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Original Article

LPS induces dopamine depletion and iron accumulation in substantia nigra in rat models of Parkinson's disease

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Abstract: Objective: Intrapallidal inflammation may lead to the pathogenesis of Parkinson's disease. Pathological changes caused by lipopolysaccharide (LPS)-induced inflammation in Parkinson's disease rat models were largely unknown. Methods: Male Sprague-Dawley rat models were intra-globuspallidus injected with saline and lipopolysaccharide and divided into two groups, the control group and the LPS-stimulation group. The locomotor activity of the rat models was recorded for 4 consecutive weeks by trajectory analysis software for animal behavior. For the evaluation of pathological profiles, the expression levels of tyrosine hydroxylase and OX-42 in the substantia nigra tissues were detected by immunohistochemical staining. Also, the concentrations of dopamine at specific sites were detected through high-performance liquid chromatography. Perl's iron staining was used to evaluate iron accumulation in substantia nigra tissues. Results: LPS-stimulation reduced the locomotor capacity of the rat models compared with the control group. The density of tyrosine hydroxylase-positive cells was reduced and the secretion of striatal dopamine in the substantia nigra pars compacts was lower in the LPS group than it was in the control group. OX-42 positive microglia and ferritin levels were enhanced in the LPS group. Conclusion: Intrapallidal inflammation by LPS induced dopamine depletion and iron accumulation in the substantia nigra of Parkinson's disease rat models. The management of cerebral inflammation might be pivotal for PD pathogenesis and prognosis.

Keywords: Parkinson disease, lipopolysaccharide, inflammation, iron

Introduction

Parkinson's disease (PD) is a neurological disorder characterized by a progressive degeneration of dopamine neurons in the substantia nigra pars compacta (SNc) and the loss of predominant dopamine in the striatum [1, 2]. Based on the fact that dopamine modulates neuronal function in the striatum [3, 4], in clinical practice, therapies including dopamine replacement and updated neurosurgical techniques by subthalamic nucleus (STN) high-frequency stimulation in PD have been developed [5-9]. In the past over 2 decades, data from clinical trials have shown the involvement of dopamine in the functional regulation of the basal ganglia [10, 11]. However, dopamine-associated pathophysiological profiles in PD still remain a great challenge.

In PD patients, abnormal activations of substantia nigra and increased levels of inflammatory cytokines have been observed. Increased superoxide dismutase (SOD) and decreased reduced-glutathione (GSH) and catalase are characteristic evidence of local inflammatory oxidative stress. Free radicals generated by oxidative stress play a major role in the dampening of adjacent tissues. On the other hand, the enhancement of cerebral iron storage in PD patients is widely recognized [12-14]. Iron promotes the generation of oxidative free radicals and the process of neuronal degenerative diseases [15-17]. A high concentration of iron can accelerate neuron death by enhancing the processes of lipid peroxidation and free radical generation. Thus, inflammation associated iron accumulation might induce oxidative stress and contribute to the pathogenesis of PD.

The lipopolysaccharide (LPS)-induced PD rat model is a feasible and powerful instrument for laboratory investigation [18, 19]. Dopamine controls both GABAergic and glutamatergic sig-

naling inputs via D1 and D2 receptors [20-24]. Meanwhile, GP neurons transmit inhibitory GABAergic projections to the STN and the substantia nigra pars reticulata (SNr) [25, 26]. Recently, Abedi et al. reported that intrapallidal administration of 6-hydroxydopamine would mimic in large part the electrophysiological and behavioral consequences of major dopamine depletion in the classical rat model of Parkinson's disease [27], supporting the notion that dopamine mediates actions in the striatum and regulates biological processes in basal ganglia circuitry. However, inflammation-induced iron-mediated pathological changes in substantia nigra have not been well investigated in these PD models.

The present study investigated the effects of dopamine neurons on free iron and ferritin expression in substantia nigra by a classical rat model, aiming to decipher the impacts of LPS-induced inflammation on the pathogenesis of PD.

Materials and methods

Animals

Adult male Sprague-Dawley rats that weighed 190-210 g were used in this study. The animals were housed three per cage, kept under artificial light (12 h light/dark cycle, lights on at 7:00 A.M.), with a temperature of 24°C and the humidity at 45%, with food and water available *ad libitum*. All efforts were made to minimize the number of animals used and their suffering. The Experiments were carried out in accordance with the European Communities Council Directive (*EU Directive 2010/63/EU*) and the National Institutes of Health's Guide for the Care and Use of Laboratory Animals.

LPS injection into the GP

The rats were placed in a stereotaxic frame (Kopf, Unimecanique, Beijing, China) under xylazine (xylazine hydrochloride, 10 mg/kg, i.p, Sigma, China) and ketamine anesthesia (ketamine hydrochloride 75 mg/kg, i.p., Sigma, China). Each animal received a lateral injection of 4-μl LPS (2.5 g/L in sterile NaCl, 0.9%; Sigma, China) into the GP at coordinates 0.9 mm posterior to bregma, 3.0 mm lateral to the midline and 6.5 mm below the dura, according to the brain atlas of Paxinos and Watson (1998). The LPS injection was made over a 5-min period using a cannula connected by polyethylene tubing to a 10-μl Hamilton microsyringe. At the end of each injection, the cannula was left in place for an addition 5 min to prevent a reflux of the solution and to allow for a toxin diffusion, and then was withdrawn slowly. For the sham group, the vehicle (saline plus 0.01% ascorbic acid) was infused into the GP under the same conditions as the LPS. Twenty rats were used and distributed into two groups, the LPS-treated group (n=10) and the sham group (n=10). All the rats were tested for locomotor activity before surgery and at 7 days, 14 days, 21 days, and 28 days after surgery. At the end of the locomotor data recording period, all the animals were sacrificed, and their brains were used for histological and biochemical studies.

Locomotor activity

Spontaneous horizontal motor activity on lidless cages (50 cm×50 cm) was recorded using digital photography (Sony, Japan). Images from 30 min of the active trajectories of the rats were collected. We tested the 30-minute movement distance (cm) and the movement speed (cm/s) using trajectory analysis software for animal behavior (EthoVision, version 2.3, Noldus, Holland). One frame was recorded every six seconds. All testing was done in an isolated room at 10:00 A.M.

Preparation of immunohistochemical sections

The sham rats (n=5) and the LPS-treated rats (n=5) were anesthetized with ureethane (1.2 g/kg). Then we opened the sternum and exposed the heart of each rat. The puncture needle was directly inserted into the ascending aorta through the left ventricle, and the right atrial appendage was cut. Then we rapidly washed each rat with 200 ml of saline, and the color of the liver became white. Then at 4°C, 400 ml 40 g/L of paraformaldehyde was perfused and fixed for 1 hour, and then the rats was sacrificed, and the midbrain was cut. After the midbrain was soaked in a 40 g/L poly formaldehyde solution fixation for three or four hours, the midbrains were placed in a 0.1 mol/L phosphate buffer containing 30% sucrose at 4°C overnight, allowing the specimen to sink to the bottom. The brain tissue samples were washed in a 0.01 mol/L phosphate buffer with continuous coronal sections (the thickness of 30 μm) prepared by a frozen section machine (Leica Corporation, Germany). From the onset of sub-

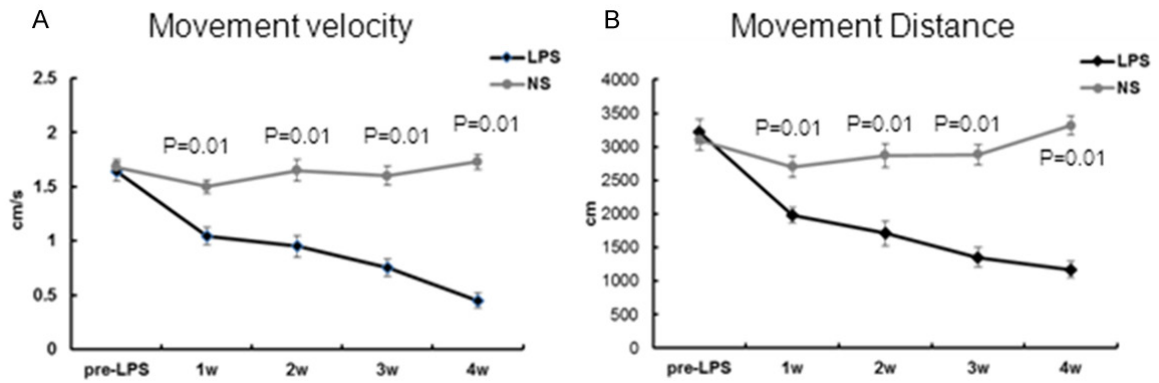

Figure 1. The locomotor capacity of rat models was dampened by LPS stimulation over time. A. The mean movement velocity was reduced in the LPS-treated group. B. The mean movement distance was also reduced in the LPS-treated group.

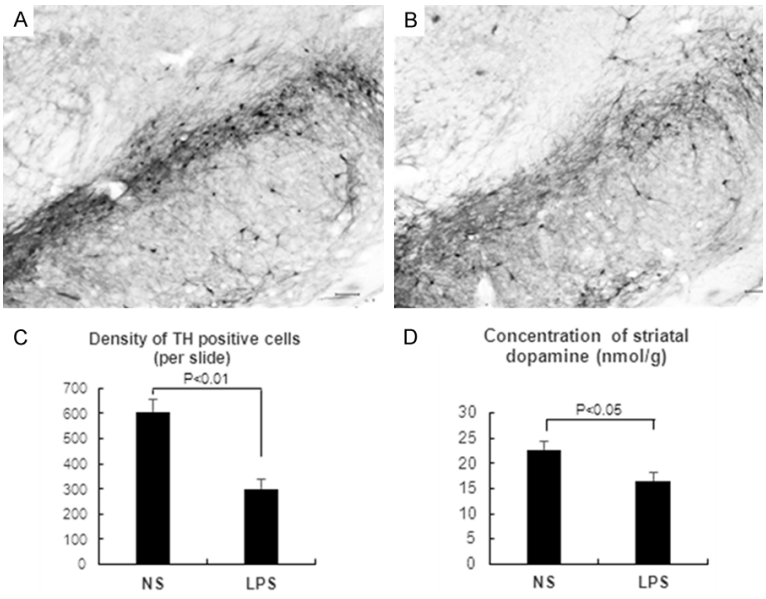

Figure 2. LPS-stimulation reduced tyrosine hydroxylase positive cell amounts in the substantia nigra pars compacts. A. TH positive cells in the control group; B. TH positive cells in the LPS group (×50). C and D. The density of TH positive cells and the concentration of striatal dopamine in the control and LPS groups.

stantia nigra, every 6th slice was treated with TH, OX-42, iron staining.

Immunohistochemical analysis

Immunohistochemistry detection was applied for the expression of TH, OX-42, and ferritin in the tissues. Briefly, antigens were unmasked by microwaving sections in a 10 mmol/L citrate buffer for 15 minutes, and immunostaining was undertaken using the avidin-biotinylated enzyme complex method with antibodies against TH at 1 mg/mL, OX-42 at 1 mg/mL, and equivalent concentrations of polyclonal nonimmune IgG controls. After incubation with a biotin-conjugated secondary antibody and subsequently with a streptavidin solution, color development was performed with 3,3-diaminobenzidinetetrahydrochloride (Vector Laboratories) as a chromogen. The sections were counterstained using Gill-2 hematoxylin (Thermo-Shandon, Pittsburgh, PA). After staining, the sections were dehydrated through increasing concentrations of ethanol and xylene. The Leica Qwin image analysis system was used to observe and count the morphology.

High-performance liquid chromatography (HPLC) measurements

The effectiveness of the LPS stimulation was confirmed by measuring the DA concentrations at specific brain sites. Sham (n=5) and LPS-treated (n=5) rats were sacrificed, the brain removed and stored at -80°C until analysis. The fresh frozen brains were cryostat-cut at -20°C and the bilateral regions of the striatum were punched. Punches were then stored at -80°C until biochemical assessments were done. The samples were homogenized in perchloric acid. Homogenates were centrifuged, and the supernatant was assessed for dopamine content by means of reverse phase HPLC with electro-

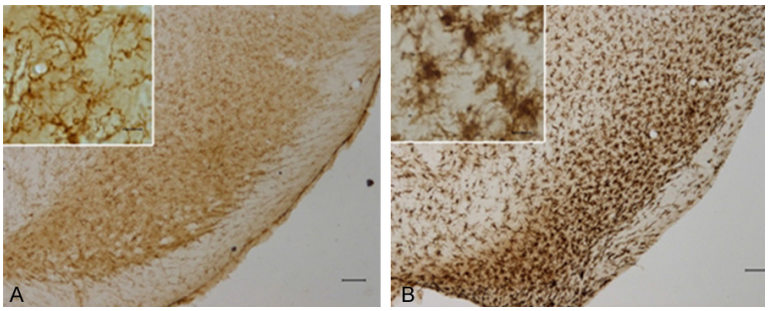

Figure 3. OX-42 positive microglia cells in the substantia nigra pars compacts. The left panels (A) showed a representative result of the OX-42 staining of the substantia nigra pars compacts from the control group, while the right panels (B) showed a representative result of the OX-42 staining of the substantia nigra pars compacts from the LPS group (×50 for the base images, and ×400 for the zoomed images).

chemical detection, as previously described [28, 29]. The mobile phase consisted of NaH_2PO_4, disodium EDTA, octane sulfonic acid and methanol, adjusted to PH 3.9 with orthophosphoric acid. The potential of the electrodes was set at +350 and -270 mV, and the results were expressed as ng/g of tissue. Each value corresponds to the mean ± SEM. Values were compared using the Mann-Whitney *U* test (Prism, GraohPad software, San Diego, CA, USA).

Perl's iron staining

Iron staining was performed as previously described [30]. Briefly, the slides were transferred to 100% ethanol. Individual lobe biopsy materials were placed in processing cassettes for 30-60 seconds, and then hydrated through a serial alcohol gradient at 100%, 100%, 95%, 80%, 70%. The tissue sections were immersed in xylene for 2 min. Then for 30 min they were incubated with 20 g/L hydrochloric acid and ferric ferrocyanide in a solution of 1:1. After rinsing with water and neutral red complex dyeing, the sections were dehydrated through serial ethanol solutions (70%, 80%, 95%, 100%, 100%) each for 30 seconds, respectively. Finally, the slides were input for xylene transparentation and neutral balata fixation.

Results

LPS-stimulation reduced locomotor capacity of rat models

After the intra-globus pallidus injection of saline, the movement velocity of the rat models in the control group showed no significant change at 1 week, 2 weeks, 3 weeks and 4 weeks, compared with the velocities before injection. For the rats in LPS group, after an intra-globus pallidus injection of LPS, the velocity of movement was gradually and significantly reduced from 1.04± 0.08 cm/sec at 1 week, to 0.95±0.1 cm/sec at 2 weeks, to 0.75±0.08 cm/sec at 3 weeks and 0.45±0.07 cm/sec at 4 weeks, compared to a median velocity of 1.5 cm/sec before injection and in the control group (P=0.01, **Figure 1A**). Accordingly, the 30-minute movement distance was significantly shortened in the LPS group, from 1700 cm at 1 week to 1500 cm at 2 weeks, 1000 cm at 3 weeks and 800 cm at 4 weeks in the LPS group, while the distance was maintained at 3000 cm in the control group at those timepoints (P=0.01, **Figure 1B**). These data demonstrated that LPS-stimulation dampened the movement capacity of the rat models, and such an change was progressively devastating over time.

Tyrosine hydroxylase positive cell amounts in substantia nigra pars compacts and the striatal dopamine concentration deceased after LPS-stimulation

Immunohistochemistry of the substantia nigra pars compacts at 4 weeks showed that the density of the TH positive cells in the LPS group was 300.2±3 6.2 per slide, significantly less than the level of 607.9±51.2 per slide in the control group (P<0.01, **Figure 2**). The concentration of striatal dopamine was 22.79±1.56 nmol/g in the LPS group, significantly lower than the level of 16.49±1.79 nmol/g in the control group (P<0.05). Tyrosine hydroxylase positive cell amounts in the substantia nigra pars compacts and the striatal dopamine concentration deceased after LPS-stimulation.

LPS stimulation promoted the proliferation of microglia in substantia nigra pars compacts

After 4 weeks of LPS-stimulation, the OX-42 positive microglia cells were morphologically

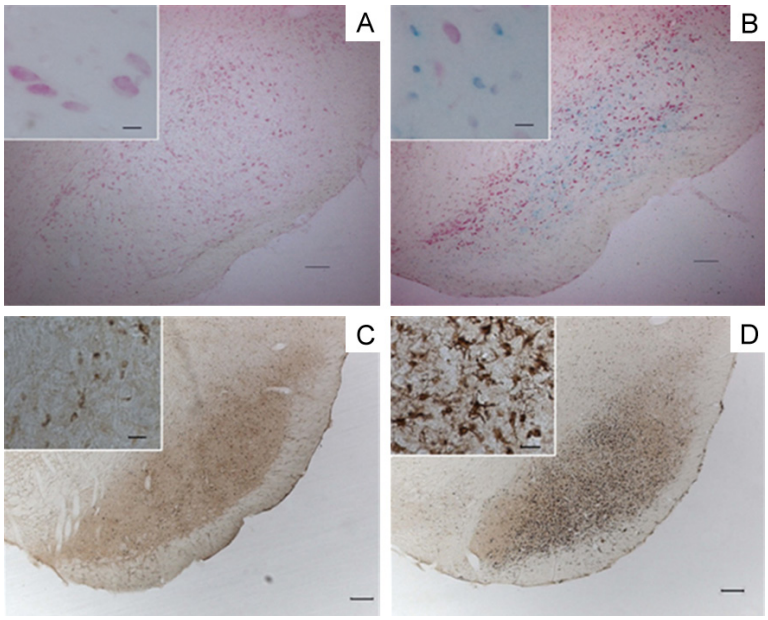

Figure 4. Iron metabolism altered by LPS stimulation. A. Free iron staining showed a pale color in the substantia nigra pars compacts of the control group. B. Free iron staining was obviously increased in the LPS group. C. The immunohistochemical staining of ferritin showed sporadic dots in the substantia nigra pars compacts from the control group. D. Immunohistochemical staining of ferritin showed thick dots in the substantia nigra pars compacts from the LPS group. The data were obtained from representative samples (×50 for the base images, and ×400 for the zoomed images).

enlarged, with shortened axons and hyperchromatic nuclei on the slides. The OX-42 positive microglia cells showed longer axons with pale nuclei. The density of the OX-42 positive cell was more enhanced in the LPS group than it was in the control group (**Figure 3**).

LPS stimulation caused iron accumulation in the substantia nigra pars compacts

After 4 weeks of LPS stimulation, free ions increased in the substantia nigra pars compacts in the LPS group (**Figure 4A**), compared with almost no iron stains in the control group (**Figure 4B**). Meanwhile, the expression of ferritin in the substantia nigra pars compacts in the LPS group was also enhanced (**Figure 4C**), compared with the control group (**Figure 4D**).

Discussion

In this study, we established a rat model through the intrapallidal administration of LPS to explore the effects of inflammation-induced pathological changes in the substantia nigra. Our results showed that LPS-induced inflammation caused dopamine depletion and iron accumulation in the substantia nigra and dampened the movement capacity of rat PD models.

Our data clearly demonstrate that LPS-induced inflammation gradually dampened the capacity of movement in the rat models. Inflammation in the peripheral nervous system and the central nervous system (CNS) is believed to be one of the causes underlying PD. Loss of midbrain dopaminergic (DA) neurons and a decrease in dopamine levels, accompanied by a concomitant local neuroinflammatory response, contribute to PD pathogenesis. Toxin-based animal models have been crucial for elucidating PD pathophysiology, as well as developing therapeutic approaches to alleviate motor symptoms. Studies have proposed that inflammatory dysfunctions are associated with psychiatric disorders and neurodegeneration in both animal models and human patients [31-33]. In this study, we applied LPS intrapallidal administration to triggering neuroinflammation in rat models. The behavioral alternations implied that inflammation caused by LPS was efficacious in mimicking PD symptoms.

Neuroinflammation always triggers downstream signaling pathways and causes subsequent pathological changes. We further confirmed that LPS-induced inflammation reduced the neuron cell amount in substantia nigra pars compacts and suppressed the secretion of striatal dopamine. Inflammatory molecules can induce the recruitment of peripheral leukocytes into CNS. This neuroinflammatory process could be partially beneficial for neuronal tissues since it promotes the clearance of cell debris. Conversely, inflammatory mediators could also act on neurons to cause neurodegeneration. The neuronal death further activates inflammatory mechanisms, resulting in a vicious cycle composed of local inflammation

and neuronal death. A systemic inflammation due to infection or peripheral injury could exacerbate symptoms and promote neuronal damage in PD. Pro-inflammatory cytokines secreted by leukocytes act on brain tissues through several pathways, such as the endothelial cells and leakage through damaged BBB. In addition, microglial activation has also been linked with cerebral disorders [34]. Microglia are CNS-resident immune cells activated in response to cerebral injury [35]. As reported, microglial activation induces the release of pro-inflammatory cytokines including interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF)-α, leading to neuronal damage and cellular loss [36, 37]. These cytokines further stimulate microglia to secrete chronic inflammation mediators, maintaining neuroinflammation status. The acute and chronic inflammations might synergistically reduce the neuron cell amount and the suppressed secretion of striatal dopamine in the substantia nigra pars compacts, contributing to the pathogenesis of PD.

Our data showed that LPS-induced inflammation altered iron metabolism in the substantia nigra pars compacts. Enhanced iron and ferritin levels in the substantia nigra pars compacts reflects an inflammatory status quo caused by LPS. Iron accumulation in the substantia nigra pars compacts might directly result from LPS injection, but it also contributes to the pathogenesis of PD through its toxic effects on dopamine neutrons. As a consequence of LPS-stimulation, iron accumulation leads to slow and progressive neuronal death, which is supported by a gradual reduction in movement capacities over time in our PD models. Despite being essential for tissue homeostasis, our data confirm that inflammatory responses contribute to neuronal injury when they are not controlled and/or chronic, and dopaminergic neurons from substantia nigra are particularly vulnerable to microglial mediated neurotoxicity, which is in line with previous reports [38, 39].

Collectively, in this study, we investigated the effects of intrapallidal administration of LPS on dopamine depletion and iron accumulation in substantia nigra in a PD rat model. We found that inflammation-induced iron accumulation promotes PD pathogenesis through subsequent cell damage. Thus, taking measures to prevent the onset of inflammation and to control the magnitude of cerebral inflammation would be pivotal for PD patients.

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Disclosure of conflict of interest

None.

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