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Research article

Involvement of brain-gut axis in treatment of cerebral infarction by β-asaron and paeonol

Xiaogang He[a,*], Qiufang Cai[a], Jianxiang Li[b], Weifeng Guo[c]

[a] Department of Neurology, Kunshan Hospital of Traditional Chinese Medicine, Affiliated to Nanjing University of Traditional Chinese Medicine, Kunshan, 215300, PR China
[b] Department of Neurology, Nanjing Hospital of Traditional Chinese Medicine, Affiliated to Nanjing University of Traditional Chinese Medicine, Nanjing, 210004, PR China
[c] First Clinical Medical College of Nanjing University of Traditional Chinese Medicine. Nanjing, 210004, PR China

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ABSTRACT

Cerebral infarction (CI) causes severe brain damage with high incidence. This study aimed to investigate the involvement of brain-gut axis in the treatment of CI by combined administration of β-asaron and paeonol. Rat middle cerebral artery occlusion (MCAO) model was established, the interleukin-1beta (IL-1β) and tumor necrosis factor α (TNF-α) in the rat peripheral blood were determined by ELISA assay, and brain tissue damage was evaluated by TUNNEL assay. The correlation of cholecystokinin (CCK) and nuclear factor-kappaB (NF-κB) signaling components between intestinal mucosa and prefrontal cortex of MCAO rats treated with β-asaron and paeonol were analyzed by quantitative RT-PCR and western blotting. In vitro transwell co-culture was performed to confirm the correlated expression. The expression of CCK and NF-κB signaling components were closely correlated between the intestinal mucosa and prefrontal cortex of MCAO rats treated with β-asaron and paeonol. The combined administration also regulates the IL-1β and TNF-α in the MCAO rat peripheral blood and ameliorate the brain damage in MCAO rats. Elevated expression of related genes was observed in the cortical neurons co-cultured with intestinal mucosal epithelial cells treated by β-asaron and paeonol. The brain-gut axis mediates the therapeutic effect of β-asaron and paeonol for cerebral infarction through CCK and NF-κB signaling.

1. Introduction

Cerebral infarction (CI), also known as ischemic stroke, refers to the situation when brain tissue necrosis occurs due to the interrupted blood flow caused by the blockage or narrowing in the arteries. Thrombus, embolus and atheromatous plaque and stenosis in the arteries are the most common causes of cerebral infarction, usually resulting into cerebral infarction within a short time [1]. The brain-gut axis, alternatively known as the brain-gut interaction, is composed of complex bidirectional regulations between the brain and gut, and has been gradually shown to an possible mediator of multiple brain diseases [2–4]. Recent studies also suggested potential role of brain-gut interaction in the pathological process of ischemic stroke [5–7]. The involvement of brain-gut interaction in pathological process of cerebral infarction and the underlying mechanisms remain largely unknown.

Cholecystokinin (CCK) was first identified from jejunal extracts as a gallbladder contraction factor, and listed as one of classical gut hormonal peptides [[8],9]. Comprehensive studies over the past decades have showed that, as an important regulator of the digestion system, cholecystokinin could also act as growth factor, neurotransmitter, spermatozoa fertility factor, anti-inflammatory cytokine and cardiac marker in various physiological and pathological processes among multiple organs. Outside the gastrointestinal tract, the CCK gene was found to be expressed and functioning in endocrine cells, neurons and epithelial cells in cell-specific manners, leading to the synthesis and release of different forms of peptides belonging to the cholecystokinin (CCK) family [8]. CCK-8 and CCK-5 are identified as potent neurotransmitters in neurons [10]. CCK performs multiple regulatory roles in different organs by binding two G protein-coupled receptors, CCK-1 and CCK-2, and CCK-2 was found to be mainly expressed in the brain tissues [11]. Specially, cholecystokinin has been established as major component of the brain-gut axis due to its expression and regulatory effects in both the two organs and associated with ischemic stroke [12,13]. The expression of CCK-8 was detected in a number of patients with cerebral hemorrhage and infarction [14]. However, effects of the brain-gut axis on ischemic stroke, and the potential mediation by CCK-related signaling, need further investigation.

Previous research demonstrated that β-asaron, the major

* Corresponding author at: Department of Neurology, Kunshan Hospital of Traditional Chinese Medicine, Affiliated to Nanjing University of Traditional Chinese Medicine, No. 189 Chaoyang Road, Kunshan, 215300, Jiangsu, PR China.
E-mail address: 13962641937@163.com (X. He).

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component of *Acorus Tatarinowii Schott*, could attenuate ischemia-reperfusion-induced autophagy in rat brains [15,16]. Paeonol protects memory and attenuate cerebral ischemic injury by inhibiting β-secretase activity and cell apoptosis and upregulating downstream signaling component expression [17,18]. In the present study, the rat middle cerebral artery occlusion (MCAO) model was established to test the effects of β-asaron and paeonol on brain function after CI, as well as the CCK expression and related signaling pathways in both the brain and gut tissues, combined with in vitro cell co-culture assay, providing novel insights into the involvement of brain-gut axis in cerebral infarction especially in treatment of CI with β-asaron and paeonol.

2. Material and methods

2.1. Animals and cell lines

The SPF Sprague-Dawley (SD) rats in this study were purchased from SLAC Laboratory Animal Co., Ltd. (Shanghai, China), and fed at clean environment with a 12:12 h light: dark cycle. All procedures of this study were approved by the Institutional Animal Care and Use Committee of Nanjing University of Traditional Chinese Medicine. Rat intestinal mucosal epithelial IEC18 cells (CRL 1589) were obtained from the American Type Culture Collection (ATCC) and cultured in Dulbecco's modified Eagle's medium (DMEM) supplied with 10% fetal calf serum, 2 mM glutamine, 100 U mL^{-1} penicillin, 0.1 and mg mL^{-1} streptomycin. The rat cortical neurons (CP-R105) were purchased from the Procell company (Wuhang, China) and cultured in DMEM containing fetal calf serum, glutamine, penicillin and streptomycin.

2.2. Middle cerebral artery occlusion (MCAO) model establishment

Totally 24 SD rats were randomly divided into the sham group (n = 8), the model group (n = 8) and the treatment group (n = 8). The sham group was used as the negative control without middle cerebral artery ischemia-reperfusion operation and administration of β-asaron and paeonol. The model group indicates rats that underwent middle cerebral artery ischemia-reperfusion operation but no intragastric administration of β-asaron and paeonol. Rats in the treatment group were subjected to intragastric administration of β-asaron and paeonol for consecutive seven days before the model establishment, and treated with β-asaron and paeonol by intragastric administration at 24 h, 48 h and 72 h after the model establishment surgery respectively. The establishment of middle cerebral artery occlusion (MCAO) model, also known as middle cerebral artery (MCA) ischemia-reperfusion model, was carried out by suturing the middle cerebral artery twice as previously described [19,20]. The rats were subjected to intraperitoneal injection anesthesia with 10% chloral hydrate (0.4 ml/100 g weight), and the surgery was carried out following the Zea Longa method and the suture was removed 90 min later. A five-point scale was applied for scoring the neurologic findings as previously described [19]. Specifically, the score of 0 indicates no neurologic deficit, 1 indicates failure to fully extend left forepaw, 2 indicates circling to the left, 3 indicates falling to the left during walking, 4 indicates coma, and 5 indicates death.

2.3. Quantitative RT-PCR analysis

The relative mRNA levels were measured by quantitative RT-PCR method. Briefly, the total RNA samples were extracted using the Trizol reagent according to the manufacturer's instructions (Life Technologies, UK). The synthesis of cDNA with random primers was done using the Prime Script RT Master Mix kit following the manufacturer's instructions (Cat. #RR036a; Takara). The quantitative real-time polymerase chain reaction (qRT-PCR) was then performed using the SYBR Select Master Mix kit (Cat. #4472908; Applied Biosystems) by the manufacturer's instructions. GAPDH expression was applied as the internal control. The sequences of primers in this study were listed as follows: CCK forward: 5′- ATGAAGTGCGGCGTGTGT −3′; CCK reverse, 5′-AGAGGGAGCTTTGCGGAC-3′; CCKBR forward: 5′-GTGAAAATGACA GCGAGACC-3′; CCKBR reverse: 5′-AGCCAACACAGGAAGAAAAG −3′; Three biological replicates were performed for statistical analysis.

2.4. Western blotting

Rat tissues were homogenized in liquid nitrogen and used for protein extraction using RIPA supplied with 1 × Halt Protease Inhibitor Cocktail (Thermo Fishier Scientific, USA). Protein samples from cultured cell lines were extracted by lysing the cells using the same extraction buffer. Proteins were boiled at 100 °C for 5 min in sample buffer consisting of 300 mM Tris-HCl, 5% glycerol, 1% SDS, 3.5 M β-mercaptoethanol, 4 mM PMSF and 0.06% bromophenol blue, and then separated by SDS-PAGE, transferred onto PVDF membranes, blocked with 5% fat-free milk solution, incubated with primary antibodies, incubated with secondary antibodies in TBST buffer, and finally developed using enhanced chemiluminescence (ECL) solution (Amersham, USA). The anti-CCK-8 (#ab27441), anti-CCKBR (#ab77077), anti-PKA (#ab75991), anti-IkB (#ab32518), anti-NF-kB p65 (#ab16502) and anti-β-actin (#ab8227) antibodies used in this study were purchased from the Abcam company. Three biological repeats were done for quantitative ananlysis.

2.5. Enzyme-linked immunosorbent assay

The IL-lβ and TNF-α levels in rat peripheral blood were determined by enzyme-linked immunosorbent assay using the IL-1β Rat ELISA Kit (Cat. #BMS630; Thermo Fisher Scientific) and the TNF alpha Rat ELISA Kit (Cat. #KRC3011; Thermo Fisher Scientific) respectively following the manufacturer's instructions. Briefly, all reagents were kept to the room temperature before use, and blood samples were added into the 96-well microtiter plates. After the ELISA procedure, the absorbances of each well in the plates were read at 450 nm after the stop solution was added. Values were read within 2 h after adding the stop solution. For reliable determination, three biological repeats and technical repeats were performed for IL-lβ and TNF-α measurement.

2.6. TUNNEL apoptosis assay and H&E staining

The tissue damage extent of rat brain was analyzed with the Colorimetric TUNEL Apoptosis Assay Kit (Cat. #C1098; Beyotime, China) according to the manufacturer's instructions. Briefly, rat brain slides were first dewaxed in xylene and ethanol, incubated with DNase-free proteinase K, washed with PBS buffer, incubated with 3% H_2O_2, and then added with 50 μl TUNEL detection solution and kept at 37 °C in dark for 60 min, added with 0.2 ml stop solution, incubated with 50 μl streptavidin-HRP working solution, incubated with 0.4 ml DAB solution, and finally with hematoxylin and eosin solution. Three biological replicates were performed.

2.7. Transwell co-culture assay

The Transwell co-culture system was used to test the interaction between rat intestinal mucosal epithelial IEC18 cells and rat cortical neurons. The isolated rat cortical neurons cell were pre-treated by hypoxia-reoxygenation injury was carried out as previously described [21]. A semipermeable membrane with a pore size of 0.4 μm (BD Biosciences) was used to separate the two chambers filled with DMEM buffer supplied with β-asaron and paeonol. IEC18 cells were then seeded in the upper chamber, and rat cortical neurons were seeded at the lower chamber. Cells analyzed in the medium containing no β-asaron and paeonol and neurons cultured alone were used as the control groups. After cultured for 48 h, gene expression in the IEC18 cells and cortical neurons were analyzed separately by quantitative RT-PCR