Note

Continual Treatment with the Peels of *Citrus kawachiensis* (Kawachi Bankan) Protects against Dopaminergic Neuronal Cell Death in a Lipopolysaccharide-Induced Model of Parkinson’s Disease

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Summary Our previous study showed that the subcutaneous administration of auraptene (AUR) suppresses inflammatory responses including the hyperactivation of microglia in the substantia nigra (SN) of the midbrain of lipopolysaccharide-induced Parkinson’s disease (PD)-like mice, as well as inhibits dopaminergic neuronal cell death in this region. We also showed that the oral administration of the dried peel powder of *Citrus kawachiensis*, which contains relatively high amounts of AUR, suppresses inflammatory responses including the hyperactivation of microglia in the systemically inflamed brain. In the present study we showed that the oral administration of this dried peel powder successfully suppressed microglial activation and protected against dopaminergic neuronal cell death in the SN, suggesting its potential as a neuroprotective agent for the treatment of patients with PD.

Key Words citrus, anti-inflammation, microglia, neuroprotection, Parkinson’s disease

Parkinson’s disease (PD) is an age-related progressive neurodegenerative disorder, and it is caused by the selective loss of dopaminergic neurons in the substantia nigra (SN) of the midbrain (1). Accumulating evidence indicates that hyperactivated inflammation in the brain, particularly hyperactivation of microglia in the SN, is the initial factor that triggers neurodegeneration (2). Thus a PD mouse model was produced by the subacute injection of lipopolysaccharide (LPS), a pro-inflammatory agent, into the SN (3). Using these PD-like model mice, we recently showed that subcutaneously (s.c.) administration of natural prenyloxyphenyl-propanoids such as auraptene (AUR) suppresses the LPS-induced inflammatory responses in the SN and the dopaminergic neuronal cell death in this region (4).

On the other hand, we also showed that the dried peel powder of *Citrus kawachiensis*, a citrus species in Japan, is abundant in AUR (5) and that it has the potent ability to suppress the hyperactivation of microglia in the systemically inflamed brain, which was produced by single intraperitoneal (i.p.) administration of LPS (1 mg/kg of mice) (6). These findings prompted us to investigate here whether the oral (p.o.) administration of the dried peel powder of *C. kawachiensis* would be effective for neuroprotection against LPS-induced PD-like symptoms in model mice. The dose of the dried peel powder in the present study was designed to be 1.2 g/kg/d or 2.4 g/kg/d, which was the same dose as used in our previous study on systemically inflamed brain (6).

Materials and Methods

The dried peel powder was prepared from *C. kawachiensis* grown in Yawatahama (Ehime, Japan) as previously described (6). LPS was purchased from Sigma-Aldrich (St. Louis, MO, USA).

Nine-week-old male C57BL/6 strain mice (about 25 g) purchased from Japan SLC, Inc. (Hamamatsu, Japan) were housed in controlled conditions (4, 6). Experiments were carried out in accordance with the Guidelines for Animal Experimentation specified by the Animal Care and Use Committee of Matsuyama University (Approval No. 9002).

A total 36 mice were randomly divided into the following 4 groups: PBS-injected control group (CON group; \(n = 7\)), LPS-injected group (LPS group; \(n = 9\)), LPS-injected and dried peel (1.2 g/kg/d)-treated group (LPS+P1.2 group; \(n = 10\)), and LPS-injected and dried peel (2.4 g/kg/d)-treated group (LPS+P2.4 group; \(n = 10\)). The procedure for the single intranigral injection of LPS (2 \(\mu\)g/2 \(\mu\)L) or vehicle (PBS) on Day 1 was performed as described previously (4). Immediately after their intranigral injection, p.o. administration of samples into mice was started and continued for 20 d (namely, from Day 1 to Day 20). Mice in the 2 sample-treated groups (LPS+P1.2 and LPS+P2.4 groups) received the sample solution (0.75 mL) containing each amount of the dried peel powder. Mice in the CON and

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LPS groups were received vehicle solution (distilled water) in the same way. During the experimental period, the mice were given free access to tap water and food until 08:30 and then deprived of food until the time of administration of samples or vehicle (at 17:00). At Day 21 (one day after the last administration of each sample), the mice were anesthetized and perfused via the heart with ice-cold PBS prior to removal of their brains, as previously reported (4). Coronal sections of 20-μm thickness were prepared and then immunostained using a rabbit polyclonal antibody against ionized calcium-binding adaptor molecule 1 (Iba1; Wako Pure Chemical Industries, Ltd., Osaka, Japan) to stain microglia or a rabbit polyclonal antibody against tyrosine hydroxylase (TH; Cell signaling, Woburn, MA, USA) to stain dopaminergic neurons. Data for the individual groups were expressed as the mean±SE and analyzed by performing the t-test (between CON and LPS) and one-way ANOVA with the post-hoc Dunnett’s multiple comparison test (among LPS, LPS+P1.2, and LPS+P2.4). Significance was defined as p<0.05.

**Results and Discussion**

The body weight was measured at Day 1 and Day 21 and observed that there were no statistical effects of LPS/sample on body weight (data not shown). At Day 21, we examined the behavior of the animals using the open-field test and observed that there was no statistical difference in total distance among the 4 groups (data not shown). On the other hand, mice administered a higher dose of LPS (10 μg/mice) were reported to display behavioral abnormality (3). On the basis of these findings, we speculated that the LPS treatment in the present experimental condition (single intranigral injection with 2 μg of LPS/mice) did not affect on body weight and not induce motor system dysfunction yet in the SN and striatum dopaminergic pathway system.

As neuroinflammation, in particular microglial activation, is reported to be a key player in PD (2), we investigated the effects of the dried peel powder on the brains of LPS-induced PD model mice. Figure 1A shows representative photographs of the SN region stained with antibody against Iba1, a marker of microglia. Only a few Iba1-positive cells were seen in the CON group, and their shape indicated the inactivated form (smaller cell bodies; ramified microglia). In contrast, many Iba1-positive cells were detected in the LPS group, and their shape had changed to the activated form (larger cell bodies and short ramifications; ameboid microglia). In the 2 sample-treated groups (LPS+P1.2 and LPS+P2.4 groups), the number of Iba1-positive cells decreased; and their shape returned to that of the inactivated form. Figure 1B shows the results of a quantitative analysis of the cell density of Iba1 staining. The Iba1-signal density of the cells in the LPS group was significantly (**p<0.001) higher than that of the CON group cells, whereas in the LPS+P1.2 group and LPS+P2.4 group it was significantly lower (##p<0.01 and ###p<0.001, respectively) than that of the cells in the LPS group. These results indicate that the dried peel powder had the ability to suppress LPS-induced microglial hyperactivation in the SN in a dose-dependent manner.

Dopaminergic neurons in the SN of the brain are well known to be selectively vulnerable and their number to decline in PD (7). Figure 2A shows representative photographs of the SN region stained with an antibody against TH, a selective marker of dopaminergic neurons. In the LPS group, the number of TH-positive neurons was apparently reduced compared with that in the CON group. On the other hand, the number of these neurons...
Neuroprotective Effect of Peel of *Citrus kawachiensis*

The dried peel powder used in the present study contained AUR delivered at 4.88 mg/kg/d (for LPS+P1.2 group) or 9.77 mg/kg/d (for LPS+P2.4 group). These AUR contents were rather lower than that in the previous case of the single s.c. dose of AUR (25 mg/kg/d) (4, 6).

This peel is also a rich source of naringin (NGIN), delivered at 52.8 mg/kg/d for the LPS+P1.2 group and 106 mg/kg/d for the LPS+P2.4 group. NGIN exerts neuroprotective effects against various brain injuries by reducing oxidation- and inflammation-mediated alterations (8), and intraperitoneal injection (80 mg/kg for 4 d) of this molecule has been revealed to protect the nitrostriatal dopaminergic projection through the induction of glial cell line-derived neurotrophic factor (GDNF) in 1-methyl-4-phenylpyridine (MPP⁺)-induced PD model rats (9). The neuroprotection seen in the present model mice is likely to be caused by not only AUR but also NGIN.

As microglial activation is observed during the development of symptoms of neurodegenerative diseases including Alzheimer's disease and multiple sclerosis as well PD, inflammation has been suggested to be implicated in the progression of neurodegeneration (10, 11). In conclusion, the findings in the present study suggested that the dried peel powder of *C. kawachiensis* might be useful as a neuroprotective agent for the treatment of neurodegenerative diseases as well as systemic inflammatory diseases (4).

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