Lipopolysaccharide-induced cerebral inflammatory damage and the therapeutic effect of platelet activating factor receptor antagonist

Wen-Chao LIU, Wen-Long DING, Hong-Yu GU, Ming-Feng CHEN, Jin-Jia HU

Department of Anatomy, Medical College of Shanghai Jiaotong University, Shanghai 200025, China

Abstract: Objective To investigate lipopolysaccharide (LPS) induced acute cerebral inflammatory damage and the therapeutic effect of ginkgolide B (BN52021). **Methods** Thirty Sprague-Dawley rats were randomly divided into 3 groups ($n = 10$ for each group): Control group, Model group and Treatment group (treated with BN52021). LPS were injected into the fourth ventricle of rat to make a neuroinflammatory murine model. Morris water maze was used to detect the learning and memory ability of rats; changes of synapse number and subcellular ultrastructures were observed under a transmission electron microscope; OX-42 positive microglia in the brain was detected by immunohistochemical method. **Results** The average escape latency in the Treatment group were significantly shortened than that in the Model group; and the percentage of swimming distance traveled in platform quadrant accounting for total distance increased markedly. The rough endoplasmic reticulum and polyribosomes in the Treatment group were more than that in the Model group, but the number of synapses seemed to have no obvious change. The number of OX-42 positive microglia in the Treatment group decreased markedly than that in the Model group, and the grey density of OX-42-positive cells increased significantly. **Conclusion** LPS can induce inflammatory damages to the brain, but the damage could be antagonized by BN52021. Platelet activating factor receptor antagonist may offer an effective therapy for neurodegeneration diseases.

Keywords: brain inflammation; platelet activating factor; ginkgolide B; ultrastructure; microglia

1 Introduction

The incidence of Alzheimer's disease (AD) is rising continually in recent years, and AD is becoming one of commonly encountered diseases in elders^[1]. Although the pathogenesis of AD remains unclear, microglia-mediated neuroinflammation in the central nervous system is an accepted mechanism^[2]. When the toxicity substance A β 42 deposits in the brain, microglia will proliferate and release cytokines such as platelet activating factor (PAF), interleukin-1 (IL-1), tumor necrosis factor- α (TNF- α), and prostaglandins (PGs). The activated microglia and cytokines may damage the neurons and synapses in the brain and impair the learning and memory functions. Therefore, anti-inflammation is a main target for treatment of AD nowa-

days^[3].

PAF is a phospholipid substance and an important inflammatory factor and plays important roles in many brain diseases^[4]. Lipopolysaccharide (LPS) is a highly immunogenic component in the cell wall of Gram-negative bacteria. It activates gliocytes in the brain and induces the expression of many cytokines. Injection of LPS into the cerebral ventricles can activate gliocytes to produce the cytokines IL, TNF- α , PAF and PGs^[5]. The activated microglia and cytokines induced by LPS, similar to that induced by A β 42, may result in neuropathological and neurodegenerative disorders. In the present study, we want to investigate the morphological and functional changes of brain in rat suffering the LPS-induced acute cerebral inflammatory damage and to explore the neuroprotective effect of the PAF receptor antagonist ginkgolide B (BN52021).

2 Materials and methods

2.1 Animals, reagents and instruments Sprague-Dawley (SD) rats from the medical animal center of Shanghai

Corresponding author: Jin-Jia HU
Tel: 86-21-63846590-776474
E-mail: jjhu2609@126.com
Article ID: 1673-7067(2007)05-0271-06
CLC number: R742; R338.64
Document code: A
Received date: 2007-03-09

Jiaotong university, China; LPS (Sigma); mouse anti-rat CD11b monoclonal antibody (MRC OX-42, Sigma), horse-radish peroxidase enzyme labeling rabbit anti-mouse IgG (Bio-technology); Morris water video frequency analysis system (DigBehv-MM, Jiliang Soft Tech. Co.); stereotaxic instrument (NARISHIGE, Japan); frozen section instrument (AS620, England); DM2500 microscopy (Leica Co.); RS IMAGE analyzing software (Roper scientific). Ultrastructural observation and analysis were done by the Electron Microscopy Department of Shanghai Jiaotong University, China.

2.2 Animal preparation Thirty SD rats, approximately 5 months old and 250 g weight, were divided into 3 groups ($n = 10$ for each group): Control group, Model (LPS) group and Treatment group (treatment with BN52021). Rats were anesthetized with 20% urethane (1 300 mg/kg, i.p.) and placed in a stereotaxic instrument. The scalp was incised and retracted at 3 mm below the earbars (i.e. plat skull). A hole was drilled at appropriate location in the skull with a dental drill. LPS (10 μ g / 100 g) was dissolved in artificial cerebrospinal fluid (CSF) (120 mmol/L NaCl, 3.0 mmol/L KCl, 1.2 mmol/L CaCl_2 , 1.2 mmol/L MgCl_2 , 0.67 mmol/L NaH_2PO_4 , 0.3 mmol/L Na_2HPO_4 ; pH 7.4; 5 μ g/ μ L) and injected into the fourth ventricle of rat. Ten control rats were injected with the artificial CSF only. Subsequently, the treatment group rats were treated with BN52021 (dissolved in DMSO and then diluted in 0.01 mol/L PBS; 5 mg/kg, i.p.) once a day. Rats in Control group and Model group were treated with PBS only. The treatment persisted until the behavioral test was completed.

2.3 Behavioral test Morris water maze testing^[6] began at 3 d post-injection. Rats were introduced into the perimeter of a circular water-filled tank which was 180 cm in diameter and 30 cm in height, with a video camera suspended 170 cm above the liquid surface. A tracking software recorded the path of rat by light/dark contrast detection for the subsequent analysis. A square "escape platform" (9 cm $\times$ 9 cm) was placed 2 cm below water level in the second quadrant. The detection system was programmed to terminate the trial when animals reached the escape platform. Water temperature was maintained at 15 °C to 17 °C.

2.3.1 Navigational test Rats were put into the pool at four compass points (N, S, E, and W). The release positions around the perimeter of the maze were different for each trial, and the order was determined randomly. Rats who found the platform within 90 s remained on the platform for

30 s; those who did not find the platform were also placed on the platform for 30 s, and the time was counted as 90 s. The evasive latency was used for statistics. The test was carried out once a day, and lasted for 4 d.

2.3.2 Probe test The platform was removed at the day following the navigational test. Rats were placed in the center of the pool and made to swim for 90 s to locate the platform by memory. The percentage of swimming distance traveled in platform quadrant and the times of rat passing through the platform was recorded.

2.4 Processing of brain tissue Following water maze testing, rats were killed under pentobarbital anesthesia. The rats were intracardially perfused with cold saline, then perfused with 4% paraformaldehyde / 0.1 mol/L sodium phosphate buffer (pH 7.3). After fixation, brains were removed and post-fixed in 4% paraformaldehyde / 0.1 mol/L sodium phosphate buffer for 1 h, then cryoprotected in 0.1 mol/L of sodium phosphate buffer containing 20% sucrose for 24 h at 4 °C, and finally snap-frozen by transferred into isopentane (-50 °C) and stored at -70 °C. Each frozen brain was cut at 15 μ m on the coronal plane with a cryostat for histological analysis.

Two rats in each group were decapitated for ultrastructural observation. The brains were removed, sectioned into 2-mm slabs, and then placed in fixative solution of 2% sodium cacodylate-buffered glutaraldehyde (pH 7.4) for 6 h. The tissues were then rinsed in buffered solution of saccharose, post-fixed with 1% phosphate-buffered OsO_4 (pH 7.4), dehydrated with acetone, and embedded in Epon by using flat molds. The semi-thin sections which were cut by an ultramicrotome were stained with toluidine blue solution and photographed under a light microscope. The ultra-thin sections of selected areas were cut by a diamond knife and stained with alcoholic solution containing uranyl acetate, followed by concentrated bismuth subnitrate solution. These sections were examined and photographed under an electron microscope. Ten ranges of vision were randomly selected from two slices of each group, and the number of synapses were counted under a transmission electronic microscope (24 500 $\times$).

Since activated microglia rapidly upregulates the expression of tissue antigens following inflammation, it could be visualized by a monoclonal antibody against OX-42 (1:400 dilution). Two slices in each rat for OX-42 immunohistochemical reaction were ever used, two visual fields in the cortex around olfactory bulb, hippocampus (hippo),

dentate gyrus (DG), diagonal band (DB), amygdala (AG), and septal nuclei (SN) of each slice were selected to be photographed under the DM2500 Leica microscope. Cell number and cell density were analyzed by the RS IMAGE software.

2.5 Data analysis All of the experimental data were disposed by SPSS11.5 software. Data of image analysis including number of synapses and grey density were presented as mean $\pm$ SD and analyzed with one way ANOVA. $P < 0.05$ was considered statistically significant.

3 Results

Rats lived normally and no obviously weight lost after LPS injection.

3.1 Behavioral testing

3.1.1 Navigational test The average escape latencies of three groups following the 4-d test are shown in Fig. 1. With the learning time extension, the average escape latency for the Control group and the Treatment group decreased obviously compared with the Model group. Rats in the Model group was also showed gradually decreased average escape latency as the learning time increasing, but they showed lower learning abilities than the Control group and the Treatment group rats.

3.1.2 Probe test The percentages of swimming distance traveled in platform quadrant in the three groups are shown in Fig. 2; and the times of rat passing through platform are shown in Fig. 3. The percentages of swimming distance traveled in platform quadrant for rats in Control group and Treatment group were obviously higher than that in Model group. Times of rat passing through platform in Control

Fig. 1 Mean escape latency of rats in each group. On the fourth day, the average latency in Control group or Treatment group decreased obviously compared with that in Model group. $*P < 0.01$ vs Model group.

Fig. 2 Percentage of swimming distance traveled in platform quadrant in Control group or Treatment group was much higher than that in Model group. $*P < 0.05$, $**P < 0.01$ vs Model group.

Fig. 3 Times of rat passing through platform in Control group or Treatment group increased significantly than in Model group. $*P < 0.05$ vs Model group.

group and Treatment group were also obviously more than that in Model group. The results indicated that those rats in Control group and Treatment group had higher memory abilities than the rats in Model group.

3.2 Ultrastructural changes In Control group, the cytoplasm of the hippocampal neurons was filled with polyribosomes and rough endoplasmic reticulum (RER) cisternae. The Golgi complexes were numerous and prominent. In Model group, on the contrary, the cytoplasm of the hippocampal neurons had an empty aspect and contained much fewer polyribosomes and RER cisternae. The Golgi complexes remained small. In Treatment group, the cytoplasm of the hippocampal neurons was similar to the control, there were many polyribosomes and RER cisternae, and the Golgi complexes were prominent as well. The appearance of the mitochondria, lysosomes, lipofuscin pigment and cytoskeleton apparatus did not differ among the three groups. The number of synapses in Control group, Model group and Treatment group under the $24\ 500\times$ electron microscope visual field were 18.0 ± 2.3 , 16.3 ± 2.3 , and 17.3 ± 2.2 , respectively, having no obviously differences among the

Fig. 4 Ultrastructure of the hippocampal neurons. **A:** Control group, many polyribosomes and rough endoplasmic reticulum (RER) cisternae in the cytoplasm. **B:** Model group, fewer polyribosomes and RER cisternae than in the Control group. **C:** Treatment group, polyribosomes and RER cisternae more than in Model group. **D, E and F** shows the intercellular space of Control group, Model group and Treatment group, respectively. We can see from the pictures that there are many synapses in the three groups but no obvious differences among them. Scale bar, 2 μ m.

Fig. 5 Microglia stained by OX-42 antibody in the cortex around olfactory bulb. **A:** Control group, fewer microglia, and fewer but long processes. **B:** Model group, bigger and more microglia, and more but shorter processes of microglia. **C:** Treatment group, similar morphological features of microglia to the Control group. Scale bar, 50 μ m.

Tab. 1 Comparison of the grey density and the number of OX-42-positive microglia in different parts of the brain among groups

	Grey density			Cell number		
	Control	Model	Treatment	Control	Model	Treatment
Cortex	164.49 $\pm$ 11.36**	145.08 $\pm$ 6.49	159.28 $\pm$ 7.78**	17.30 $\pm$ 1.49**	21.50 $\pm$ 2.22	18.50 $\pm$ 1.58**
Hippo	159.23 $\pm$ 14.29**	147.24 $\pm$ 6.36	156.10 $\pm$ 2.99*	13.53 $\pm$ 1.87*	15.89 $\pm$ 1.57	14.39 $\pm$ 1.41
DG	159.08 $\pm$ 13.68*	147.46 $\pm$ 9.64	157.26 $\pm$ 8.96*	13.80 $\pm$ 1.69**	17.30 $\pm$ 1.34	14.40 $\pm$ 1.58**
DB	153.97 $\pm$ 4.92**	143.42 $\pm$ 5.81	151.75 $\pm$ 6.91**	15.67 $\pm$ 1.58**	20.22 $\pm$ 2.22	15.89 $\pm$ 1.90**
AG	161.67 $\pm$ 11.05**	150.04 $\pm$ 8.75	156.16 $\pm$ 10.53*	14.20 $\pm$ 1.75**	17.60 $\pm$ 1.26	14.20 $\pm$ 1.32**
SN	162.91 $\pm$ 7.70**	147.06 $\pm$ 9.34	152.37 $\pm$ 9.04**	10.11 $\pm$ 1.36**	14.56 $\pm$ 1.51	10.89 $\pm$ 1.69**

* $P < 0.05$, ** $P < 0.01$ vs Model group.

three groups (Fig. 4).

3.3 Expression of OX-42 in microglia Immunohistochemical staining for OX-42 revealed that the microglia within the cerebral cortex around olfactory bulb, hippo, DG, DB, AG

and SN developed greatest inflammatory responses following acute exposure to LPS. There were relatively fewer microglia in the Control group, and the processes of microglia were fewer but longer. In the Model group, the micro-

glia were numerous and characterized by contraction of highly ramified processes, giving them a bushy morphological feature. The Treatment group appeared to have fewer positive microglia than the Model group. Figure 5 shows the positive microglia in the cerebral cortex around olfactory bulb.

4 Discussion

4.1 Neuroinflammation and AD The underlying mechanisms of AD involve the neuroinflammation induced by amyloid- β . The neuroinflammation gives rise to impairments in neurons, including cholinergic neurons^[7], and inhibiting neuroinflammation seems the main therapy of AD in clinical practice at present.

It is reported that chronic infusions of LPS may impair spatial memory, destroy cholinergic cells, activate astrocytes and microglia, thus induce neuroinflammation^[8]. The central inflammatory response induced by infusion of LPS into the fourth ventricle is associated with an increase of activated microglia, which distributes throughout the brain. The greatest inflammatory response occurs within the cortex around olfactory bulb, hippo, DG, DB, AG, and lateral SN, all of which are the areas closely associated with learning and memory. The activated microglia release inflammatory factors such as IL-1 α , IL-1 β , TNF- α , PAF and prostaglandins, and induce neuron apoptosis. The combination of LPS with CD-14 receptor in cell membrane can activate microglia, and then startup a series of signal transmits mechanism^[9]. Neuron apoptosis often appears in the hippocampus, temporal cortex and basal forebrain, similar to the impairment areas of AD.

In this study, the neuroinflammatory murine model was used to explore the relationship between the cognitive function and the neuropathological changes after injection of LPS into the forth ventricle^[10]. The results showed that LPS infusions impaired the spatial memory ability of experimental rats.

Immunohistochemical test showed a significant increase of the activated microglia in the brain of LPS-treated rat. Our data are consistent with the hypothesis that long-term inflammation within the brain may lead to degenerative changes, particularly within the cortex around olfactory bulb, hippo, DG, DB, AG and SN. These degenerative changes might underlie the age-associated deficit in spatial memory ability, which has been widely documented in rats^[11]. The greatly enhanced inflammatory response within

the cortex around olfactory bulb, hippo, DG, DB, AG and SN in this report might explain why these regions are associated with such profound pathological changes in AD patients.

The electron microscopic observation revealed numerous changes in the cellular components which are involved in protein synthesis within the hippocampal neurons of LPS-treated rats. These changes probably underlie the significant learning and memory impairments. The cytoplasm of microglia in the LSP-induced group contained few polyribosomes, few rough endoplasmic reticulum cisternae and a small Golgi apparatus, all of which suggested that the function of protein synthesis was impaired or reduced. These ultrastructural changes inside cytoplasm may represent an attempt of compensation for impaired protein synthesis^[8].

4.2 Relationship of PAF and its receptor with AD PAF exerts many physiological functions, such as relaxing blood vessels and decreasing blood pressure^[11]. As an important inflammatory factor, PAF activates many kinds of inflammatory reactive cells, produces cytokines, and amplifies inflammatory reaction. PAF production increasing is implicated in the pathogenesis of AD^[12]. PAF directly and indirectly increases glutamate content in the extracellular space, leading to stimulation of glutamate and NMDA receptors, which result in ultimate neuron death^[13]. So theoretically, PAF receptor inhibitor can provide an effective treatment to protect neurons in the AD brain. In the present study, PAF receptor inhibitor BN52021 was used as the treatment medicine. After BN52021 was administrated to the LPS-treated rats, the average escape latency decreased, while the percentage of swimming distance traveled in platform quadrant and the times of rat passing through platform increased. The results proved that PAF receptor antagonist could prevent the cognition impairment induced by LPS.

In the BN52021-administrated rats which were pretreated with LPS, the number of polyribosomes and rough endoplasmic reticulum cisternae in the hippocampal neurons increased and the size of Golgi apparatus was enhanced, indicating that BN52021 may attenuate the dysfunction of protein synthesis. The results explained the protective effect of PAF receptor antagonist on the learning and memory functions of AD model rats.

OX-42 immunohistochemistry showed that BN52021 decreased the number of LPS-activated microglia, indicating that PAF receptor could control the neuroinflammation

in the brain, which might be the pharmacological mechanism of BN52021's therapeutic effect on AD.

Since PAF receptor antagonist provides an effective protection against the neurotoxicity induced by LPS, it may be a potential therapy for neuroinflammatory diseases and will be widely used in clinical trials for neurodegenerative diseases such as AD.

Acknowledgements: This work was supported by the Research Foundation of the Medical College of Shanghai Jiaotong University, China (No. 2005JY05).

References:

- [1] Pavlik VN, Doody RS, Massman, Chan W. Influence of premorbid IQ and education on progression of Alzheimer's disease. *Dement Geriatr Cogn Disord* 2006, 22: 367-377.
- [2] Walker DG, Link J, Lue LF, Dalsing-Hernandez JE, Boyes BE. Gene expression changes by amyloid β peptide-stimulated human postmortem brain microglia identify activation of multiple inflammatory processes. *J Leukoc Biol* 2006, 79: 596-610.
- [3] Gasparini L, Ongini E, Wilcock, Morgan D. Activity of flurbiprofen and chemically related anti-inflammatory drugs in models of Alzheimer's disease. *Brain Res Brain Res Rev* 2005, 48: 400-408.
- [4] Herschkowitz M, Adunsky A. Binding of platelet-activating factor to platelets of Alzheimer's disease and multiinfarct dementia patients. *Neurobiol Aging* 1996, 17: 865-868.
- [5] Pang Y, Cai Z, Rhodes PG. Disturbance of oligodendrocyte development, hypomyelination and white matter injury in the neonatal rat brain after intracerebral injection of lipopolysaccharide. *Brain Res Dev Brain Res* 2003, 140: 205-214.
- [6] Zink WE, Anderson E, Boyle J, Hock L, Rodriguez-Sierra J, Xiong H, *et al.* Impaired spatial cognition and synaptic potentiation in a murine model of human immunodeficiency virus type 1 encephalitis. *J Neurosci* 2002, 22: 2096-2105.
- [7] Xu J, Qian CY. Excitotoxic neurodegeneration in Alzheimer disease. *Chin J Neurosci* 2000, 16: 379-382.
- [8] Hauss-Wegrzyniak B, Vannucchi MG, Wenk GL. Behavioral and ultrastructural changes induced by chronic neuroinflammation in young rats. *Brain Res* 2000, 859: 157-166.
- [9] Liu Y, Walter S, Stagi M, Cherny D, Letiembre M, Schulz-Schaeffer W, *et al.* LPS receptor (CD14): a receptor for phagocytosis of Alzheimer's amyloid peptide. *Brain* 2005, 128 (Pt 8): 1778-1789.
- [10] Hauss-Wegrzyniak B, Lukovic L, Bigaud M, Stoeckel ME. Brain inflammatory response induced by intracerebroventricular infusion of lipopolysaccharide: an immunohistochemical study. *Brain Res* 1998, 794: 211-224.
- [11] Leutgeb A, Mizumori SJ. Excitotoxic septal lesions result in spatial memory deficits and altered flexibility of hippocampal single-unit representations. *J Neurosci* 1999, 19: 6661-6672.
- [12] Kondratskaya EL, Pankratov YV, Lalo UV, Chatterjee SS, Krishtal OA. Inhibition of hippocampal LTP by ginkgolide B is mediated by its blocking action on PAF rather than glycine receptors. *Neurochem Int* 2004, 44: 171-177.
- [13] Xu Y, Tao YX. Involvement of the NMDA receptor/nitric oxide signal pathway in platelet-activating factor-induced neurotoxicity. *Neuroreport* 2004, 15: 263-266.

脂多糖诱导的大鼠脑急性炎性损伤及血小板活化因子受体拮抗剂的干预治疗效果

刘文超, 丁文龙, 顾红玉, 陈明峰, 胡金家

上海交通大学医学院解剖学教研室, 上海 200025

摘要: 目的 研究脂多糖(lipopolysaccharide, LPS)诱导的脑内炎性损害以及银杏内脂B(BN52021)的干预治疗效果。方法 Sprague-Dawley 大鼠 30 只, 随机分为对照组, 模型组和治疗组(BN52021 治疗), 每组 10 只。第 IV 脑室注射 LPS 造模, Morris 水迷宫检测实验动物学习和记忆能力; 透射电子显微镜观察海马神经元突触数量及亚细胞结构的变化; 免疫组织化学法检测脑内 OX-42 在小胶质细胞内的表达。结果 治疗组大鼠的水迷宫逃避潜伏期比模型组显著缩短, 平台象限游泳距离百分比显著增加; 治疗组大鼠海马神经元内质网和核糖体数量比模型组明显增加, 突触数量则无明显变化; 治疗组大鼠脑内的 OX-42 阳性小胶质细胞数量比模型组明显减少, 染色灰度上升。结论 LPS 可诱导脑内炎性损害, 血小板活化因子受体拮抗剂 BN52021 对 LPS 诱导的脑内炎性损害具有保护作用, 提示血小板活化因子受体拮抗剂对以中枢炎症为病理特征的神经退行性变有治疗作用。

关键词: 神经炎症; 血小板活化因子; 银杏内脂 B; 超微结构; 小胶质细胞