Salvianolic acid B improves the disruption of high glucose-mediated brain microvascular endothelial cells via the ROS/HIF-1 α /VEGF and miR-200b/VEGF signaling pathways

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Abstract

The study investigated the roles and mechanisms of Salvianolic acid B (Sal B) on permeability of rat brain microvascular endothelial cells (RBMECs) exposed to high glucose. The results demonstrated that Sal B greatly up-regulated the expression of tight junction (TJ) proteins and decreased the permeability of RBMECs compared with the control group. And the increase of reactive oxidative species (ROS) production, the upregulation of hypoxia-inducible factor-1 alpha (HIF-1 α) and vascular endothelial growth factor (VEGF) protein induced by high glucose were antagonized by Sal B. In addition, a great decrease of microRNA-200b (miR-200b) was observed in the RBMECs under high-glucose condition, which was significantly increased by Sal B pretreatment. And overexpression of miR-200b markedly attenuated the RBMECs permeability and inhibited the expression of VEGF protein by targeting with 3'-UTR of its mRNA. This led to the conclusion that Sal B-mediated improvement of blood-brain barrier dysfunction induced by high-glucose is related to the ROS/HIF-1 α /VEGF and miR-200b/VEGF signaling pathways.

Keywords: Tight junction proteins, Salvianolic acid B, High glucose, HIF-1 α , miR-200b

1. Introduction

Endothelial dysfunction has been implicated in the development of diabetic microvascular diseases. The blood-brain barrier (BBB) is mainly composed of the brain microvascular endothelial cells from the central nervous system (CNS), which tightly regulates the movement of cells, molecules, and chemical substances between

the blood and brain parenchyma [1]. It has been demonstrated that diabetes increased the risk of neurological dysfunction due to the considerable alteration of BBB permeability [2]. The drugs that attenuate the BBB disruption would tend to improve the neurological complication induced by diabetes [3]. The dried root of *Salvia miltiorrhiza* Bunge (Danshen) is a kind of traditional Chinese medicines and remains an extensive treatment for various diseases [4,5]. Salvianolic acid B (Sal B) is the most abundant and bioactive constituent of salvianolic acid in Danshen [6]. Fan et al. had demonstrated that Sal B could significantly attenuate the disruption of blood-spinal cord barrier (BSCB) permeability induced by spinal cord injury [7]. In addition, previous studies also indicated that Sal B decreased the high glucose-induced MMP-2 and MMP-9 secretion [8] and then improved the BBB dysfunction after cerebral ischemia-reperfusion in rats by inhibiting the MAPK signal pathway [9]. Sal A protected against vascular endothelial dysfunction in diabetes [10]. However, the effect and mechanism of Sal B on the permeability function of BBB exposed to high glucose still remains unclear.

It is well known that vascular endothelial growth factor (VEGF) causes the disruption of BBB, possibly via altering the expression and distribution of tight junction (TJ) proteins [11]. Recently, it was reported that high glucose induced the up-regulation of HIF-1 α expression in brain vascular endothelial cells and then HIF-1 α was involved in regulating the BBB permeability through inducing the expression of VEGF [12]. Sal B specifically inhibited the expression of HIF-1 α in oral squamous cell carcinoma [13]. Reactive oxidative species (ROS) influenced the expression and function of

HIF-1 α under normoxic conditions and Sal B inhibited the high glucose-induced oxidative stress, activation of the mitochondrial pathway and apoptosis in Schwann cells [14]. In addition, Sal B also repressed the expression of VEGF in TNF- α induced human umbilical vein endothelial cells [15]. Therefore, we hypothesize that Sal B could protect the permeability of brain endothelial cells exposed to high glucose through inhibiting the ROS/HIF-1 α /VEGF signaling pathway. In addition, high-glucose treatment significantly decreased the expression of miR-200b in the human retinal endothelial cells. MiR-200b mimic prevented diabetes-mediated upregulation of VEGF mRNA and protein [16]. Therefore, we also investigated that the effect of Sal B on miRNA-200b.

The purpose of the present study was to determine the effect and mechanism of Sal B on high glucose-induced BBB permeability. We provided a direct evidence that Sal B improved the brain endothelial dysfunction induced by high glucose.

2. Materials and methods

2.1. Establishment of BBB model *in vitro* and Drug treatments

Primary rat brain microvascular endothelial cells (RBMECs) were isolated and cultured as described [17]. And the cell cultures were maintained in DMEM with various glucose concentrations (5.5, 30 or 50 mM) for 1, 3, 6 or 10 days. Salvianolic acid B (purity 99%) was purchased from Sigma Aldrich. RBMECs were pretreated with a series of concentrations of Sal B (10, 20, 100 μ M) for 24 h and then stimulated with 5.5 or 30 mM glucose for 3 days.

2.2. Measurement of Transendothelial Electric Resistance

Transendothelial electrical resistance (TEER) of RBMEC monolayers cultured on 24-well Transwell filters was measured with a resistance meter (Millicell ERS-2, Millipore). The TEER values were calculated as ohms per square centimeter ($\Omega \cdot \text{cm}^2$, $R_{\text{cell monolayer}} = (R_{\text{sample}} - R_{\text{blank}}) \times 0.6$) by the surface area of the Transwell insert.

2.3. Horseradish Peroxidase Flux Measurement

The RBMECs monolayers in Transwell inserts were cultured and then transferred to a new 24-well plate. Horseradish peroxidase (HRP, 0.5 μM , Sigma-Aldrich) was added into the upper compartment of the Transwell system. And then the content of HRP in the samples from the lower chamber was measured [18].

2.4. ROS measurements in RBMECs

Confluent RBMECs were treated with glucose concentrations (30 mM) for 1, 3, 6 or 10 days. According to the instruction, fluorescence of Dihydroethidium (DHE, Vigorous Biotechnology, Beijing) was measured using a flow cytometry.

2.5. Cell Transfection of MicroRNA-200b

MiR-200b agomir or antagomir were synthesized by GenePharma based on mature miRNA sequences of Rno-miR-200b (MIMAT0000875). Cells were transfected using lipofectamine 3000 reagent (Life Technologies Corporation, USA). Transfection complexes were prepared according to the manufacturer's instructions.

2.6. Quantitative RT-PCR

Total RNA was extracted from cells with Trizol reagent (Life Technologies Corporation, USA). For measuring miR-200b, RNA samples were reverse transcribed

using Taqman MicroRNA Reverse Transcription Kit, and real-time PCR analysis was performed using Taqman Universal Master Mix II with the TaqMan MicroRNA Assay of miR-200 b and U6 (Life Technologies Corporation, USA). For quantification of the VEGF mRNA level, reverse transcription and real-time PCR amplification were performed using AccuPower 2X GreenstarTM qPCR Master Mix (vazyme). Relative expression values were calculated using the relative quantification ($2^{-\Delta\Delta Ct}$) method.

2.7. Western blots analysis

The RBMECs were homogenized in lysis buffer on ice. And then samples were centrifuged at 12000 g for 20 min. Protein lysates (20 µg per lane for each sample) were fractioned using the BoltTM Mini Gel Tank . And then we perform dry blotting of protein from mini gels (10%) with iBlot Gel Transfer Device for 7-9 min. The protein bands were scanned using Chemi Imager 5500 V2.03 software, and IDVs were calculated by Fluor Chen 2.0 software.

2.8. Immunofluorescence Assays

The RBMECs were fixed with 3% paraformaldehyde for 30 minutes, permeabilized with 0.2% Triton X-100 for three minutes and then were incubated with primary antibodies against Occludin (1:50) and ZO-1 (1:50) overnight at 4°C. with rhodamine-labeled or FITC-goat anti-rabbit (diluted 1:1000, Thermofisher Biotechnology) was treated for 1 hr at room temperature. The images were observed using immunofluorescence microscopy .

2.9. Luciferase Reporter Assay

The pmirGLO vectors (Promega, USA) constructed with either 3'-UTR fragments or

mutation of 3'-UTR fragments, and miRNAs were cotransfected into HEK 293T cells using Lipofectamine 3000. Luciferase activity was analyzed 48 hours after transfection using the Dual-Luciferase Reporter System (Promega, USA). The relative luciferase activity was presented as the ratio of firefly luciferase activity to renilla luciferase activity [19].

2.10. Statistical analysis

All values are evaluated as the mean $\pm$ SD. One-way analysis of variance (ANOVA) was performed to compare the differences among groups for all of data. $P < 0.05$ was considered significant difference.

3. Results

3.1. Effect of Sal B on the permeability of BBB induced by High glucose

The effects of high glucose on TEER and HRP flux were significant and time-dependent both in 30 μ M and 50 μ M groups (Fig. 1A and 1B). SalB (10, 20, 100 μ M) did not have a significant effect on TEER (Fig.1C-D). However, the effects of Sal B on HRP flux were more significant in 20 μ M and 100 μ M groups than in the 10 μ M when RBMECs were exposed to 30 mM glucose for 3 days (Fig.1F, ** $P < 0.01$). Sal B at the dose of 20 μ M was administrated in the following experiments for 3 days.

3.2. Sal B prevented the High glucose-Induced increase in ROS production

ROS levels in RBMECs were accessed using flow cytometry via evaluating the ROS-detecting fluorescent dye, DHE. Our results demonstrated that high glucose induced a time-dependent increase in ROS formation in RBMECs, which could be greatly attenuated by Sal B treatment for 3days (Fig. 2).

3.3. Sal B or HIF-1 α inhibitor or ROS inhibitor prevented the High glucose-Induced Alteration of Occludin and ZO-1

Western blot analysis showed that Sal B (20 μ M) partially prevented the down-regulation of Occludin and ZO-1 induced by high glucose treatment for 3 days (Fig. 3 A-C). In the control cells, Occludin and ZO-1 were localized to the cell–cell boundaries with continuous distribution (Figs. 3D-H). Fig 3E and 3H. showed much stronger staining of Occludin in the nucleus and the immunoreactivity of ZO-1 were attenuated in high glucose group and HIF-1 α inhibitor (2ME2) group. Sal B or 2ME2 or ROS inhibitor (N-2-mercaptopropionyl glycine, MPG) significantly reversed the distribution of Occludin and ZO-1 induced by high glucose. There is not significant difference in the expression of Occludin and ZO-1 between 5.5 mM group and Sal B+5.5 mM group (data not shown).

3.4. Sal B attenuated the up-regulation of HIF-1 α and VEGF induced by High glucose in brain endothelial cells

The levels of HIF-1 α was increased gradually after high glucose administration for 3 days, which was greatly inhibited by Sal B or ROS inhibitor. Increases in VEGF expression were observed in the cells exposed to 30 mM glucose for 3, 6 and 10 days. And Sal B treatment significantly reduced the expression of VEGF protein induced by high glucose. High glucose-induced the increase of VEGF could be also attenuated by HIF-1 α inhibitor and ROS inhibitor (Fig. 4).

3.5. Sal B attenuated VEGF mRNA induced by high glucose in brain endothelial cells by up-regulating the miR-200b expression

As shown in Figure 5A, the miR-200b expression of RBMECs was lower in high glucose group than that in control group. the increase of BBB permeability induced by high glucose was significantly attenuated by antagomiR-200b. Mechanistic investigations defined VEGF as a direct and functional downstream target of miR-200b (Fig. 5D).The level of VEGF mRNA and protein was reduced or increased by overexpression or inhibition of miR-200b, respectively.

4. Discussion

Sal B is one of the most bioactive components of *Salvia miltiorrhiza*, a traditional Chinese herbal medicine that has been commonly used for prevention and treatment of cerebrovascular disorders. In order to further understand the related mechanism of Sal B on the permeability function of RBMECs exposed to high glucose condition, we investigated the effect of Sal B on the BBB dysfunction induced by high glucose. Our results demonstrated that Sal B could significantly inhibit the increase of BBB permeability induced by high glucose. And a significant reduction in TJ proteins (Occludin and ZO-1) induced by high glucose administration were observed, which were partly increased by Sal B. Moreover, the significant increases in ROS, HIF-1 α and VEGF induced by high glucose administration were also demonstrated, which was also inhibited by Sal B. Our results suggest for the first time that the protective effects of Sal B on high glucose-induced BBB dysfunction are mediated by inhibiting the ROS/HIF-1 α signaling pathway.

BBB represents a tight barrier between circulating blood and the central nerves system (CNS) and it consists of dense TJs. It is well-known that the integrity of TJs is

mainly responsible for the brain endothelial permeability [20]. TJs are composed of complicated TJ proteins including transmembrane proteins, members of the peripheral membrane protein family and adhesion molecules [21]. In the present study, it was found that there was a decrease in the expression of TJ-associated proteins Occludin and ZO-1 in RBMECs after high glucose treatment for 3 days, which were correspondence with the previous study [11]. And Sal B could significantly inhibit the down-regulation and distribution of TJ-associated proteins induced by high glucose. However, the mechanism of Sal B on permeability function of RBMECs exposed to high glucose remains largely unknown.

Previous studies *in vitro* and *in vivo* have shown that high glucose induces upregulation of HIF-1 α expression in brain vascular endothelial cells [11]. It is well known that HIF-1 α protein is rapidly degraded under normoxia and that the degradation pathway includes HIF-1 α hydroxylation by prolyl hydroxylases (PHDs), binding with von Hippel-Lindau tumor suppressor and degradation by the ubiquitin-proteasome pathway [22,23]. It was reported that ROS stabilize the HIF-1 α in cancer cells by inhibiting the activity of PHDs. The previous results have demonstrated that Sal B showed a protective role against the ischemia-reperfusion injury in rat brain by scavenging free radicals and improving the energy metabolism [24]. Sal B inhibited the high glucose-induced oxidative stress, activation of the mitochondrial pathway and apoptosis in Schwann cells [13]. In the present study, the results demonstrated that high glucose induced a time-dependent increase in ROS formation in RBMECs, which could be greatly attenuated by Sal B. In addition, ROS

inhibitor, MPG, could greatly attenuate the expression of HIF-1 α induced by high glucose. Moreover, Sal B could inhibit the increase of ROS and HIF-1 α in RBMECs after high glucose administration for 3 days. HIF-1 α induces the expression of VEGF under hypoxic condition, which is involved in paracellular permeability of brain endothelial cells. It is well established that VEGF is a key regulator of BBB permeability and could alter the expression and arrangement of TJ proteins [25]. Our results have shown that high glucose-induced the increase of VEGF could be attenuated by Sal B or HIF-1 α inhibitor (2ME2) or ROS inhibitor (MPG), suggesting that Sal B up-regulates the expression of TJ protein through the ROS/HIF-1 α /VEGF signaling pathway.

MicroRNAs are endogenous regulators produced as small, nonprotein-coding RNAs with 21-25nt, negatively regulate gene expression at the post-transcriptional level by targeting with their 3'-UTR of mRNA and have an important modulatory role in diabetes [26]. Transfection of endothelial cells with agomiR-200b prevented diabetes-induced increased VEGF expression and high glucose-induced increased permeability and angiogenesis [16]. In the present study, we demonstrated that high glucose could reduce the expression of miR-200b and increase the expression of VEGF mRNA and protein. And transfection of RBMECs with agomiR-200b normalized high glucose-induced increase of VEGF mRNA and protein. Sal B greatly induced the expression of miR-200b in RBMECs under high-glucose treatment, suggesting that Sal B could inhibit the expression of VEGF via inducing the miR-200. The mechanism by which Sal B improves the BBB dysfunction induced by high

glucose is complex. Our results provide a experimental evidence that Sal B up-regulates the expression of TJ protein through the ROS/HIF-1 α /VEGF pathway and miR-200b/VEGF signaling pathway, and Sal B may be a novel and preventive agent for human diabetic brain microvascular diseases in the future.

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Conflict of interest

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

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Fig. 1. The permeability of blood-brain barrier (BBB) *in vitro* was assessed by transendothelial electric resistance (TEER) and horseradish peroxidase (HRP) flux in different groups. Time course of high glucose-induced changes in TEER (A) and HRP flux (B) of BBB *in vitro*. At different time-point group, RBMECs were treated with normal glucose (5 mM) or high glucose (30 mM or 50 mM) administration for 1 day, 3 days, 6 days, 10 days, respectively. Data are presented as mean $\pm$ S.D. ($n=6$, each). $**P<0.01$ vs. 5.5 mM group. The effects of Sal B (10, 20, 100 μ M) on TEER (C and D) and HRP flux (E and F) in RBMECs with 5.5 mM or 30 mM. Data are presented as mean $\pm$ S.D. ($n=6$, each). $**P<0.01$ vs. 30 mM group.

Fig. 2. High glucose (30 mM) induced a time-dependent increase in reactive oxygen species (ROS) production as determined by Dihydroethidium (DHE) fluorescence measurements in rat brain microvascular endothelial cells (A) (RBMECs) ($n=6$). (B) The effect of Sal B (20 μ M) on ROS production induced by high glucose (30 mM) for 3 days. Changes of DHE fluorescence in different group were shown. Data are presented as mean $\pm$ S.D. ($n=6$, each). $**P < 0.01$ vs. 0 day group; $^{##}P < 0.01$ vs. 30 mM group.

Fig. 3. Effects of Sal B or inhibitors on tight junction protein expression in rat brain microvascular endothelial cells (RBMECs) after high glucose (30 mM) administration for 3 days (A). 2-methoxyestradiol(2ME), HIF-1 α inhibitor. N-2-mercaptopropionyl glycine (MPG), Lane 1: 5.5 mM group; lane 2: 30 mM group; Lane 3: SalB+30mM group; Lane 4: 2ME+30 mM group; Lane 5: MPG+30mM group; Changes of relative integrated density value (IDV) of occludin (B) and ZO-1 (C) protein ($n=6$, each). $**P < 0.01$ vs. 5.5 mM group, $^{##}P < 0.01$ vs. 30 mM group. Immunofluorescence localization of Occludin and ZO-1 protein in rat brain microvascular endothelial cells (RBMECs) after high glucose (30 mM) administration with/without specific inhibitors treatment. D: 5.5 mM group; E: 30 mM group; F: SalB+30mM group; G: 2ME+30 mM group; H: MPG+30mM group; Scale bar: 20 μ m.

Fig. 4. High glucose (30 mM) induced a time-dependent increase in HIF-1 α and VEGF protein expression in rat brain microvascular endothelial cells (RBMECs). (A) Lane 1: 1 day group; Lane 2: 3 days group; Lane 3: 6 days group; Lane 4: 10 days group. Representative Western blots illustrating differences in the bands of HIF-1 α and VEGF protein. Changes of relative integrated density value (IDV) of HIF-1 α (B) and VEGF (C) protein ($n=6$, each). ** $P < 0.01$ vs. 1 day group. Effects of Sal B or inhibitors on HIF-1 α and VEGF protein expression in RBMECs after high glucose (30 mM) administration for 3 days (D). Lane 1: 5.5 mM group; lane 2: 30 mM group; Lane 3: SalB+30mM group; Lane 4: 2ME+30 mM group; Lane 5: MPG+30mM group. Changes of relative integrated density value (IDV) of HIF-1 α (E) and VEGF (F) protein ($n=6$, each). ** $P < 0.01$ vs. 5.5 mM group, ### $P < 0.01$ vs. 30 mM group.

Fig. 5. Effects of high glucose-induced miR-200b downregulation in the rat brain microvascular endothelial cells (RBMECs). A: The expression of miR-200b in different group. ** $P < 0.01$ vs. 5.5 mM group, # $P < 0.05$ vs. 30 mM group. B. The expression of VEGF mRNA in different group. C. The TEER in BBB in different group. * $P < 0.05$ vs. MiR-200b (+)-NC group, # $P < 0.05$ vs. MiR-200b (-)-NC group. ## $P < 0.05$ vs. MiR-200b (-) group. D. MiRNA-200b inhibits the expression of VEGF by targeting its 3'-untranslated region. E. The expression of VEGF protein in different group and Changes of relative integrated density value (F). Data represent means $\pm$ sd ($n=5$, each). ** $P < 0.01$ versus MiR-200b(+)-NC group, ### $P < 0.01$ vs. MiR-200b(-)-NC group.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5