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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).