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Effects of colistin on amino acid neurotransmitters and blood-brain barrier in the mouse brain

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ABSTRACT

Neurotoxicity is one of the major potential side effects of colistin therapy. However, the mechanistic aspects of colistin-induced neurotoxicity remain largely unknown. The objective of this study was to examine the effects of colistin on the blood-brain barrier (BBB) and amino acid neurotransmitters in the cerebral cortex of mouse. Mice were divided into four groups (n = 5) and were administrated intravenously with 15 mg/kg/day of colistin sulfate for 1, 3 and 7 days successively while the control group was administrated intravenously with saline solution. The permeability and ultrastructure of the BBB were detected using the Evans blue (EB) dye and transmission electron microscopy (TEM), and the expression of Claudin-5 were determined by real-time PCR examination and western blotting. The brain uptake of colistin was measured by high-performance liquid chromatography (HPLC). The effects of colistin on amino acid neurotransmitters and their receptors were also examined by HPLC and real-time PCR. The results of EB extravasation, TEM and expression of Claudin-5 showed that colistin treatment did not affect the BBB integrity. In addition, multiple doses of colistin could induce accumulation of this compound in the brain parenchyma although there was poor brain uptake of colistin. Moreover, colistin exposure significantly increased the contents of glutamate (Glu) and gamma aminobutyric acid (GABA), and enhanced the mRNA expression levels of gamma aminobutyric acid type A receptor (GABAAR), gamma aminobutyric acid type B receptor (GABABR), *N*-methyl-D-aspartate 1 receptor (NR1), *N*-methyl-D-aspartate 2A receptor (NR2A) and *N*-methyl-D-aspartate 2B receptor (NR2B) in the cerebral cortex. Our data demonstrate that colistin is able to accumulate in the mouse brain and elevate the levels of amino acid neurotransmitters. These findings may be associated with colistin-induced neurotoxicity.

1. Introduction

The emergence of *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, and carbapenemase-producing Enterobacteriaceae strains that are resistant to lactams, fluoroquinolones, and aminoglycosides has led to renewed interest in polymyxin antibiotics as therapeutic agents (Landman et al., 2008). Colistin (also known as polymyxin E), a cationic polypeptide antibiotic, has been used as the last-line therapy against multidrug-resistant gram-negative bacteria such as *Pseudomonas aeruginosa*, *Acinetobacter baumannii* and *Klebsiella pneumonia* (Li et al., 2006; Velkov et al., 2013). However, the use of colistin has largely been limited by neurotoxicity and nephrotoxicity. Patients administered with intravenous colistin methane-sulfonate, the inactive prodrug of colistin, may experience confusion, facial and peripheral paresthesia, muscles weakness, vertigo and ataxia (Bergen et al., 2006). Our previous studies have also shown that colistin sulfate could induce depression, ataxia, neuronal and axonal degeneration of brain and sciatic nerves when intravenously administrated in mice at a rate of 15 mg/kg/day for 7 days (Dai et al., 2012; Dai et al., 2013a). Currently, the exact mechanism of colistin-induced neurotoxicity is still unclear.

Amino acid neurotransmitters are extensively scattered in the central nervous system, especially in cerebral tissues (Chen et al., 2014). Glu and GABA are the major excitatory and inhibitory amino acids in the brain, respectively and exert their functions by binding to the receptors. The Glu receptors such as NR1, NR2A, NR2B and GABA receptors, which mainly include GABAAR and GABABR are crucial to the function of the central nervous system. The balance between Glu and GABA is disturbed when the contents of amino acid neurotransmitters change significantly due to the damage in brain tissue (Hinzman et al., 2010). The effects of colistin on amino acid neurotransmitters are still unclear.

The BBB formed by endothelial cells lining the cerebral microvessels is a boundary between the peripheral circulation and the central

Abbreviations: BBB, blood-brain barrier; EB, Evans blue; TEM, transmission electron microscopy; HPLC, high-performance liquid chromatography; Glu, glutamate; GABA, gamma aminobutyric acid; GABAAR, gamma aminobutyric acid type A receptor; GABABR, gamma aminobutyric acid type B receptor; NMDA, *N*-methyl-D-aspartate; NR1, *N*-methyl-D-aspartate1receptor; NR2A, *N*-methyl-D-aspartate 2A receptor; NR2B, *N*-methyl-D-aspartate 2B receptor; FMOC-CL, 9-fluorenylmethyl chloroformate; SPE, solid-phase extraction; PEPT, polypeptide transporter; OCTN1, organic cation transporter 1; OCTN2, organic cation transporter 2; RIPA, radioimmunoprecipitation assay.

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Table 1
Primers used for quantitative real-time PCR.

Target gene	Primer	Sequence (5′–3′)	Length (bp)
GABAAR	Forward	GTGTTTGGATGGCAAGGACT	139
	Reverse	AAAGGCGGTAGGGAAGAAGA	
GABABR	Forward	TCTGGTTGTGCTCTTTGTGC	111
	Reverse	TCCTCATTGTTGTTGGTGGA	
NR2A	Forward	TGCCACAACGAGAAGAATGA	118
	Reverse	GCTCCCAGATGAAGGTGA	
NR2B	Forward	AAAGATGCCCACGAGAAA	106
	Reverse	AGATGCGGGTGATTATGCTC	
NR1	Forward	TGTTATGGCTTCTGCGTTGA	115
	Reverse	TGTTGTTTACCCGCTCCTGT	
Claudin-5	Forward	CAGTTAAGGCACGGGTAGCA	124
	Reverse	GGCACCGTCGGATCATAGAA	
β-actin	Forward	GGCTGTATTCCCCTCCATCG	154
	Reverse	CCAGTTGGTAACAATGCCAT	

nervous system (Jin et al., 2009). Tight junctions play a significant role in the integrity of the BBB (Liu et al., 2012). The main functions of tight junctions is to prevent the most harmful substances from invading the central nervous system, thus preventing damage and, maintaining stability and normal physiological functions of the central nervous system (Cardoso et al., 2010; Tam and Watts, 2010). Claudins are now believed to be a major constituent of tight junctions. A study of Claudin-5-deficient mice revealed that Claudin-5 is indispensable for the barrier function of neural blood vessels to small molecules (Nitta et al., 2003). The impact of colistin on the BBB is still unclear. Our recent study has shown that the half-life of colistin was longest in the brain (27.9 ± 9.1 h), suggesting that colistin may be accumulating in the brain (Lin, 2012). In the present study, we used a mouse model to investigate the brain uptake of colistin with repeated intravenous administration of this drug.

2. Materials and methods

2.1. Chemicals and reagents

Colistin sulfate (Lot: 095K1048, 20,195 U/mg), 9-fluorenylmethyl chloroformate (FMOC-CL), trichloroacetic acid, EB, Glu and GABA were obtained from Sigma-Aldrich (St. Louis, MO, USA). Acetonitrile, methanol, tetrahydrofuran and acetone of HPLC grade were all purchased from Sigma-Aldrich (St. Louis, MO, USA). Solid-phase extraction (SPE) cartridges (C18 Sep-Pak; 100 mg) were purchased from Waters Corporation, Milford, MA.

Primary antibodies against Claudin-5 were purchased from Wuhan Boster Bio-engineering Co. Ltd. (Wuhan, China). Anti-β-actin Rabbit monoclonal and secondary antibodies (horseradish peroxidase-labeled goat anti-rabbit IgG) were obtained from Beijing Zhongshan Golden Bridge Biotechnology Co. Ltd. (Beijing, China).

2.2. Experimental animals

All procedures involving animals were approved by the Animal Care and Use Committee of Heilongjiang Province, PR China. Female Kunming mice (supplied by the Harbin Veterinary Research Institute, China) at 8 week of age and weighing 18–20 g were kept in separate cages, had free access to feed and water and were adapted to housing and environmental conditions (temperature, humidity, light and ventilation) for at least 1 week prior to commencing treatments. Animal room temperature and relative humidity were set at 22 ± 2 °C and 55 ± 10%, respectively. Artificial light was provided for 12 h (7 a.m.–7 p.m.) followed by a period of 12 h in the dark. Mice were divided into four groups (n = 5) – one control group which received intravenous injection of saline solution, and three colistin-treated groups intravenously injected with 15 mg/kg/day of colistin sulfate for 1, 3 and 7 days successively. After 24 h of the last administration of colistin sulfate, mice were anesthetized with sodium pentobarbital (40 mg/kg, i.p.) and decapitated. The whole brain, the skull and cerebral dura mater were removed. Microsurgical tweezers were used to remove the cortex from the brain and the cortex was stored at −20 °C for the studies described below.

2.3. Assessment of EB extravasation

The assay of EB extravasation was used to evaluate BBB disruption after colistin treatment, as described previously (Huang et al., 2013). Briefly, 2% EB (4 mL/kg) was injected via the tail vein at 24 h after colistin treatment. At 2 h after EB injection, mice were anesthetized and perfusion was then performed through the left ventricle with phosphate-buffered saline to remove the intravascular EB dye, and this was continued until the fluid from the right atrium turned colorless. Tissue samples were collected from the cortex, mixed with 50% trichloroacetic acid and centrifuged. The absorbance of supernatants was measured at 620 nm with infinite M200PRO (TECAN, Austria).

2.4. Observation of the BBB ultrastructure

On days 1, 3 and 7 of colistin treatment, the cerebral cortices were obtained from each group for ultrastructural studies and examined, as has been described previously (Dai et al., 2013a). Briefly, small pieces of tissue were dissected from the cerebral cortex and cut into approximately1 mm cubes, then fixed in 2.5% glutaraldehyde and kept overnight at 4 °C in the fixative. Post-fixation was performed with 2% osmium tetroxide in 0.1 M cacodylate buffer (pH 7.4) for 2 h at 4 °C. The specimens were then dehydrated in graded acetone solutions and embedded in araldite. Ultrathin sections (50 nm) were prepared and counterstained with uranyl acetate and lead citrate, and examined with the JSM25610LV transmission electron microscope.

2.5. Real-time PCR examination

Total RNA was isolated using the TRIzol extraction method according to the manufacturer's instructions (Invitrogen Inc., Carlsbad, CA). The quality of RNA was verified by evaluating the optical density at 260 nm and 280 nm. The extracted RNA was reverse-transcribed into cDNA using a Prime Script reverse transcription-PCR kit (TaKaRa, Dalian, China) for Quantitative PCR. The primers (Table 1) used for all of the assayed genes were designed by Sangon Biotech (Shanghai, China). The mouse β-actin gene was used as an internal control. Data were analyzed and quantified using the $2^{-\Delta\Delta}$Ct method (Livak and Schmittgen, 2001).

2.6. Western blotting

The cortices from each group were obtained and total protein was prepared in radioimmunoprecipitation assay (RIPA) lysis buffer followed by centrifugation at 12,000*g* for 30 min at 4 °C. Equal amounts of protein, estimated using the BCA protein assay kit (Wuhan Boster Bio-engineering Limited Co., Wuhan, China), were separated by SDS-PAGE electrophoresis (12%) and transferred to nitrocellulose membranes (Millipore, Bedford, MA). The blots were blocked and then labeled with primary antibodies to Claudin-5 and β-actin (1:200 dilution) at room temperature for 1.5 h. The membranes were subsequently incubated with diluted horseradish peroxidase-conjugated anti-rabbit antibody at room temperature for 1 h (1:2000 dilution) at room temperature for 2 h. The immunoblots were visualized by enhanced chemiluminescence reagent. The autoradiogram was scanned and the protein bands were analyzed by densitometry using Image J (Version 1.42, National Institutes of Health, USA).