.2.1. Establishment of BTB model in vitro

Primary rat brain microvascular endothelial cells (RBMECs) were isolated and cultured as described (Scott and Bicknell, 1993). We established the BTB model in vitro as described by Hurst and Fritz (1996). Initially, rat C6 glioma cells were plated onto the under-side of collagen-coated polytetrafluoroethylene polyester polystyrene transwell membrane (Costar) with suitable culture medium containing 10% cells. When C6 glioma cells were 80% confluent, RBMECs were plated on the upper side of the transwell membrane.

2.2.2. Drug treatment

To produce the specific inhibition of the activity of ROS/RhoA/PI3K/PKB, MPG, ROS inhibitor (10 μM, Sigma–Aldrich) or C3 exoenzyme, RhoA inhibitor (5 μg/ml, Sigma–Aldrich) or LY294002, PI3K inhibitor (2 μM, Sigma–Aldrich) or triciribine, PKB inhibitor (12.5 μM, Sigma–Aldrich) was added to the upper compartment of the Transwell for 2 h. And then MS (10 μM, Sigma–Aldrich) was added to upper compartment of the Transwell after the inhibitor was removed completely. The cultured cells were then rinsed with cold phosphate-buffered saline (PBS) at different time points after MS treatment. The RBMECs were used by the following experiments.

2.2.3. Measurement of transendothelial electric resistance

Transendothelial electrical resistance (TEER) of RBMECs monolayers cultured on Transwell filters was measured with a Millicell-electrical resistance instrument (Millicell-ERS; Millipore, USA). The final TEER values were calculated as ohms per square centimeter (Ω cm²) by the surface area of the Transwell insert.

![Fig. 1. Schematic overview. We selected 0 min, 15 min, 30 min, 1 h, 2 h and 4 h after the start of minoxidil sulfate (MS) infusion as the time points for the investigation. 2% Evans blue (EB) in saline (2 ml/kg) was injected intravenously. Two hours after EB injection, brain tumor tissue were extracted for the following experiments.](Image)
2.2.4. Horseradish peroxidase flux measurement
The RBMECs monolayers in Transwell inserts were co-cultured with C6 glioma cells. Horseradish peroxidase (HRP, 0.5 mM, Sigma-Aldrich) in serum-free DMEM was added into the upper compartment of the Transwell filters. And then the medium from the under-side chamber was collected and the content of HRP in the samples was evaluated by Multifunctional Microplate Reader (Varioskan Flash, Thermo Scientific). The HRP flux was expressed as picomoles passed per square centimeter surface area (Xie et al., 2011).

2.2.5. ROS measurements in BTB model in vitro
Confluent RBMECs co-cultured with C6 glioma cells were treated with MS for different time points (0 h–4 h). MS was diluted to the desired concentration (100 mM) using culture medium and then directly added to the wells. The generation of ROS was measured using Dihydroethidium (DHE, Vigorous Biotechnology, Beijing) as the fluorescent probe, which is oxidized into ethidium bromide in the presence of ROS. RBMECs were incubated with the dye for 30 min, and then replaced with PBS. Fluorescence was measured immediately at an excitation wavelength of 485 nm and an emission wavelength of 535 nm using a flow cytometry (FACSCalibur, BD, American), and data were acquired using control software.

2.2.6. Western blot analysis
The effect of specific inhibitor and MS on occludin, claudin-5, PKB protein and phosphorylation protein expression in RBMECs was analyzed via Western blotting. RBMECs from different groups were obtained, respectively. Protein homogenates of cells were prepared by rapid homogenization in 10 volumes of lysis buffer. Samples were centrifuged at 17,000 g for 1 h. The protein concentration of soluble materials was determined by the BCA protein assay kit (Bicin-choninic Acid, Kangwei Biotechnology, Inc). The Protein lysates (20 mg per lane for each sample) were fractioned on 4% and 12% SDS-polyacrylamide gels, followed by transferring to nitrocellulose membranes (Santa Cruz Biotechnology, Inc.). The Protein lysates were blocked in blocking buffer (5% non-fat dairy milk dissolved in Tween-Tris-buffered saline, TTBS) for 2 h at room temperature. The blots were then incubated with rabbit polyclonal antibody anti-occludin (Invitrogen, Life technology), anti-claudin-5 (Invitrogen, Life technology), anti-β-actin (dilution 1:8000; Santa Cruz

![Figure 2](image-url)

**Fig. 2.** The permeability of blood–brain tumor barrier (BTB) was assessed by Evans blue (EB) extravasation (µg/g brain tissue) in different groups (A). Data are presented as mean ± S.D. (n = 6, each). *P < 0.05, **P < 0.01 vs. minoxidil sulfate (MS) 0 min group. Immunofluorescence localization of EB in brain tumor vascular. B: MS 0 min group; C: MS 15 min group; D: MS 30 min group; E: MS 1 h group; F: MS 2 h group; G: MS 4 hr group.
Biotechnology, Inc.), anti-PKB and anti-phosphoserine-PKB (Cell Signaling Technology Inc., Danvers, MA, USA) overnight at 4 °C. The occludin, claudin-5, PKB and phosphoserine-PKB protein bands on these immunoblots were visualized using enhanced chemiluminescence (ECL kit, Santa Cruz Biotechnology, Inc.). The occludin, claudin-5, PKB, p-PKB and β-actin protein bands were scanned using ChemiImager 5500 V2.03 software, and IDVs were calculated by Fluor Chen 2.0 software and normalized with that of β-actin.

2.2.7. Immunofluorescence assays

The RBMEC monolayers grown on 24-well plate were permeabilized with 0.2% Triton X-100 in PBS on ice for two minutes, then fixed with 3% paraformaldehyde for 30 min and permeabilized with 0.05% Triton X-100 for 5 min. After blocking with 5% bovine serum albumin in PBS for 20 min, cells were incubated with primary antibodies against occludin (10 μg/ml) and claudin-5 (10 μg/ml) to assess the expression of occludin and claudin-5. The cells were incubated with rhodamine-labeled or FITC-donkey anti-rabbit for 1 h at room temperature (diluted 1:500, Invitrogen, Life technology). The images were analyzed using immunofluorescence microscopy (IX71, Olympus).

2.2.8. RhoA activation assay

Confluent RBMECs were lysed and then were collected for protein quantification. Equal amounts of protein were incubated with GST-rhotekin (Upstate Biotech, Waltham, MA, USA) at 4 °C for 1 h. Affinity-precipitated RhoA proteins were detected by Western blot analysis. And the lysates were blotted with anti-RhoA (diluted 1:500, Santa Cruz Biotechnology, Inc.) to determine total RhoA levels.

2.3. Statistical analysis

All data are presented as the mean ± SD. One-way analysis of variance (ANOVA) was used to compare the group differences in the measurements of protein expression levels. Dunnett’s post hoc tests were applied to compare specific group differences if the ANOVA revealed a significant difference. For other measurements, the data were analyzed by paired Student’s t test. \( P < 0.05 \) was considered statistically significant.

3. Results

3.1. MS increased the permeability of in vivo and in vitro BTB

In the present study, the permeability of BTB in vivo was evaluated by Evans blue leakage. The brain tissue with tumor was stained in blue, while non-tumoral brain tissues had no staining. Evans blue content in the brain tumor tissue was significantly increased in the MS group (15 min, 30 min, 1 h, 2 h, and 4 h) infusion compared with the control group (MS 0 min group). There are two peaks in MS 15 min and 2 h groups (Fig. 2A). As shown in Fig. 2B, there is strong red fluorescence surrounding the vascular of brain tumor in MS group.

In this study, the permeability of BTB in vitro was measured by TEER assays and HRP flux. MS time-dependently reduced TEER across a monolayer of RBMECs. The effects of MS on TEER and HRP flux were more significant in 10 μM, 50 μM and 100 μM groups than in the 1 μM group (Fig. 3A and B, \( \# P < 0.05 \)). MS at the dose of 10 μM was administrated in the following experiments. The value of TEER was significantly reduced at 2 h after MS treatment (Fig. 3C, **\( P < 0.01 \)). We further evaluated the paracellular permeability of the RBMECs monolayer toward HRP. Addition of MS to a RBMEC monolayer on Transwell filters greatly enhanced the leakage of HRP (Fig. 3D, **\( P < 0.01 \)) at 2 h after MS administration.
3.2. MS-induced a time-dependent increase in ROS production

In RBMECs co-cultured with C6 glioma cells, intracellular ROS levels were accessed using flow cytometry to measure the ROS-detecting fluorescent dye DHE. DHE is oxidized into quantifiable ethidium bromide when the presence of ROS. We demonstrated that MS induced a time-dependent increase in ROS formation in RBMECs. The level of fluorescent probe-DHE reached its maximum concentration after MS administration for 2 h (Fig. 4).

3.3. Effects of ROS/RhoA/PI3K/PKB on MS-induced BTB permeability

MS-induced increase in BTB permeability was reduced by pretreatment with the MPG (ROS inhibitor) or C3 exoenzyme (RhoA inhibitor) or LY294002 (PI3 kinase inhibitor) or triciribine (PKB inhibitor) for 2 h. The inhibition was dose-dependent, and reached a plateau at 10 μM (MPG), 5 μg/ml (C3 exoenzyme), 2 μM (LY294002), 12.5 μM (triciribine), respectively. In addition, we demonstrated that MPG or C3 exoenzyme or LY294002 or triciribine alone did not significantly alter baseline BTB permeability (data not shown). As shown in Fig. 5, the MS-induced increase in BTB permeability after treatment for 2 h was greatly attenuated by pretreatment with MPG or C3 exoenzyme or LY294002 or triciribine.

3.4. Inhibition of ROS/RhoA/PI3K/PKB prevented the MS-induced alteration of TJ proteins

We first examined the protein expression of claudin-5 and occludin in brain tumor tissue by western blot analysis. Compared to MS treatment for 0 min group, the protein expression levels of occludin and claudin-5 in brain tumor tissue of rats showed no changes within 1 h and began to decrease significantly after 2 h of MS infusion (Fig. 6A and C). Based on these findings, we then used an in vitro BTB model and selective inhibitors of diverse signaling pathways to investigate whether ROS/RhoA/PI3K/PKB pathway play a key role in the process of the increase of BTB permeability induced by MS. MPG or C3 exoenzyme or LY294002 or triciribine partially prevented the down-regulation of claudin-5 and occludin in RBMECs induced by MS treatment for 2 h (Fig. 6E). The IDV ratios of claudin-5 and occludin for western blot in different groups were shown in Fig. 6B, D, F and G.

The localization of claudin-5 and occludin were observed by immunofluorescence microscopy. In MS 0 min group, claudin-5 and occludin were localized to the cell–cell boundaries with continuous distribution (Fig. 7A and 8A). The expression of claudin-5 and occludin was intact and high on the cell surface. After MS administration for 2 h, the immunoreactivity of claudin-5 and occludin were attenuated, and they were discontinuously distributed in the cellular boundaries (Figs. 7B and 8B). And MPG or C3 exoenzyme or LY294002 or triciribine partially prevented the change of claudin-5 and occludin induced by MS treatment for 2 h (Figs. 7C–F and 8C–F).

3.5. Inhibition of ROS/RhoA/PI3K/PKB prevented the activation of RhoA and PKB phosphorylation induced by MS

As shown in Fig. 9A, the levels of RhoA activation in brain tumor tissue began to increase by 1 h and the highest level appeared at 2 h after MS treatment. And the pretreatment with MPG significantly inhibited the activation of RhoA induced by MS treatment for 2 h. MS significantly induced transient PKB phosphorylation at Ser-473, which is essential for PKB activation, without affecting total PKB levels. PKB phosphorylation was maximal at 2 h after MS treatment (Fig. 10A). However,
pretreatment of MPG or C3 exoenzyme or LY294002 partly prevented the phosphorylation of PKB induced by MS. The IDV ratios of PKB phosphorylation for western blot were shown in Fig. 10B.

4. Discussion

The use of MS as a K\textsubscript{ATP} channel activator in a rat glioma (C6) model has been shown to significantly increase the permeability of BTB (Ningaraj et al., 2003). In order to further understand the related mechanism, we extended the observation time for BTB permeability to 4 h post MS infusion. Our results demonstrated that MS significantly increased the permeability of the BTB in a rat brain glioma (C6) model starting at 15 min and lasting until 4 h post MS infusion. A significant reduction in TJ proteins occludin and claudin-5 was observed after MS administration for 2 h, which were partly prevented by specific inhibitors for ROS, RhoA, PI3 kinase and PKB. Our results therefore indicate that ROS/RhoA/PI3 kinase and PKB is involved in the increase of BTB permeability after MS treatment for 2 h.
Tight junctions are composed of complicated TJ proteins including transmembrane proteins (occludin and claudins), members of the peripheral membrane protein family (zonula occludens) and adhesion molecules, etc, all of which play an important role in regulating the opening of TJs (Gloor et al., 2001; Wolburg and Lippoldt, 2002). In vivo and in vitro studies have shown that TJ dysfunction leads to an increase of BBB permeability and vasogenic edema (Stamatovic et al., 2006). Whereas occludin was found to be involved in the formation of BBB function of the intercellular seal (Bamforth et al., 1999). Claudin-5 is critical to the formation of the TJ and is a major cell adhesion molecule found in TJs located mainly in brain endothelial cells. There is some evidence to suggest that it may be directly involved in the establishment and regulation of the BBB (András and Toborek, 2011). We found that MS had no effect on the protein expression levels of occludin and claudin-5 after MS treatment for 15 min, but that it significantly decreased the expression of the TJ proteins after MS administration for 2 h, indicating that MS-induced BTB permeability increase is also a time-dependent process. It was well known that the delivery pathways of antineoplastic drugs into brain tumors through the BTB can be classified as being either paracellular or transcellular (Komarova and Malik, 2010). Ningaraj et al. (2003) demonstrated that MS-induced BTB permeability increase without TJs opening was associated with the transcellular pathway at 15 min post MS infusion. In our previous study, we demonstrated that the infusion of MS for 15 min could up-regulate the expression of caverolin-1 and then increase the permeability of the BTB by augmenting the quantity of transendothelial pinocytotic vesicles (Gu et al., 2011). However, our results in the present study provide new evidence to support the idea that the paracellular pathway may play an important role in MS-induced BTB permeability increase at later time points (i.e. after 2 h of MS infusion).

Recently, potassium channels (K<sub>ATP</sub> channels) expressed in the mitochondrial membrane have become new research targets for MS-induced BTB permeability increase. It has been demonstrated that MS could regulate the function of K<sub>ATP</sub> channels in both sarcolemmal and mitochondrial membrane (Goñi-Allo et al., 2008). Activation of K<sub>ATP</sub> channels depolarized the mitochondrial membrane, which rapidly induced ROS production (Cao et al., 2007). Previous studies have also shown that oxidative stress can induce increases in BBB permeability by modulating the expression and distribution of TJ proteins (Schreibelt et al., 2007; Pun et al., 2009). We therefore hypothesized that MS might have an effect on ROS production, which could possibly be mediated by K<sub>ATP</sub> channels in the mitochondrial membrane, and accompanied by BTB opening. In the present study, it has shown that MS induced a time-dependent increase in ROS production and BTB permeability, which reached its maximum value at 2 h post MS incubation, in RBMECs co-cultured with C6 glioma cells. We demonstrated MPG as the ROS scavenger could significantly reduce the increase of BTB permeability induced by MS infusion. Moreover, MS-induced down-regulation of TJ-related proteins was also significantly abolished in the presence of MPG, suggesting that ROS could be a class of important signaling molecules involved in MS-induced BTB permeability increase.

ROS specifically activate the Protein kinase B (PKB) signaling pathway via RhoA, PI3K in brain microvascular endothelial cells (Schreibelt et al., 2007). PI3 kinase signal transduction pathway, has been suggested to be involved in the regulation of TJ (Massoumi
Fig. 9. Effects of Minoxidil sulfate (MS) on RhoA activation in rat brain microvascular endothelial cells (RBMECs) at different time points after treatment (A). Lane 1: MS 0 min group; lane 2: MS 15 min group; Lane 3: MS 30 min group; Lane 4: MS 1 h group; Lane 5: MS 2 h group; Lane 6: MS 4 h group; Lane 7: MPG + MS 2 h group. The Densitometric ratios of RhoA-GTP/Total RhoA are shown (B). Data are presented as mean ± S.D. (n = 6, each). *P < 0.05, **P < 0.01 vs. MS 0 h group. #P < 0.01 vs. MS 2 h group.

Fig. 8. Immunofluorescence localization of occludin protein in RBMECs after Minoxidil sulfate (MS) administration with/without specific inhibitors pretreatment. A: MS 0 min group; B: MS 2 h group; C: MPG + MS 2 h group; D: C3 exoenzyme + MS 2 h group; E: LY294002 + MS 2 h group; F: triciribine + MS 2 h group. And the reduction of occludin protein induced by MS was greatly inhibited by specific inhibitors pretreatment. Scale bar: 20 μm.

Fig. 10. Effects of specific inhibitors and Minoxidil sulfate (MS) on PKB phosphorylation in rat brain microvascular endothelial cells (RBMECs) at different time points after treatment. Lane 1: MS 0 min group; lane 2: MS 15 min group; Lane 3: MS 30 min group; Lane 4: MS 1 h group; Lane 5: MS 2 h group; Lane 6: MS 4 h group; Lane 7: MPG + MS 2 h group; Lane 8: C3 exoenzyme + MS 2 h group; Lane 9: LY294002 + MS 2 h group. The Densitometric ratios of PKB phosphorylation/Total PKB are shown (B). Data are presented as mean ± S.D. (n = 6, each). *P < 0.05, **P < 0.01 vs. MS 0 min group. ##P < 0.01 vs. MS 2 h group.

References

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and Sjolander, 1998; Wang et al., 2011). PI3K, as downstream target of RhoA, could phosphorylate many substrate proteins, such as PKB phosphatase. And it is generally believed that phosphorylation of PKB could induce the rapid internalization of TJ proteins, this could result in the disassembly of TJ (Ivanov et al., 2004). In the present study, we demonstrated that MS activated RhoA and the downstream target PKB of PI3 kinase in a transient manner in brain tumor tissue. Moreover, inhibition of PI3 kinase contributed to the disappearance of occludin and claudin-5, indicating that the PI3 kinase and PKB signaling pathway could play a key role in the increase of BTB permeability after MS treatment for 2 h. And the inhibition of RhoA activation prevented MS-induced down-regulation of occludin and claudin-5 protein. MPG could greatly attenuate the activation of RhoA. C3 exoenzyme or LY294002 or triciribine could significantly inhibit the increase in BTB permeability at 2 h post MS treatment. These data indicate that MS activates ROS, which time-dependently affect the tight junction via RhoA, PI3 kinase and PKB pathway. Due to the complexity of the BBB and the general limitation of in vitro experiments in the field, the in vivo study should be further investigated.

In conclusion, the mechanism by which MS induces BTB permeability changes is complex. Our results provide evidence that MS down-regulates the expression of TJ protein in a time-dependent manner through a ROS/RhoA/PI3K/PKB signaling pathway, and provide a new target for understanding the operative mechanisms surrounding MS-regulated BTB permeability and the delivery of molecules across brain tumor microvessels following biochemical modulation.

Disclosure

The authors confirm that there are no disclosures, no conflicts of interest and no financial supports to declare.

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