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Bradykinin increased the permeability of BTB via NOS/NO/ZONAB-mediating down-regulation of claudin-5 and occludin

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ABSTRACT

After demonstrating bradykinin (BK) could increase the permeability of blood-tumor barrier (BTB) via opening the tight junction (TJ), and that the possible mechanism is unclear, we demonstrated that BK could increase the expressions of eNOS and nNOS and promote ZONAB translocation into nucleus. NOS inhibitors L-NAME and 7-NI could effectively block the effect of BK on increasing BTB permeability, decreasing the expressions of claudin-5 and occludin and promoting the translocation of ZONAB. Overexpression of ZONAB could significantly enhance BK-mediating BTB permeability. Meanwhile, chromatin immunoprecipitation verified ZONAB interacted with the promoter of claudin-5 and occludin respectively. This study indicated NOS/NO/ZONAB pathway might be involved in BK's increasing the permeability of BTB.

1. Introduction

Glioma is the most common tumor in central nervous system (CNS) in adults [1]. Treatments for glioma include surgical resection followed by radiotherapy and chemotherapy which is assuming an increasingly important role in the treatment [2]. However, it has severely affected the chemotherapeutic effect that blood-tumor barrier (BTB) has restricted the anti-tumor drug from entering the CNS [3]. Studies demonstrated small dose of bradykinin (BK) can selectively open BTB without affecting the permeability of normal brain tissue [4]. Studies indicated BK could increase the permeability of BTB by transcellular and paracellular pathways (opening the tight junction (TJ)) [5,6]. However, the precise molecular mechanisms are not illustrated. Most drugs are transported transcellularly depending on their physicochemical properties; however, the paracellular pathway is usually the main way to absorb hydrophilic drugs [7]. Therefore, we will further study the molecular mechanism of BK increasing the BTB permeability via opening the TJ.

It is known that Nitric oxide (NO) synthesis results from the oxidation of L-arginineby a family of NO synthase (NOS). NOS and NO play an important role in increasing the permeability of blood–brain barrier (BBB) [8]. Our research reported that BK could up-regulate the expressions of eNOS and nNOS in the RG2 rat glioma model [9]. Nakano et al. found the permeability of BTB was increased by BK infusion, which was mediated by NO [10]. However, it is unclear whether BK can open the TJ through NOS/NO signaling pathway.

ZO-1 associated nucleic acid binding proteins (ZONAB) is one of ZO-1 related Y-box transcription factors. With transcriptional repression function, it lies in the nucleus and the TJ of the endothelial cells and epithelial cells [11]. It can regulate the proliferation and differentiation of epithelial cells [12]. Research revealed overexpression of ZONAB could regulate the paracellular permeability of epithelial cells [13], but the mechanism is unclear. Estradiol could decrease the expression of epidermal maker occludin and CRB3, which is correlated with the ZONAB nuclear translocation [14]. However we still have no idea whether ZONAB is involved in regulating the permeability of BTB.

The aim of this study is to address whether BK can open the TJ through NOS/NO signaling pathway, and further elucidate whether ZONAB is involved in this process by mediating the expression of TJ associated proteins claudin-5 and occludin.

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2. Materials and methods

2.1. Experiments in vivo

2.1.1. Establishment of rat brain C6 tumor model

The adult female Wistar rats (180–200 g) were provided by the Experimental Animals Center of China Medical University. All animal experiments were carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals. Rat brain C6 tumor models were prepared according to the previously described procedure [6]. Fourteen days after the tumor implantation, the rats were prepared for agents infusion.

2.1.2. Agents administering and experimental groups

BK (Sigma–Aldrich, Inc) was was pumped into brain via the proximal end of common carotid artery at a speed of 53.5 μl/min (10 μg/kg/min), which was established in previous studies [6]. We selected 0, 5, 10, 15, 30 and 60 min after the start of BK infusion as the time point for our investigation. Rats were divided into 6 groups randomly (n = 8/group): control group (infusion of saline for 15 min), BK 5 min group, BK 10 min group, BK 15 min group, BK 30 min group and BK 60 min group.

In order to test the effect of NOS/NO on BK increasing the BTB permeability, the inhibitors of NOS were administered before BK. Nw-nitro-L-arginine methyl ester (L-NAME, 100 mg/kg, ip) was the non-selective NOS inhibitor, 7-nitroindazole (7-NI, 50 mg/kg, ip) and aminoguanidine (AG, 50 mg/kg ip) were the selective inhibitors of nNOS and iNOS, respectively. They were given 30 min prior to BK infusion. Rats were divided into 5 groups randomly (n = 8/group): control group (infusion of saline for 15 min), BK group, BK + L-NAME group, BK + 7-NI group and BK + AG group.

2.1.3. Measurement of BTB permeability by Evans blue (EB)

The BTB permeability was quantitatively evaluated by extravasation of Evans blue (EB) as a marker of albumin extravasation [6]. Briefly, 2% EB in saline (2 mg/kg) was injected intravenously. Two hours later, rats were transcardially perfused and then both hemispheres were weighed and put into formamide (1 ml/100 mg) at 60 °C for 24 h. The supernatant was obtained, and its optical density was determined by spectrophotometer at 620 nm. The EB concentration was expressed as microgram of EB per gram of brain tissue.

2.1.4. Reverse transcription and quantitative real-time PCR (qRT-PCR)

The mRNA expressions were detected by SYBR Green-based real-time PCR (TaKaRa, Dalian, China). The microvessel fractions were isolated from the tumor tissue by centrifugation in 15 ml with 18% (w/v) dextran solution at 10,000 g and 4 °C for 10 min. The primers of rat claudin-5 (NM_031701), occludin (NM_031329) and GAPDH (NM_017008) are as follows: claudin-5 Forward: 5′-TTGACCGACCTTTTCTTCTATGC-3′, Reverse: 5′-TTCATCGGTCCTTTGAC GGC-3’; occludin Forward: 5′-TCCAATGGCAAAGTGAATGACAAG-3′, Reverse: 5′-TTACCACCGCTGCTGTAACGAG-3’; GAPDH Forward: 5′-AAATCCCAT CACCATCTTCCAG-3′, Reverse: 5′-TGATGACCCTTTTGGCTCCC-3’. PCR was performed for 40 cycles with the following parameters: 10 s at 95 °C, and for each cycle 10 s at 95 °C for denaturation and 20 s at 60 °C for annealing. All quantitative RT-PCR analyses were conducted by means of a 7500 Fast Real-Time PCR System (Applied Biosystems). Expressions of claudin-5 and occludin were normalized to that of endogenous control GAPDH with the $2^{-\triangle\triangle Ct}$ formula.

2.1.5. Western blot assessment

Total protein from microvessel fractions was extracted in lysis buffer (Pierce, Rockford, IL, USA) and quantified by using the BCA method. Equal amounts of protein (20–40 μg) were separated by SDS-PAGE and processed for immunoblotting with antibodies for nNOS, iNOS, eNOS, claudin-5, occludin, ZONAB and GAPDH (diluted 1:300, 1:300, 1:300, 1:500, 1:500, 1:1000, respectively) All the protein bands were scanned and relative integrated density values (IDV) were calculated by Fluor Chen 2.0 software and normalized to that of GAPDH.

2.1.6. Immunohistochemistry assay

Rats were fixed by infusing heparinized saline through the cardiac ventricle, followed by 4% paraformaldehyde. The sections of tumor tissues were obtained and blocked by incubation in PBS solution containing 10% normal goat serum for 1 h at 4 °C, and the following procedures conformed to the standard procedures. The sections were then incubated with claudin-5 and occludin antibodies (diluted 1:300, 1:300, 1:1000, respectively) at 4 °C overnight. For semi-quantitative measurements of claudin-5 and occludin average optical density, the slides were photographed and analyzed by using a computer-aided image-analysis system (Motic Images Advanced 3.2).

2.2. Experiments in vitro

2.2.1. Culture of cells and establishment of BTB model in vitro

The immortalized human cerebral microvascular endothelial cell line hCMEC/D3 was kindly supplied by Dr. Couraud (Institut Cochin, Paris, France). The cells were cultured with endothelial basal medium (EBM-2) (Lonza, Walkersville, MD) supplemented with 5% FBS. The human glioblastoma cell line U87 MG was purchased from Cell Bank of Shanghai Institutes for Biological Sciences. The cells were cultured with high glucose DMEM with 10% FBS at 37 °C and 5% CO_2.

BTB models in vitro were established as described [15]. First, 2×10^6 U87 MG cells were plated onto the lower chamber of a 6-well Transwell inserts (0.4 μm pore size; Corning, USA). After the U87 MG cells were confluent, 2×10^5 hCMEC/D3 were seeded on the upper chamber of the Transwell insert. Our previous results of trans-endothelial electric resistance (TEER) of BTB showed that the cells were co-cultured for 4 days, an in vitro BTB model was established successfully [15]. Therefore, 4 days was considered as the optimum co-cultured duration in the subsequent experiments.

2.2.2. Agents administering and experimental groups

The overexpression and silencing of ZONAB were respectively performed with pGCMV/EGFP/Neo vector and pGPU6/GFP/Neo vector (GenePharma, Shanghai, China). The normal hCMEC/D3 cells were seeded onto 24-well plates and transfected with overexpression or silence plasmid of ZONAB by using Lipofectamine LTX and Plus Reagents according to the manufacturer's instructions. After G418 selecting, the cell clones of ZONAB overexpression or silencing were established. After that, BK (1 μmol/L) was administrated for 15 min. The experiments were divided into 8 groups (n = 8/group): control group (infusion of saline for 15 min), BK group, ZONAB (+) blank group (transfected with blank control of ZONAB overexpression plasmid), ZONAB (+) group (transfected with ZONAB overexpression plasmid), BK + ZONAB (+) group (BK was administrated to the cells that transfected with ZONAB overexpression plasmid), ZONAB (−) blank group (transfected with blank control of ZONAB silence plasmid), ZONAB (−) group (transfected with ZONAB silence plasmid), BK + ZONAB (−) group (BK was administrated to the cells that transfected with ZONAB silence plasmid).

2.2.3. TEER value

TEER was determined with a millicell-ERS instrument (Millipore, Billerica, MA). To avoid fluctuations in temperature, TEER was measured at 37 °C with the aid of a heating plate. Background electrical resistance including filter and medium was subtracted from each reading. The final TEER values were calculated as $\Omega \cdot cm^2$ by multiplying by the surface area of the Transwell insert.

2.2.4. Chromatin immunoprecipitation (ChIP) assay

ChIP assays were performed by using Simple ChIP Enzymatic Chromatin IP Kit (Cell signaling Technology, Danvers, MA). In brief, hCMEC/D3 cells of BTB model in vitro were fixed with 1% formaldehyde, and lysed with cold buffer containing PMSF. Then, chromatin was digested with Micrococcal Nuclease and incubated for 20 min at 37 °C with frequent mixing to digest DNA. Immunoprecipitation was carried out overnight at 4 °C by using 3 μg antibody against ZONAB with gentle shaking and the following procedures according to the manufacturer's instruction. The primers of each PCR set, the size of PCR products, and annealing temperatures were listed in Table 1. In each PCR reaction, corresponding input was taken in parallel for PCR validation.

2.3. Statistical analysis

All data were expressed as the mean ± SD for each group. Data were analyzed with SPSS 19.0 software. A Student's *t*-test was performed to determine the significant difference between two groups. One-way analysis of variance (ANOVA) was utilized to determine the significant difference between multiple groups. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Studies in vivo

3.1.1. Effect of BK on the protein expressions of nNOS, eNOS and iNOS

As shown in Fig. 1, in control group, nNOS and eNOS had slight protein expression in tumor tissues. After BK infusion, the protein expressions increased by 5 min. The peak appeared at 10 min and decreased thereafter. There was no significant change of iNOS after BK infusion.

3.1.2. L-NAME and 7-NI blocked the effect of BK on increasing the BTB permeability

EB assay result showed, after BK infusion, the BTB permeability significantly increased compared with control group. Pretreatment of L-NAME completely blocked BK's effect on increasing the BTB permeability, leading the BTB permeability to the level of control group. After 7-NI pretreatment, BTB permeability significantly decreased compared with that of BK group, but increased in comparison with that of control group. AG had no effect on BK regulating the BTB permeability, and there was no significant difference between BK and AG + BK groups (Fig. 1B).

3.1.3. L-NAME and 7-NI blocked the effect of BK on down-regulating the mRNA and protein expression levels and the distribution of claudin-5 and occludin

In control group, claudin-5 and occludin had certain mRNA and protein expressions which decreased significantly after BK infusion. In L-NAME + BK group, they significantly increased compared with those of BK group, while there wasn't marked difference from control group. The pretreatment of 7-NI could increase the mRNA and protein expressions in comparison with BK group, but decrease them compared with control group. However, after AG pretreatment, there was no marked difference from BK group (Fig. 1C and D).

In glioma rat model, immunohistochemistry analysis indicated claudin-5 and occludin were mainly expressed on tumor capillaries and tumor cells in tumor tissue. After BK infusion, the immunoreactivity of claudin-5 and occludin was attenuated on tumor capillaries. Similar to the result of qRT-PCR and Western blot assays, the effect of BK on the immunoreactivity of TJ associated proteins could be attenuated by the pretreatment of L-NAME and 7-NI (Fig. 1E).

3.1.4. L-NAME and 7-NI blocked the effect of BK on regulating the protein expression of ZONAB

As shown in Fig. 2A, in control group, in cell nucleus, ZONAB had slight expression which increased by 5 min after BK infusion. The peak appeared at 15 min and decreased thereafter. On the contrary, there were totally opposite changes of protein expression of ZONAB in cell membrane. Compared to BK 15 min group, the expression of ZONAB in nucleus significantly decreased, while that in cell membrane significantly increased after L-NAME pretreatment. Similar to BK + L-NAME group, 7-NI could partly block BK's effect on regulating the expression of ZONAB. However, AG didn't have the similar blocking effect.

3.2. Studies in vitro

3.2.1. BK increased the permeability of BTB via up-regulating ZONAB

The data demonstrated that TEER value of ZONAB (+) significantly decreased compared with ZONAB (+) blank group while that

Table 1
Primers used for ChIP assay.

Gene	Binding site and control	Sequence	Amplicon size	Annealing temperature
Claudin-5	PCR1	(F) 5′-CGGCACGATGACCCGCGCAC-3′ (R) 5′-CTCTTGGCCCCAGTCCGTT -3′	161	62 °C
	PCR2	(F) 5′-CCGTGCGCGGGTCATCGT-3′ (R) 5′-AATGCGCTCGGCCGCGAC-3′	162	62 °C
	PCR3	(F) 5′-CTGAAGGCCAGAGCAGGCT-3′ (R) 5′-AGGGGCTGCTCCTCTTCCT-3′	160	62 °C
Occludin	PCR1	(F) 5′-GGTTCCTTTAACAGCGCGCT-3′ (R) 5′-GGAGTGTAGGTGTGGTGTGT-3′	123	62 °C
	PCR2	(F) 5′-AGCAATGCTGACATTCCAGAT-3′ (R) 5′-AGTCTGCACTTAACACTGTGT-3′	183	60 °C
	PCR3	(F) 5′-CTGCCATCATCTGAAATACCT-3′ (R) 5′-ATCTGGAATGTCAGCATTGCT-3′	169	60 °C
	PCR4	(F) 5′-GACAGACACAAAGGGCACAT-3′ (R) 5′-CCATGCCCATTAATAGTCACT-3′	153	60 °C

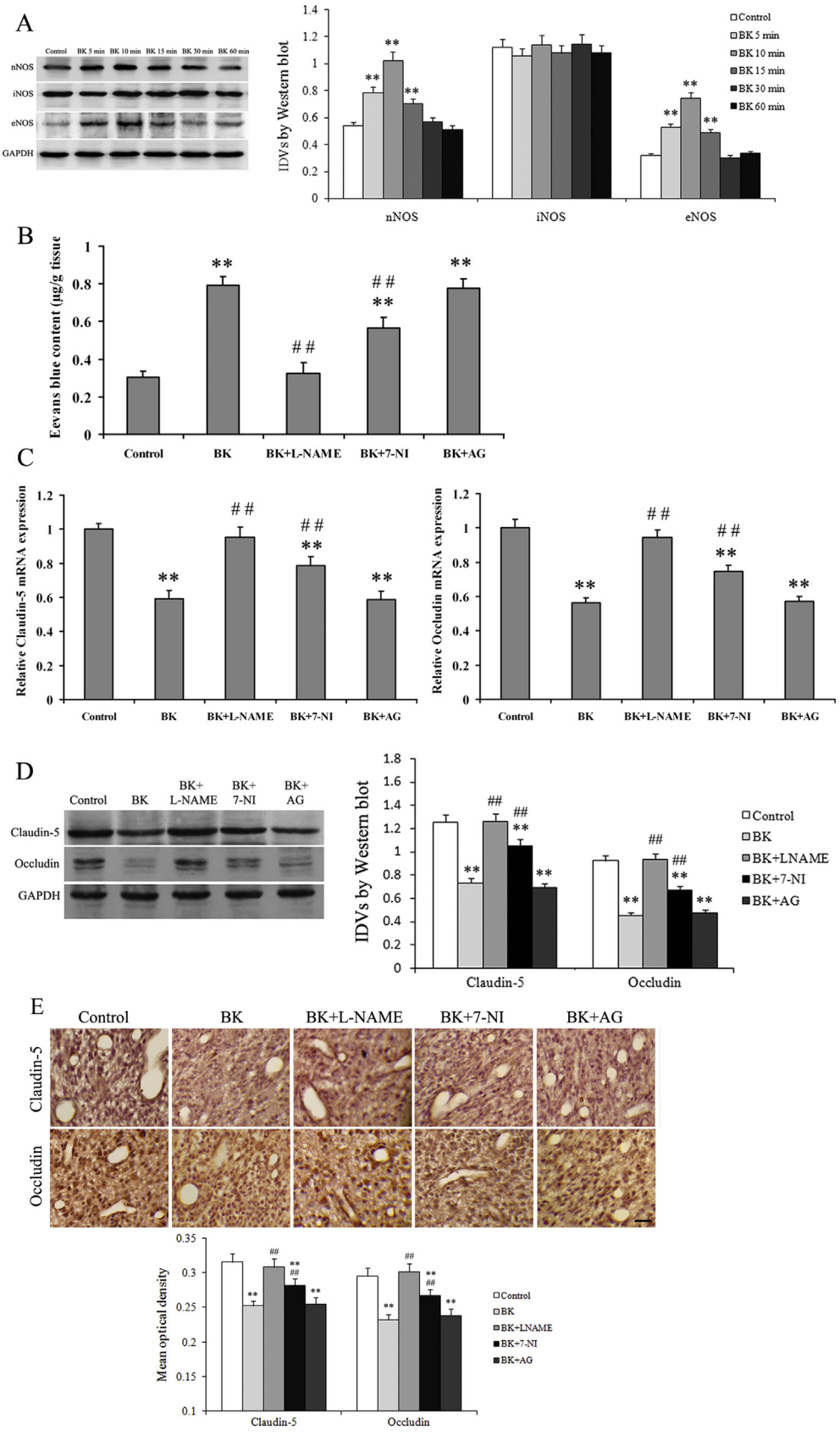

Fig. 1. (A) Effect of BK on the protein expressions of nNOS, eNOS and iNOS were detected by Western blot. (B) Effects of L-NAME and 7-NI on the EB extravasated through the BTB in vivo after BK infusion. (C, D) The mRNA and protein expression levels of claudin-5 and occludin were determined by qRT-PCR and Western blot. (E) The expressions and distribution of claudin-5 and occludin were analyzed by immunohistochemistry. Scale bar = 20 μm. Data represented the means ± SD (n = 8, each). $^{**}P < 0.01$ vs. control group, $^{\#\#}P < 0.01$ vs. BK group.

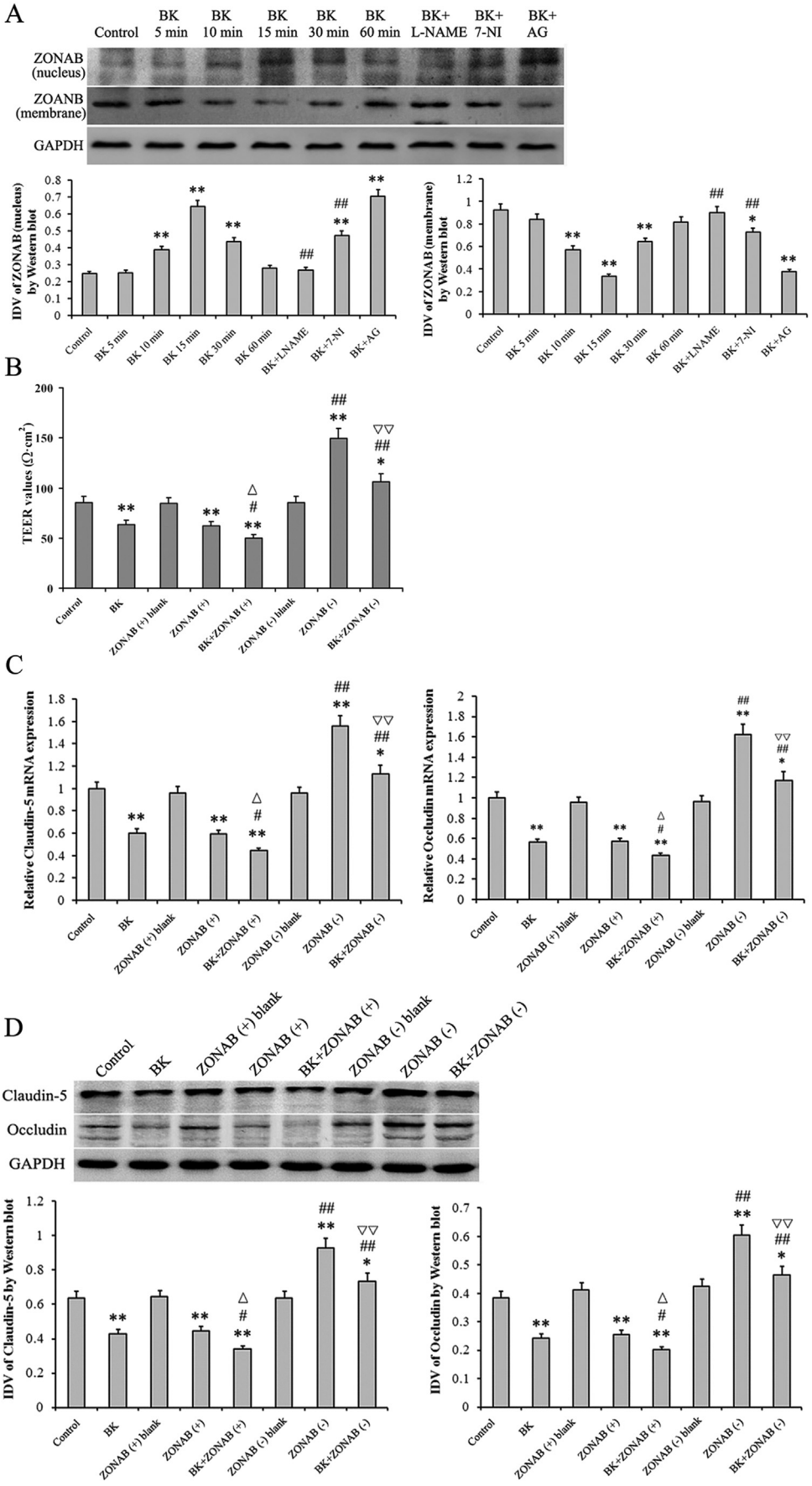

Fig. 2. (A) The protein expression levels of ZONAB in nucleus and cell membrane were determined by Western blot. Data represented the means ± SD (n = 8, each). $^{**}P < 0.01$ vs. control group, $^{\#\#}P < 0.01$ vs. BK group. (B) TEER values of BTB models in vitro were expressed as $\Omega \cdot cm^2$. (C, D) The relative mRNA and protein expression levels of claudin-5 and occludin were determined by qRT-PCR and Western blot. Data represented the means ± SD (n = 8, each). $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. control group, $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ vs. BK group, $^{\triangle}P < 0.05$ vs. ZONAB (+) group, $_{\triangledown}{}_{\triangledown}P < 0.01$ vs. ZONAB (−) group.

of ZOANB (−) group markedly increased in comparison with ZOANB (−) blank group. TEER value of BK + ZONAB (+) group significantly decreased compared with BK and ZOANB (+) group. Correspondingly, TEER value of BK + ZONAB (−) group was significantly enhanced compared with BK group, but was evidently reduced compared with ZOANB (−) group (Fig. 2B).

3.2.2. BK decreased the expressions of claudin-5 and occludin via up-regulating ZONAB

The results demonstrated both mRNA and protein expressions of claudin-5 and occludin were significantly down-regulated in BK group compared with control group, the same as that in ZONAB(+) group in contrast to those of ZONAB(+) blank group; conversely, those of claudin-5 and occludin evidently increased in ZONAB (−) group in contrast to those of ZONAB(−) blank group. The mRNA and protein expressions of claudin-5 and occludin in BK + ZONAB (+) group were significantly reduced compared with those of BK group and ZONAB (+) group. Furthermore, the mRNA and protein expressions of claudin-5 and occludin in BK + ZONAB (−) group increased in comparison with those of BK group while decreasing compared with those of ZONAB (−) group (Fig. 2 C and D).

3.2.3. ZONAB bound to the promoters of claudin-5 and occludin

There are two putative ZONAB binding sites at +843 and + 813 positions in claudin-5, three putative ZONAB binding sites at +229, −744 and −781 positions in occludin. Primers were designed to bind sequence flanking the putative ZONAB binding sites (Table 1). As a negative control, PCR were conducted to amplify the upstream region of the putative ZONAB binding site that was not expected to associate with ZONAB. The results revealed there was an association between ZONAB and putative binding site 1 of claudin-5 and putative binding site 1 of occludin. There was no association between ZONAB and all of the control regions (Fig. 3).

4. Discussion

The present study demonstrated after BK, the expressions of eNOS and nNOS significantly increased without significant change in iNOS expression. NOS inhibitors L-NAME and 7-NI could effectively block BK increasing BTB permeability and decreasing the mRNA and protein expressions of claudin-5 and occludin, while AG had no such effect. After BK administering, the expression of ZONAB increased significantly in the nucleus of the tumor microvascular endothelial cells, while significantly decreasing in the cell membrane. After the pretreatment of L-NAME and 7-NI, ZONAB expression in the nucleus was reduced, while increased in the cell membrane. Meanwhile, overexpression of ZONAB significantly increased the permeability of BTB, and vice versa. Most importantly, it dramatically promoted BK-mediating increasing of BTB permeability. Whereas, silencing of ZONAB markedly blocked BK-mediating increasing of BTB permeability. ChIP verified ZONAB was bound to the inverted CCAAT box of claudin-5 and occludin promoter. Altogether, all of these results indicated BK promoted ZONAB translocation into nucleus via increasing the expressions of eNOS and nNOS and further down-regulating TJ associated proteins claudin-5 and occludin.

TJ is an important structural and functional basis of maintaining the blood brain barrier (BBB) and BTB integrity in vivo and it localizes cells of brain capillary endothelium and is made up of transmembrane proteins of occludin and claudins, cytoplasmic proteinszonulaoccludens (ZOs) linked to an actin-based cytoskeleton that forms a seal-like structure [16]. It is generally believed that the alteration of TJ-associated protein contributes to regulating the function of TJ in physiological and pathological conditions. Our previous study results demonstrated BK could open TJ by down-regulating the protein expressions of claudin-5 and occludin, but the molecular mechanisms wasn't clarified.

NO can open TJ through the NO/sGC/cGMP/PKG signaling pathway, nitration and oxidation of cytoskeleton F-actin pathway and activation of the Rho kinase pathway [17–19]. But whether NOS/NO signaling pathway is involved in the process of BK regulating the expression of TJ associated proteins need further researches. To this end, the protein expression levels of three NOS subtypes in C6 rat brain glioma model were detected. The results showed the protein expressions of eNOS and nNOS were significantly up-regulated after BK infusion, with no obvious changes in the expression of iNOS, which is consistent with our results in RG2 rat glioma model [9]. To further analyze the effect of NOS on BK opening TJ, L-NAME, 7-NI and AG were pretreated before BK infusion. The results showed the effect of BK on increasing the permeability of BTB and decreasing the expressions of caludin-5 and occluding was completely blocked by L-NAME, and partially by 7-NI. But AG had no similar effect to them. These suggested nNOS and eNOS participated in the process where BK regulated the expression of TJ associated proteins. Molecular mechanism of NOS regulating the expressions of TJ associated proteins has not been reported.

ZONAB is an epithelial-specific transcription factor and the localization and activities of it are partially regulated by the density-dependent assembly of epithelial junctions [20]. The research on the function of ZONAB is mainly focused on ZONAB regulating cellular proliferation and differentiation. But there are few researches on ZONAB's function on regulating the TJ in the BTB. Research revealed overexpression of ZONAB could regulate the paracellular permeability of epithelial cells [13]. Estradiol could decrease the expression of epidermal maker occludin and CRB3, which is correlated with the ZONAB nuclear translocation [14]. These results suggest ZONAB may regulate the BTB permeability through paracellular pathway. To study whether ZONAB acts in BK opening TJ and increasing BTB permeability, the protein expressions of ZONAB in nucleus and cell membrane of tumor microvascular endothelial cells were tested. Results showed BK could increase the protein expression of ZONAB in the nucleus and decrease it in cell membrane. The result suggested, after BK, ZONAB was promoted into the nucleus, playing a role of increasing the BTB permeability.

To make the effect and mechanism of ZONAB participating in BK opening TJ clear, the overexpression or silencing of ZONAB was performed in hCMEC/D3 cells of BTB model in vitro separately. As expected, overexpression of ZONAB increased the permeability of BTB and decreased the mRNA and protein expression levels of claudin-5 and occludin, and vice versa. More importantly, overexpression of ZONAB dramatically promoted BK-mediating increasing of BTB permeability and decreasing of TJ associated proteins, but silencing of ZONAB markedly blocked both of them. Although silencing of ZONAB was not returned BTB permeability and TJ associated proteins expression levels to the control level, it blocked the effect of BK was still significant. Therefore, these results suggest ZONAB might regulate the expression of TJ associated proteins to control the permeability of BTB. Nevertheless, little is known about the relationship between ZONAB and claudin-5 and occludin. On the basis of these above findings, ChIP assay was carried out to elucidate whether ZONAB interacted with the promoters of claudin-5 and occludin. The result demonstrated ZONAB could be bound with claudin-5 and occludin, indicating there was a direct interaction between ZONAB and promoters of TJ associated proteins. Thus up-regulation of ZONAB could directly reduce the expression of TJ associated proteins to increase the permeability of BTB.

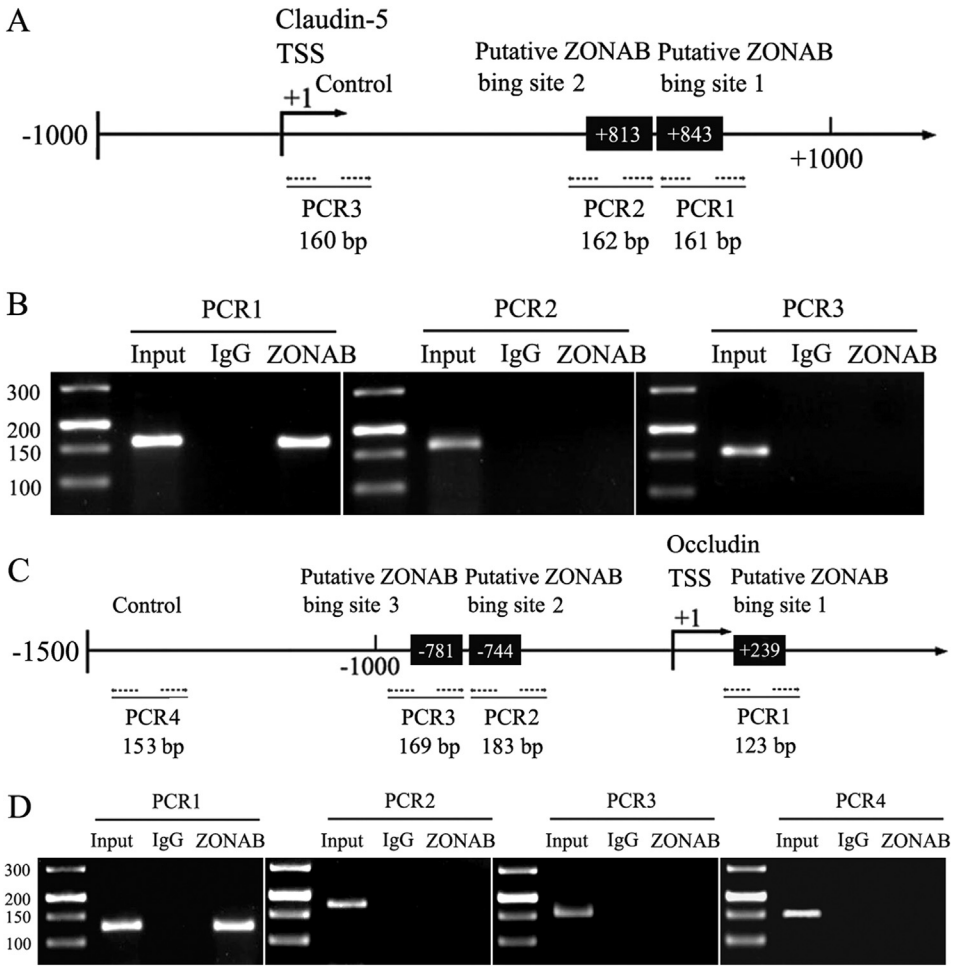

Fig. 3. ZONAB binds to promoters of TJ associated proteins claudin-5 and occludin. Schematic representation of human claudin-5 (A, B) and occludin (C, D) promoter region in 1500 bp upstream or 1000 bp downstream of the transcription start site (TSS, designated as +1).

It's unclear whether BK controlled the expression of ZONAB and affected its function by NOS. Nie et al. found that the transcriptional activity of ZONAB was Rho-dependent [21,22]. NO can increase the permeability of BBB by activating Rho kinase pathway [19]. Therefore, we hypothesize NOS/NO pathway might participate in BK regulating the expression of ZONAB. Thus, the NOS inhibitors were applied to detect the effect of NOS on BK regulating ZONAB. The results showed the protein expression of ZONAB in nucleus significantly decreased after L-NAME and 7-NI pretreatments, but markedly increased in cell membrane. Results showed both eNOS and nNOS participated in BK regulating ZONAB.

In summary, this study proves NOS/NO is an important signaling pathway for BK to control the permeability of BTB. The expressions of eNOS and nNOS in tumor cells are up-regulated by BK, which leads to the increase of NO concentration. NO diffuses to the tumor microvascular endothelial cells to activate ZONAB transcription inhibition activity, resulting in decreased expressions of TJ associated proteins claudin-5 and occludin, and increased permeability of BTB. This study has clarified the molecular mechanism of BK regulating the expressions of TJ associated proteins, providing a target for the delivery of antitumor drugs into the tumor tissue as well as novel strategies for glioma treatment.

Conflicts of interest

The authors disclose no potential conflicts of interest.

Acknowledgments

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References

[1] L.W. Xu, K.K. Chow, M. Lim, et al., Current vaccine trials in glioblastoma: a review, J. Immunol. Res. 2014 (2014) 796856.
[2] F. Lefranc, N. Sadeghi, I. Camby, et al., Present and potential future issues in glioblastoma treatment, Expert. Rev. Anticancer. Ther. 6 (2006) 719–732.
[3] B.K. Hendricks, A.A. Cohen-Gadol, J.C. Miller, Novel delivery methods by passing the blood-brain and blood-tumor barriers, Neurosurg. Focus 38 (2015). E10.
[4] T. Inamura, K.L. Black, Bradykinin selectively opens blood-tumor barrier in experimental brain tumors, J. Cereb. Blood. Flow. Metab. 14 (1994) 862–870.
[5] L.B. Liu, Y.X. Xue, Y.H. Liu, Bradykinin increases the permeability of the blood-tumor barrier by the caveolae-mediated transcellular pathway, J. Neurooncol 99 (2010) 187–194.
[6] L.B. Liu, Y.X. Xue, Y.H. Liu, et al., Bradykinin increases blood-tumor barrier permeability by down-regulating the expression levels of ZO-1, occludin, and claudin-5 and rearranging actin cytoskeleton, J. Neurosci. Res. 86 (2008) 1153–1168.
[7] N.N. Salama, N.D. Eddington, A. Fasano, Tight junction modulation and its relationship to drug delivery, Adv. Drug. Deliv. Rev. 58 (2006) 15–28.
[8] W.A. Banks, S. Dohgu, J.L. Lynch, et al., Nitric oxide isoenzymes regulate lipopolysaccharide-enhanced insulin transport across the blood-brain barrier, Endocrinology 149 (2008) 1514–1523.

[9] Y. Liu, K. Hashizume, K. Samoto, et al., Repeated, short-term ischemia augments bradykinin-mediated opening of the blood-tumor barrier in rats with RG2 glioma, Neurol. Res. 23 (2001) 631–640.
[10] S. Nakano, K. Matsukado, K.L. Black, Increased brain tumor microvessel permeability after intracarotid bradykinin infusion is mediated by nitric oxide, Cancer. Res. 56 (1996) 4027–4031.
[11] E. Kavanagh, M. Buchert, A. Tsapara, et al., Functional interaction between the ZO-1-interacting transcription factor ZONAB/DbpA and the RNA processing factor symplekin, J. Cell. Sci. 119 (2006) 5098–5105.
[12] W.R. Lima, K.S. Parreira, O. Devuyst, et al., ZONAB promotes proliferation and represses differentiation of proximal tubule epithelial cells, J. Am. Soc. Nephrol. 21 (2010) 478–488.
[13] M.S. Balda, K. Matter, The tight junction protein ZO-1 and an interacting transcription factor regulate ErbB-2 expression, EMBO. J. 19 (2000) 2024–2033.
[14] J.E. Jiménez-Salazar, P. Posadas-Rodríguez, R.C. Lazzarini-Lechuga, et al., Membrane-initiated estradiol signaling of epithelial-mesenchymal transition-associated mechanisms through regulation of tight junctions in human breast cancer cells, Horm. Cancer 5 (2014) 161–173.
[15] J. Ma, P. Wang, Y. Liu, et al., Krüppel-like factor 4 regulates blood-tumor barrier permeability via ZO-1, occludin and claudin-5, J. Cell. Physiol. 229 (2014) 916–926.
[16] R. Paolinelli, M. Corada, F. Orsenigo, et al., The molecular basis of the blood brain barrier differentiation and maintenance. Is it still a mystery? Pharmacol. Res. 63 (2011) 165–171.
[17] N.P. Lee, C.Y. Cheng, Regulation of sertoli cell tight junction dynamics in the rat testis via the nitric oxide synthase/soluble guanylate cyclase/3',5'-cyclic guanosine monophosphate/protein kinase G signaling pathway: an in vitro study, Endocrinology 144 (2003) 3114–3129.
[18] A. Banan, L.J. Zhang, M. Shaikh, et al., Key role of PLC-gamma in EGF protection of epithelial barrier against iNOS upregulation and F-actin nitration and disassembly, Am. J. Physiol. Cell. Physiol. 285 (2003) C977–C993.
[19] T.J. Bivalacqua, T. Liu, B. Musicki, et al., Endothelial nitric oxide synthase keeps erection regulatory function balance in the penis, Eur. Urol. 51 (2007) 1732–1740.
[20] D. Spadaro, R. Tapia, L. Jond, et al., ZO proteins redundantly regulate the transcription factor DbpA/ZONAB, J. Biol. Chem. 89 (2014) 22500–22511.
[21] M. Nie, M.S. Balda, K. Matter, Stress- and Rho-activated ZO-1-associated nucleic acid binding protein binding to p21 mRNA mediates stabilization, translation, and cell survival, Proc. Natl. Acad. Sci. U. S. A. 109 (2012) 10897–10902.
[22] M. Nie, S. Aijaz, I.V. Leefa Chong San, et al., The Y-box factor ZONAB/DbpA associates with GEF-H1/Lfc and mediates Rho-stimulated transcription, EMBO. Rep. 10 (2009) 1125–1131.