Panaxytriol Inhibits Lipopolysaccharide-Induced Microglia Activation in Brain Inflammation in Vivo

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Received April 6, 2021; accepted April 21, 2021

Brain inflammation is a pathological characteristic of neurodegenerative diseases. In this condition, excessively activated microglia elevate proinflammatory mediator levels. We previously reported that panaxytriol inhibited lipopolysaccharide (LPS)-induced microglia activation in vitro. However, the effects of panaxytriol on microglia activation in vivo require confirmation. In the present study, we found that panaxytriol suppressed both microglia and astrocyte activation by injected LPS intracerebrally to mice with LPS-induced brain inflammation. Panaxytriol was more effective on microglia than astrocytes. Moreover, panaxytriol tended to reduce LPS-induced spontaneous motor activity dysfunction. These results suggested that panaxytriol could improve brain health by suppressing microglia activation in neurodegenerative diseases.

Key words microglia activation; panaxytriol; brain inflammation; lipopolysaccharide

INTRODUCTION

Brain inflammation is an important feature of neurodegenerative diseases, such as Alzheimer’s disease and Parkinson’s disease.1) In brain inflammation, microglia, the resident macrophages in the central nervous system, are excessively activated and produce an excess of proinflammatory mediators.2) Many reports indicate that dysregulation of microglial functions contributes to pathology progression in neurodegenerative diseases.3)

Our previous study showed that panaxytriol, an active Panax ginseng component, inhibits lipopolysaccharide (LPS)-induced microglia activation in BV-2 cells.3) Panaxytriol suppresses LPS-induced nitric oxide production, inducible nitric oxide synthase expression, and proinflammatory cytokine mRNA expression such as tumor necrosis factor-α, interleukin (IL)-1β, and IL-6. Panaxytriol also inhibits the LPS-induced translocation of nuclear factor-kappa B (NF-κB) into the nucleus, suggesting that NF-κB is central in the panaxytriol-induced inhibition of microglia activation. Thus, we consider that panaxytriol, which targets microglia activation, can be a new therapeutic agent against neurodegenerative diseases. However, our previous results did not confirm in vivo effects of panaxytriol. Therefore, we investigated the effects of panaxytriol on microglia activation in vivo using an LPS-induced brain inflammation mouse model.

MATERIALS AND METHODS

Materials We purchased Escherichia coli O111 (125-05201) LPS from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). We obtained panaxytriol as described in our previous report.4) The artificial cerebral spinal fluid (aCSF) contained 140 mM NaCl, 3.0 mM KCl, 2.5 mM CaCl2, 1.0 mM MgCl2, and 1.2 mM Na2HPO4, adjusted to pH 7.4. We used a mixture of three anesthetic agents: 0.75 mg/kg medetomidine hydrochloride (Domitor, Nippon Zenyaku Kogyo, Koriyama, Japan), 4.0 mg/kg midazolam (Midazolam (SANDOZ), Sandoz K.K., Tokyo, Japan), and 5.0 mg/kg butorphanol tartrate (Butorphal, Meiji Seika Pharma, Tokyo, Japan).

Animals We obtained 6-week-old, 18–22 g C57BL6/Ncr mice from Japan SLC (Hamamatsu, Japan). We kept the animals under controlled temperature and humidity conditions on a 12–12 h modified light–dark cycle with free access to water and food. We carried out all animal experiments following the Ethical Guidance of the University of Toyama Animal Experimentation Committee.

Spontaneous Behavior Before intracerebroventricular infusion, we placed the mice in an open box (about 150 mm wide, 200 mm deep, and 350 mm high) for 1 h to allow habituation. After 24 h of the intracerebroventricular injection, we photographed the mice for 120 min using SCLABA-Next (NOVEL TEC, Kobe, Japan) and analyzed their spontaneous behavior with SCLABA-Next Tracking software.

Drug Administration We randomly divided the mice into four groups: the dimethyl sulfoxide (DMSO) (vehicle), LPS (10 µg) + DMSO (LPS + vehicle), LPS + panaxytriol (7.5 µg) (LPS + panaxytriol (PNX)), and PNX groups. We coinjected panaxytriol and LPS intracerebroventricularly. We anesthetized the mice with a mixture of three anesthetic agents and woke them up with atipamezole (10 mL/kg, intraperitoneal injection). We injected a 2 µL single dose of the drug using a 5 µL Hamilton microsyringe at 0.667 µL/min. We left the needle in place for 5 min for proper dispersal of the drug from the tip following injection. The stereotaxic bregma coordinates were −0.5 mm anteroposterior, 1.0 mm lateral (right side), and −2.0 mm dorsoventral.

Immunofluorescence Staining To measure microglia activation in the hippocampus, we perfused the mice with 0.1 M phosphate-buffer solution (PBS) at pH 7.4 and immediately removed the brains, fixed them in 4% paraformaldehyde overnight, and stored them in a 30% sucrose solution. We then froze and cut the brains into 20-µm-thick sections using a cryostat microtome (Leica CM 3050S; Leica Biosystems, Vienna, Austria). We performed immunofluorescence staining using a secondary antibody and a fluorescent dye. We photographed the stained slides using a confocal microscope (Olympus FV1000). The staining intensity of microglia was evaluated using ImageJ (National Institutes of Health, USA) and the mean fluorescence intensity of the microglia was calculated. The results were analyzed using a one-way analysis of variance (ANOVA) and Dunnett’s post hoc test. Differences were considered significant at *p < 0.05.
Wetzlar, Germany). We washed the sections in PBS for 30 min and treated them with an antigen retrieval solution HistoVT One (10×, pH 7.0) (Nacalai Tesque, Kyoto, Japan) diluted 10 times with sterile water for 20 min at 70 °C. We washed the sections three times for 10 min with PBS, blocked them with PBS containing 3% bovine serum albumin for 60 min at room temperature, and washed them with PBS containing 0.2% Tween 20 (PBS-T) for 10 min.

Fig. 1. Panaxytriol (PNX) Attenuates LPS-Induced Microglia Activation and Morphological Changes
(A) Representative images of Iba1-positive activated microglia in the hippocampal CA1, CA3, and dentate gyrus (DG) regions. Scale bar = 200 µm. (B) Quantification of the average number of Iba1-positive microglia per unit area. n = 7. (C) Representative images of morphological changes in Iba1-positive activated microglia. Scale bar = 10 µm. (D) Quantification of the average change of Iba1-positive microglia in maximum process length and cell body. n = 7. *p < 0.05, **p < 0.01, ***p < 0.001, compared with vehicle; †p < 0.05, ††p < 0.01, †††p < 0.001, compared with LPS + vehicle; #p < 0.01, ##p < 0.001, compared with PNX. (Color figure can be accessed in the online version.)
1:2000 dilution; Sigma-Aldrich, St. Louis, MO, U.S.A.). We then washed them with PBS-T three times for 10 min and treated them for 120 min at room temperature with the fluorescent-labeled secondary antibody (Alexa Fluor 488 (a21206, Thermo Fisher Scientific, Waltham, MA, U.S.A.) and Alexa Fluor 568 (a11057, Thermo Fisher Scientific)). We washed the sections three times with PBS-T for 10 min and stained them with 4',6-diamidino-2-phenylindole (DAPI). We recorded fluorescence images using a confocal fluorescence microscope LSM700 (ZEISS, Oberkochen, Germany). We captured images from the hippocampus regions CA1, CA3, and dentate gyrus (DG).

Quantitation of Microglia and Astrocyte Activation in Cell Number and Intensity We counted Iba1-positive microglia cells using HCA-Vision (version 2.1.6) (Commonwealth Scientific and Industrial Research Organisation, Canberra, Australia), with fluorescence brightness and area above the threshold. We considered GFAP-positive activated astrocytes with fluorescence intensity above the threshold as the degree of activation using ImageJ-win64 software (National Institutes of Health, Bethesda, MD, U.S.A.).

Quantitation of Microglia Activation in Morphological Changes We analyzed the changes of Iba1-positive microglia using ImageJ-win64 software. We defined the “cell body” as the Iba1-positive area of cells with a DAPI-stained nucleus and the “process” as the Iba1-positive area of cells excluding the cell body.

Statistical Analysis We represented all data as the mean ± standard error of the mean. We used two-way ANOVA with Tukey’s test post hoc multiple comparisons to analyze statistical significance. We considered p values <0.05 as statistically significant.

RESULTS

Panaxytriol Inhibits LPS-Induced Microglia Activation in Vivo To investigate whether panaxytriol inhibits microglia activation in vivo, we intracerebroventricularly administered panaxytriol (7.5 µg) with LPS (10 µg) to C57BL/6Ncr mice simultaneously and performed immunofluorescent staining on hippocampal sections. We used the number of Iba1-positive cells as the index of activated microglia after LPS injection. LPS administration increased the number of Iba1-positive cells in the CA1, CA3, and DG regions. Panaxytriol significantly suppressed the LPS-induced increase in Iba1-positive cells in the CA1 and CA3 regions and slightly reduced it in the DG region (Figs. 1A, B). These results suggested that panaxytriol suppresses microglia activation.
Panaxytriol activates the nuclear factor-E2-related factor 2 (Nrf2)-antioxidant response element (ARE) signaling pathway. Nrf2 pathway activation has antioxidant and anti-inflammatory effects. Upon activation, the morphology of microglia changes. Here, LPS caused cell body hypertrophy and maximum process regression and panaxytriol suppressed these changes (Figs. 1C, D).

**Panaxytriol Tended to Improve LPS-Induced Spontaneous Motor Activity Decrease**  
Fig. 3. PNX Tended to Improve the LPS-Induced Spontaneous Motor Activity Decrease

Spontaneous motor activity measured for 60 min. \( n = 7 \). \( p < 0.05 \), compared with vehicle.

Microglia plastically and morphologically adapt to their ever-changing surroundings. Upon activation, the morphology of microglia changes. Here, LPS caused cell body hypertrophy and maximum process regression and panaxytriol suppressed these changes (Figs. 1C, D).

**Panaxytriol Inhibits LPS-Induced Astrocyte Activation in Vivo**  
Next, we investigated the effect of panaxytriol on the activation of astrocytes using immunohistochemistry. LPS significantly increased the number of GFAP-positive activated astrocytes in the CA1, CA3, and DG region (Fig. 2). Panaxytriol suppressed the fluorescence intensity of GFAP-positive cells in the CA1 and CA3 regions and slightly suppressed it in the DG region. These results suggest that panaxytriol also suppresses astrocyte activation.

**Panaxytriol Tended to Improve LPS-Induced Locomotor Impairment**  
To investigate the effect of panaxytriol on locomotor activity, we simultaneously administrated panaxytriol and LPS and measured spontaneous locomotor activity for 60 min. LPS significantly decreased spontaneous locomotor activity. Panaxytriol tended to counter the LPS-induced spontaneous locomotor activity decrease (Fig. 3).

**DISCUSSION**

Panaxytriol activates the nuclear factor-E2-related factor 2 (Nrf2)-antioxidant response element (ARE) signaling pathway. Nrf2 pathway activation has antioxidant and anti-inflammatory effects. We previously reported that in BV-2 microglia in vitro, panaxytriol inhibits microglia activation not by activating ARE but by suppressing NF-κB translocation into the nucleus. However, the effect of panaxytriol on microglia activation in vivo remains unclear. We thus investigated the effect of panaxytriol on the activation of glial cells induced by intracerebroventricular LPS administration. Panaxytriol suppressed the increase of Iba1-positive cells in the LPS-induced brain inflammation mouse model (Fig. 1). Moreover, panaxytriol improved the microglial morphology changes. Panaxytriol suppresses the increase of GFAP-positive cells. Similar to the response to LPS, microglia were more sensitive to panaxytriol than astrocytes. We attributed these results to the higher expression level of the toll-like receptor 4, an LPS receptor, in microglia than in astrocytes. Our previous report showed that panaxytriol inhibited LPS-induced microglia activation by suppressing the NF-κB signaling pathway. Since then, reports have shown that LPS-induced astrocyte activation also involves the NF-κB signaling pathway, and our results suggested that panaxytriol suppressed both microglia and astrocyte activation by suppressing the NF-κB signaling pathway in vivo.

Microglia have elongated processes that move dynamically and contact other brain cells to monitor and regulate them. Microglia exist in various activation states and can shift functionality during an inflammatory response. Pathological conditions activate microglia, which changes their morphology to the ramified form, in which the processes regress and cell body is enlarged. In this study, LPS reduced the processes and hypertrophied cell bodies, which is consistent with a previous report. Panaxytriol significantly improved process regression and prevented cell body hypertrophy, suggesting that panaxytriol also suppressed microglia activation-related morphological changes in vivo.

LPS intracerebroventricular injection decreased spontaneous motor activity. Panaxytriol tended to counter this locomotor activity decline. In brain inflammation, microglia are activated and produce excess proinflammatory cytokines such as IL-1β. Yamato et al. reported that IL-1β is a critical brain inflammation trigger and that the IL-1 receptor is the key trigger affecting spontaneous activity in brain inflammation. We previously reported that panaxytriol suppressed LPS-induced IL-1β mRNA expression in vitro. Therefore, panaxytriol may prevent spontaneous motor activity decrease by suppressing IL-1β expression. This study revealed that panaxytriol suppressed LPS-induced microglia and astrocyte activation in vivo. Moreover, panaxytriol relieved the LPS-induced spontaneous motor activity decrease. These results suggest that panaxytriol can inhibit brain inflammation and may be useful as a therapeutic agent against neurodegenerative diseases.

**Acknowledgments**  
This work was supported by JSPS KAKENHI [JP19K07119] and the Smoking Research Foundation, Japan.

**Conflict of Interest**  
The authors declare no conflict of interest.

**REFERENCES**


