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Tropisetron attenuates lipopolysaccharide induced neuroinflammation by inhibiting NF-κB and SP/NK1R signaling pathway

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ABSTRACT

Tropisetron, an antagonist of serotonin type 3 receptors (5-HT_3Rs), has been investigated in colonic inflammatory process. Since substance P/neurokinin 1 receptor (SP/NK1R) signaling pathway plays a key role in several sensory neuronal inflammatory. We evaluated the anti-inflammatory activity of tropisetron in mice cerebral cortex, and discovered that it was a potential inhibitor in LPS-mediated neuron inflammation through SP/NK1R signaling pathway. We found that tropisetron significantly reduced the increased number of iba-1 positive microglia, down-regulated the gene transcription and protein expression of IL-1β,IL-6 and TNF-α in LPS stimulated cerebral cortex. To characterize the inhibitory mechanism of tropisetron at the SP response in inflammation, we further examined the effect of tropisetron on NF-κB and SP/NK1R signaling pathway in the process of mice cerebral cortex inflammation. We found that tropisetron inhibited the gene transcription and protein expression of NF-κB, SP, NK1R via inhibiting 5-HT_3R activity. These findings might provide new insights into the anti-inflammatory activities of 5-HT_3R inhibitor tropisetron, which would be the interaction of serotonin receptor signaling and SP/NK1R pathway. These might highlight their potential to design novel therapeutic strategies to manage inflammatory diseases.

1. Introduction

Bacterial infections of the CNS are serious and often intractable conditions affecting the meninges and the brain parenchyma. Neuronal damage involves mechanisms of promoting blood brain barrier (BBB) breakdown, augmenting the inflammatory response and enhancing excitotoxicity in the acute central nerous system (CNS) injury (Corrigan et al., 2016; Rasley et al., 2002). The classical inflammatory response is characterized by glial activation, proliferation of microglia, leukocyte recruitment and up-regulation and secretion of mediators such as cytokines and chemokines (Orihuela et al., 2016; Yao et al., 2017). Substance P (SP) is widely distributed throughout the CNS nervous systems and plays a crucial role in the inflammatory process(Johnson et al., 2016). It induces and augments many aspects of the classical inflammatory response including leukocyte activation, cytokine production and mast cell activation (Quinlan et al., 1999). In microglia, binding of SP to the NK1 receptor leads to activation of PLC, which allow mobilization of calcium from internal stores (O'Connor et al., 2004). Inhibition of NK1 receptor may ameliorate neuronal damage, which confirms the effect of SP on classical inflammation(Johnson, Young, 2016).

5-HT is a critical neuron transmitter in CNS. The pleiotropic activity of 5-HT is due to the molecular complexity of 5-HT receptors (5-HTRs) and their wide tissue expression. It is found in the immune-inflammatory axis and influences the immune response in mammals (Idzko et al., 2004). 5-HT_3 receptor (5-HT_3R), which is the only ionotropic receptor permeant to cations, has been found to be expressed in cells of the immune system including T lymphocytes, monocytes and dendritic cells. Activation of 5-HT_3 receptors promotes the secretion of IL-1β and IL-6, which in turn accelerates molecular and cellular inflammatory response (Fiebich et al., 2004; Vega Lde et al., 2005). Evidence exists that 5-HT can modulate the T cells functionality through activation of 5-HT_3R (Vega Lde, Munoz, 2005). Interestingly, 5-HT_3R antagonists have been investigated in inflammatory process in pain, ileus and colitis (Faerber et al., 2007; Maehara et al., 2015; Stratz et al., 2014; Utsumi et al., 2016). However, the anti-inflammatory effect on cerebral cortex and mechanism of the effect is still unclear.

Recent study has shown that the 5-HT/5-HT_3 receptor and SP/NK1 receptor pathways play pathogenic roles in colonic inflammation. 5-HT acts via 5-HT_3 receptors to up-regulate inflammatory mediators and promote colonic inflammation. These effects may be further mediated by activation of macrophage NK1 receptors via SP released from 5-HT_3 receptor-positive nerve fibers. 5-HT_3/NK1 receptor crosstalk has also been reported by several other laboratories (Darmani et al., 2011; Hu

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et al., 2004; Minami et al., 2001). 5-HT_3R inhibitor Palonosetron does not bind to the NK1 receptor directly but it inhibits the SP response through inhibition of receptor signaling crosstalk (Rojas and Slusher, 2012).

However, the underlying effect and mechanism of the 5-HT_3 receptor involved in the pathogenesis of neuroinflammation have not yet been defined. The present study aims to investigate the anti-inflammation of tropisetron in a murine model of LPS-induced inflammation. Tropisetron promotes anti-inflammatory responses in LPS-induced neuroinflammation by inhibiting NF-κB p65 phosphorylation and SP/NK1R signaling pathway. These experiments expand our understanding of the biological function of and the possible interaction between 5-HT_3R and SP/NK1 receptor signaling pathway.

2. Methods

2.1. Animal treatments

Male c57 background mice (16–18 g, 5 to 6 weeks-old) were purchased from the Shanghai Experimental animal center of Chinese academy of Sciences. Forty eight mice were randomly divided into three groups of 16 mice per group as follows: control, LPS and LPS + tropisetron (1 mg/kg) group. Mice were injected with LPS (0.5 mg/kg, 0.2 ml)(Lin et al., 2017) or normal saline(0.2 ml) by an intraperitoneal route. After 3 h, the mice were then injected with tropisetron (1 mg/kg, 0.2 ml) or normal saline (0.2 ml). Another 3 h later, the mice brain tissues in each group were collected for the following experiments.

Mouse experimental protocols were performed according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and permitted by the guide lines of the institutional Animal Care and Use Committee of the Fudan University.

2.2. Drugs and reagents

Lipopolysaccharide (LPS) from *Escherichia coli* 026:B6 and tropisetron were purchased from Sigma Chemical Company. TNF-a, IL-1β, IL-6 and substance P ELISA kits were from eBioscience. Trizol reagent was from Invitrogen Company. cDNA synthesis kit and Taq polymerase was from the Takara Biotechnology. Nucleotide sequences of the target primers in PT-PCR see Table 1.

2.3. Immunofluorescence staining for microglia and image analysis

Mice in each group were anesthetized, perfused with 0.9% saline, followed by buffered 4% paraformaldehyde, and the whole mice brains were progressively removed to 20% and 30% glucose solution to dehydration. Then the brains were frozen on a cold stage and sliced into 30 μm thickness using a cryostat for Immunofluorescence staining for Microglia. The brain slices were fixed with 4% PFA for 10 min, and then washed 3 times for 5mins. Blocked and incubated with an rabbit antibody against C-terminus of Iba-1 over night at 2–8 °C·The preparations were labeled with 1:250 diluted goat anti-rat IgG Alexa Fluor 488 secondary antibody (Cell Signaling Technologies) for 60 min at 37 °C. The nucleus of total cells was labeled with DAPI for 5 min after washing 3 times with TBST. All the pictures were taken by a fluorescent microscope (DM2500, Leica). The number of Iba-1 and DAPI positive cells was counted in different areas of cortex section by using Image J. The number of positive cells was calculated as mean of 3 fields/coronal slide. At least two slides were analyzed per mouse, and the average of the individual measurements was used to calculate group means.

Table 1
Primer sequence used for PT-PCR.

Gene	Oligonucleotide	Sequence
IL-1β	Forword primer	TGACGTTCCCATTAGACAGC
	Reverse primer	TGGGGAAGGCATTAGAAACA
TNF-a	Forword primer	CCAGACCCTCACACTCAGAT
	Reverse primer	AACACCCATTCCCTTCACAG
IL-6	Forword primer	TCTCTGGGAAATCGTGGAAA
	Reverse primer	GATGGTCTTGGTCCTTAGCC
SP	Forword primer	TCTGACCGTTACCATGAGCA
	Reverse primer	GGCAGGAGGAAGAAGATGTG
NK1R	Forword primer	GCAGCGTGTACAAGGTCAGC
	Reverse primer	AGGAGCCATTGGAGGTGAGA
GAPDH	Forword primer	ACCACAGTCCATGCCATCAC
	Reverse primer	TCCACCACCCTGTTGCTGTA

2.4. RNA isolation and quantitative real-time polymerase chain reaction (qRT-PCR)

Mice cortex tissues were collected after 6 h stimulation with LPS. Total RNA was extracted from mice cortex tissues using Trizol Reagent (Invitrogen). cDNA was synthesized from equivalent amounts of RNA with reverse transcription by using PrimeScript Reverse Transcriptase (Takara, Shiga, Japan). Quantitative real-time RT-PCR was carried out using ABI 7500 (Applied Biosystems, Foster City, CA,USA) with SYBR Premix ExTaq II (Takara). Specificprimer sets for β-actin, TNF-α, IL-6 and IL-1β were obtained from the Perfect Real-time Supporting System (Sangon Biotech). The expression level of each mRNA was calculated using the comparative ΔΔCT method, where signals were normalized to the value observed in the normal group.

2.5. ELISA analysis for IL-1β, IL-6, TNF-α and SP release

Total protein was collected from Mice brain tissues of cortex with iced-PBS buffer and analyzed with bicinchoninic acid method (BCA) protein determination kit according to the manufacturer's instructions. For the concentration of IL-1β, IL-6, and TNF-α, the ELISA kits (eBioscience, America) were used in accordance with the manufacturer's instructions.

2.6. Protein extraction and western blot analysis

Mice brain tissues of cortex were lysed in RIPA buffer containing a protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN) and PMSF. To determine the protein concentrations, a standard bovine serum albumin (BSA, Sigma) and BCA protein determination kit was applied according to the manufacturer's instructions. The total proteins in each sample (50 μg) were subjected to10% SDS-PAGE, and transferred the proteins to membranes using a semidry transfer apparatus (Bio-Rad). We then blocked the membranes with 5% milk for 1 h at room temperature and then incubated seperately with primary antibodies against Iba-1 (1:1000; Wako), NF-κB p52 (1:1000; CST), NF-κB p65 (1:1000; Proteintech), phospho-NF-κB p65 (1:1000; CST), Substance P (1:1000; Proteintech) and NK1R (1:500; Proteintech) over night at 4 °C. These primary antibodies were diluted in Tris-buffered saline (TBS) containing 0.1% Tween 20 (TBST) and 1% bovine serum albumin (BSA, Sigma). After washing 3 times, protein detection was done with HRP-coupled goat anti-rabbit IgG (1:5000, Jackson). This was performed by using chemiluminescence (ECL) reagents. The final quantification of the Western blots was done by using Image J (National Institutes of Health). Equal protein loading and transfer were measured by subjection of each sample to a Western blot for actin (rabbit anti-actin IgG, diluted 1:5000). All Western blot experiments were performed at a minimum of three experiments.

2.7. Statistical analysis

SPSS 17.0 software was used to compare the data between groups by one-way ANOVA. For all quantitative data, statistical analyses were performed using origin 8.0 software. All data are expressed as the mean ± SEM. Values of $P < .05$ were considered significant.

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